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Contents

Weekly Volume 22 Number 35 September 21, 2016

DIAGNOSTIC ADVANCES

REVIEW

Contents

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DIAGNOSTIC ADVANCES

Role of spleen elastography in patients with chronic liver diseases

Mariangela Giunta, Dario Conte, Mirella Fraquelli

Mariangela Giunta, Dario Conte, Mirella Fraquelli, Gastroenterology and Endoscopy Unit, Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico, 20122 Milan, Italy

Mariangela Giunta, Dario Conte, Mirella Fraquelli, Department of Pathophysiology and Organ Transplantation, Università degli Studi di Milano, 20122 Milan, Italy

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Correspondence to: Mirella Fraquelli, MD, PhD, Gastroenterology and Endoscopy Unit, Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico, Via F. Sforza 35, 20122 Milan, Italy. mfraquelli@yahoo.it
Telephone: +39-25-5033369
Fax: +39-02-50320410

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Abstract

The development of liver cirrhosis and portal hypertension (PH), one of its major complications, are structural and functional alterations of the liver, occurring in many patients with chronic liver diseases (CLD). Actually the progressive deposition of hepatic fibrosis has a key role in the prognosis of CLD patients. The subsequent development of PH leads to its major complications, such as ascites, hepatic encephalopathy, variceal bleeding and decompensation. Liver biopsy is still considered the reference standard for the assessment of hepatic fibrosis, whereas the measurement of hepatic vein pressure gradient is the standard to ascertain the presence of PH and upper endoscopy is the method of choice to detect the presence of oesophageal varices. However, several non-invasive tests, including elastographic techniques, are currently used to evaluate the severity of liver disease and predict its prognosis. More recently, the measurement of the spleen stiffness has become particularly attractive to assess, considering the relevant role accomplished by the spleen in splanchnic circulation in the course of liver cirrhosis and in the PH. Moreover, spleen stiffness as compared with liver stiffness better represents the dynamic changes occurring in the advanced stages of cirrhosis and shows higher diagnostic performance in detecting esophageal varices. The aim of this review is to provide an exhaustive overview of the actual role of spleen stiffness measurement as assessed by several elastographic techniques in evaluating both liver disease severity and the development of cirrhosis complications, such as PH and to highlight its potential and possible limitations.

Key words: Cirrhosis; Spleen stiffness; Elastography; Portal hypertension; Transient elastography

Core tip: Spleen elastography is an attractive tool used as an alternative and/or complementary method to assess liver fibrosis, portal hypertension and

complications related to cirrhosis. There are several elastography techniques to measure spleen stiffness, all characterized by non-invasiveness and repeatability. Current data from the literature show the higher accuracy of spleen stiffness as compared to liver stiffness, in predicting major complications of cirrhosis. Thus, despite some limitations, spleen stiffness seems to be a better prognostic predictor in patients with chronic liver disease.

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INTRODUCTION

Elastographic techniques are among the most promising tools developed in the last decade for the non-invasive evaluation of patients with chronic liver disease. Liver stiffness (LS) measurement using transient elastography (Fibroscan®) is now a widely accepted and validated method to predict the severity and prognosis of liver disease[1-3]. However, there is mounting evidence that its diagnostic accuracy can be flawed by the presence of some confounders, such as liver cell inflammation and cholestasis[4-9] and its performances may be limited in patients with a high body mass index (BMI), narrow intercostal space, or ascites.

Besides this evidence, liver elastography measures hepatic fibrosis which only correlates with the fixed component of portal hypertension (PH) related to intrahepatic resistance but is unable to account for the dynamic component related to hyperdinamic splanchnic circulation and portal venous blood flow[10]. Given these considerations and the known relationship between advanced liver disease and spleen tissue changes, spleen elastography has been proposed and tested as an alternative and/or complementary method to LS for assessing liver fibrosis, predicting complications related to cirrhosis, detecting PH and predicting any presence of esophageal varices (EV).

The aim of this review is to provide, from the current literature data, an extensive overview of the different elastographic techniques used to measure spleen stiffness (SS), with the focus on their feasibility, reproducibility, limitations and diagnostic accuracy to better predict the prognosis of patients affected by chronic liver diseases.

DISCUSSION

Elastographic techniques for measuring SS: feasibility and reproducibility

All the elastographic techniques applied to assess SS were at first developed as non-invasive methods to assess LS for staging liver fibrosis and predicting the presence of PH.

Transient elastography: Transient elastography (TE) is the first elastographic technique: it became available 13 years ago with the development of FibroScan® (Echosens, Paris, France) used to assess LS. An ultrasound transducer probe is mounted on the axis of a vibrator. Vibrations of mild amplitude and low frequency are transmitted by the transducer, inducing an elastic shear wave that propagates through the underlying tissues. Pulse-echo ultrasound acquisitions are used to follow the propagation of the shear wave and to measure its velocity, which is directly related to the tissue stiffness of the elastic modulus: the stiffer the tissue, the faster the shear wave propagates.

A large amount of literature, consisting of both primary studies and meta-analyses using histology as the reference standard, has analyzed the role of TE in predicting the stage of fibrosis in patients with CLD caused by different etiologies, and has showed that TE accurately predicts hepatic fibrosis stage with very good accuracy especially for severe fibrosis/cirrhosis. TE cut-off values for the different fibrosis stages have been provided from many large-scale studies[11-14] and by meta-analysis. Results obtained by meta-analysis show cut-offs of TE for liver fibrosis in the range of 7.3-7.9 kPa for the diagnosis of METAVIR fibrosis stage $F \geqslant 2$ and 13.0-15.6 kPa for the diagnosis of liver cirrhosis with AUROC between 0.84-0.87 and between 0.93-0.96 respectively[15-19].

The main disadvantages of TE are that it requires a dedicated device, the region of interest (ROI) cannot be chosen, it cannot be performed in patients with ascites and can be difficult to obtain in patients with obesity.

Currently TE is also the most common technique used to assess SS. In the absence of guidelines for the measurement of SS, all studies applied the same main rules approved for the measurement of LS (*e.g.*, fasting period, success rate, IQR and the minimum number of valid measurements). To date, for the assessment of SS transient elastography is performed with the same probe used to perform LS, with the patient lying supine or prone. Besides the same technical limitations mentioned for LS which are valid also for SS assessment, in some patients the operator is unable to locate the splenic parenchyma because the spleen surface is smaller than the liver surface. In a previous study from our own group both the rate of indeterminate results and failures of SS measurements, decreased over time with operator experience. In particular, the rate of failures decreased from 8%-12%, in the first six months of the study, to 0%-2% in the subsequent 18-24 mo, whereas the rate of unreliable results was 16.6%. Also the inter-observer agreement of SS as expressed by ICC was optimal, being 0.89 in patients with chronic liver disease[20].

A relevant technical aspect is that SS measurements by TE are obtained using a probe validated only

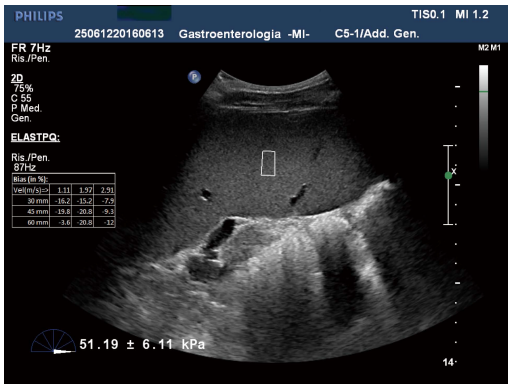

Figure 1 Spleen stiffness determination by point shear wave elastography.

for the measurement of LS. Indeed, the acquisition parameters of the FibroScan® (Echosens, Paris, France) were optimized for the stiffness assessment of liver tissues, especially in terms of low-frequency excitation. To accurately assess the stiffness of organs harder than the liver, the acquisition parameters should be probably adapted. Normal spleen stiffness, also in healthy volunteers, is higher in terms of kPa than that of the liver, ranging from 9.4 to 65.2 kPa[20]. Therefore, the use of the Fibroscan® (Echosens, Paris, France) on the spleen of patients with cirrhosis might lead to overestimated stiffness values. Often in patients with cirrhosis and portal hypertension SS measurements reach the maximum value reported by machine, *i.e.,* 75 kPa, in order to identify the correct range of SS values and to better stratify them both Calvaruso *et al*[21] and Stefanescu *et al*[22] asked Echosens to perform an analysis of their elastograms using software and calculation algorithms that allow for stiffness measurements up to 150 kPa. Using this modified spleen stiffness, the authors obtained interesting results in terms of prediction of both esophageal varices and liver decompensation[23]. However, those authors correctly pointed out that this modified SS measurement probably is not the real spleen stiffness value but only an estimation of it thus efforts should be made to develop a modified probe and software with frequency correction and depth optimization.

Acoustic radiation force impulse techniques

Point shear-wave elastography: This is an elastographic technique recently developed and integrated into several conventional ultrasound machines (*e.g.*, Siemens, Philips, Hitachi, Esaote *etc.*). It allows the quantitative assessment of tissue stiffness, providing measurements of shear-wave velocity within a small region (fixed ROI size 0.5 cm × 1.5 cm), its localization being monitored by real-time B-mode ultrasound. Results may be expressed in units of shear-wave velocity (m/s) or converted into units of Young's modulus (kPa). The advantage of point shear-wave elastography (pSWE) relates to the opportunity for the operator of positioning the region of interest where to perform measurements with an adequate ultrasound window. In addition, the exam can be performed also in presence of ascites, obesity or narrow intercostal spaces. Several recent reports and meta-analyses have demonstrated that the measurement of LS by pSWE is a valuable method of assessing liver fibrosis[24-27] while only a few studies have investigated spleen stiffness with this method[28-30]. The failure rate of acoustic radiation force impulse (ARFI) for liver stiffness determination is similar to that of TE (2.9%)[27,31] and also the reproducibility is good for LS determination whereas insufficient data is available to date regarding the rate of failure and unreliable measurements of SS by pSWE. Its feasibility seems to be similar to that for LS and inter-observer agreement seems to be acceptable but inferior to that on LS[30,32].

Figure 1 shows an example of spleen stiffness determination by pSWE.

Real-time two-dimensional SWE: Real-time two-dimensional SWE (RT 2D-SWE) has the peculiarity that the shear-wave velocities can be measured in a bi-dimensional area (a box of 2.5 cm × 3.5 cm) rather than in a single small point: this way the limitation of pSWE to accidentally investigate small regions of greater or lesser stiffness than average is overcome. Also this technique is incorporated in conventional ultrasound machines and its results are expressed either in m/sec or in kPa. Its failure rate is significantly lower than that of TE and preliminary results suggest that SWE may be better than TE at the diagnosis of clinically significant fibrosis and more reliable in patients with ascites[33-37]. SS measurement using 2D-SWE has been investigated in even fewer studies. A preliminary study by Cassinotto *et al*[38] showed excellent intra-observer (ICC 0.96) and inter-observer (ICC 0.87) agreements but the rate of failure was quite high (30%) probably due to the thinness and the more cranial position of the splenic parenchyma and to movements owed to the proximity of the left cardiac ventricle. Findings regarding the diagnostic accuracy of SS determination for the assessment of liver fibrosis are sporadic even if its diagnostic accuracy for liver cirrhosis seems good: a study by Grgurevic *et al*[39] found an AUROC of 0.82 using a cut-off value of 24 kPa.

Regarding ARFI techniques (pSWE and RT 2D-SWE) there are two relevant critical issues that should be taken into account. Firstly, the quality criteria for the correct interpretation of results (for SS and even for LS) remain to be adequately defined. Secondly, the elastographic techniques which are incorporated into conventional ultrasound devices enabling the choice of where to place the region of interest and whether to confirm or exclude each single measurement, can be more operator-dependent than TE[40].

Magnetic resonance elastography: Magnetic resonance elastography (MRE) is an imaging technique that provides full organ coverage[41] and low variability for

Table 1 Diagnostic performance of liver stiffness and spleen stiffness for liver fibrosis and esophageal varices[20]

	F ⩾ 2 (66%)		F = 4 (23%)		EV (10%)	
	L-TE	S-TE	L-TE	S-TE	L-TE	S-TE
Cut-off (kPa)	8	36	12	46	19	65
Sensitivity	83 (54-76)	76 (61-81)	92 (80-100)	89 (72-99)	73 (59-93)	91 (81-100)
Specificity	65 (76-98)	80 (65-93)	80 (72-89)	78 (82-96)	47 (34-68)	80 (65-94)
LR+	2.4	3.9	4.6	4.5	1.4	4.5
LR-	0.2	0.3	0.09	0.1	0.5	0.1
AUROC	0.85 (0.78-0.92)	0.80 (0.65-0.85)	0.93 (0.88-0.96)	0.84 (0.76-0.93)	0.62 (0.41-0.89)	0.90 (0.79-100)

TE: Transient elastography; EV: Esophageal varices.

stiffness measurement as evidenced by excellent inter-scan reproducibility[42-44] and inter-reader agreement[45]. A correlation between LS by MRE and hepatic fibrosis has been established[46] and MRE has shown to have considerably high reproducibility, feasibility and validity for assessing liver fibrosis[47-50].

SS measurement using MRE has been investigated in a few studies. Firstly, Talwalkar *et al*[51] suggested the feasibility of MRE in assessing SS as a non-invasive measure of portal pressure by assessing 38 patients with chronic liver disease. The authors found a strong linear relationship between LS and SS (r^2 = 0.75, P < 0.001) and with a mean SS valued ⩾ 10.5 kPa they noted a detection rate of 100% for EV. Similarly, Morisaka *et al*[52] found that SS had the highest correlation with EV as observed at endoscopy. For discriminating large EV *vs* no EV or small EV only SS showed a significant association (OR = 1.82) with an AUROC of 0.81. Moreover, the authors showed the reproducibility of SS measurements by MRE (Pearson's correlation coefficient 0.898). A recent retrospective study confirmed that LS and SS measured by MRE are strongly associated with the presence of EV[53]. However, all these studies cannot confirm a direct correlation between SS and hepatic vein pressure gradient (HVPG) because the measurement of HVPG was not performed and the presence of PH was only assessed indirectly by the presence of EV at endoscopy. Only in animal models a strong correlation was reported (r > 0.80) between SS measured with MRE and HVPG[54].

A more recent study[55] showed that the performance of SS by MRE for the detection of liver cirrhosis was good (AUROC of 0.87 and 0.92 using single-driver and dual-driver configuration, the latter possibly allowing the simultaneous estimation of LS and SS) but the cut-off values were based on the retrospective patient cohort and the diagnosis of liver cirrhosis was based on clinical history and imaging findings. In addition, the cost and limited availability of MRE actually restrain its use for SS determination in individual patients in the daily clinical practice.

The role of SS in the assessment of liver fibrosis

The prognosis of chronic liver diseases (CLD) of any aetiology is driven by the liver fibrosis stage, a reliable predictor of cirrhosis. Liver biopsy has traditionally been considered the reference method for evaluating and staging hepatic fibrosis but it is an expensive and invasive procedure that requires physicians and pathologists to be sufficiently trained in order to obtain adequate and representative results[56]. Recently, the determination of liver stiffness by different electrographic techniques (TE and ARFI techniques) has been shown to be a reliable non-invasive predictor of disease severity in chronic liver disease of different etiologies.

Owing to the known relationship between severe chronic liver disease and splenic involvement, in the recent years several studies investigated the role of SS in assessing liver fibrosis as an attractive alternative to liver stiffness[57,58].

Using TE several studies have showed significantly higher SS values in cirrhotic *vs* non-cirrhotic patients[20-22]. A study by our own group[20] was conducted on 132 patients with chronic liver disease, 48 patients being with hematological disease and 64 healthy controls. In the CLD patients SS showed very good diagnostic estimates in predicting fibrosis stage (Table 1). In particular for the diagnosis of liver cirrhosis using a cut-off value of 46 kPa the sensitivity was 89%, the specificity 78%, with corresponding LR+ 4.5 and LR- 0.1 and AUROC 0.84. In the study by Bota *et al*[28] using pSWE the overall diagnostic accuracy of SS in diagnosing the presence of cirrhosis showed an AUROC of 0.91. In Grgurevic *et al*[39]'s study the ratio between LS and SS is used as an additional indicator of cirrhosis because with liver fibrosis progressing the difference between LS and SS decreased. The study by Cabassa *et al*[30] showed that if spleen and liver ARFI were combined in a sequential modality even higher accuracy would be achieved. Similarly, to LS the diagnostic accuracy of SS is higher for severe liver fibrosis (F3/F4) than from early fibrosis[29,30]. All these data suggest that SS can be used as an alternative (especially in patients whose liver stiffness determination is not obtainable or is unreliable) or adjunctive diagnostic approach to stage liver fibrosis. However, as of SS, differently to LS, neither large cohort studies nor meta-analyses are available to confirm and validate this technique. Further studies are needed to confirm these interesting data.

The diagnostic estimates for SS determination reported in the primary studies are summarized in Table 2.

The role of SS in the assessment of PH and in the detection of esophageal varices

PH is a typical condition of advanced chronic liver

Table 2 Primary studies assessing the role of spleen elastography in the staging of liver severe fibrosis/cirrhosis

Ref.	Technique	Cut-off	Patients	Sens	Spec	LR+	LR-	PPV	NPV	AUROC
Chen *et al*[29], 2012 (prospective)	ARFI	3.32	163	80.0	88.4	6.9	0.23	55.5	96.0	0.93 (0.89-0.97)
Fraquelli *et al*[20], 2013 (prospective)	TE	46.00	110	89.0	78.0	4.5	0.10	54.7	95.0	0.84 (0.76-0.92)
Cabassa *et al*[30], 2015 (prospective)	ARFI	3.05	51	73.0	84.0	4.5	0.32	91.0	77.0	0.80 (0.68-0.93)
Bota *et al*[28], 2010 (prospective)	ARFI	2.51	67	85.2	91.7	10.2	0.16	73.3	87.1	0.910
Grgurevic *et al*[39], 2015 (prospective)	RT-2D SWE	24.00	66	66.7	86.7	5.01	0.38	75.0	81.3	0.821

ARFI: Acoustic radiation force impulse; AUROC: Area under receiver operative curves; LR+: Likelihood ratio positive; LR-: Likelihood ratio negative; RT 2D-SWE: Real-time two-dimensional shear-wave elastography; TE: Transient elastography; PPV: Positive predictive value; NPV: Negative predictive value.

disease leading to the formation of EV and other severe complications, such as ascites, portosystemic encephalopathy and sepsis[59,60]. Therefore, this condition is one of the most important causes of morbidity and mortality in patients with liver cirrhosis[61]. Nowadays the reference standard methods to diagnose the presence and grade of PH and EV are HVPG and upper endoscopy, respectively: however, both techniques are invasive, expensive and perceived as unpleasant by patients. Therefore, in the last years several non-invasive methods have been proposed to predict the degree of PH and the presence of esophageal varices. In this regard, several serum biomarkers and ultrasound Doppler signs have been studied. Regarding biomarkers, when used as a single test, their diagnostic accuracy was not optimal in clinical practice[62]. Platelet count combined with spleen diameter, known as Giannini's score, showed good accuracy in excluding the presence of EV[63] but not many studies have evaluated the relationship between Giannini' s score and the degree of PH. The study by Colecchia *et al*[64] found that its AUROC was inferior compared with other parameters, such as spleen stiffness (AUROC 0.857 *vs* 0.941). Regarding ultrasound signs they were highly specific to the diagnosis of cirrhosis and the presence of PH but their sensitivity was quite low especially leading to a high rate of false negative results especially in compensated cirrhosis patients[65]. In addition, several studies have showed a relevant inter-observer and inter-equipment variability among different diagnostic centers[66-68].

More recently, there has been considerable interest in the potential use of LS and SS measurements in the detection of significant PH and the prediction of any presence of esophageal varices. One of the determinants of PH is liver fibrosis and LS has proved to correlate strongly with portal pressure as measured by HVPG[69,70] and it may predict clinical decompensation in compensated cirrhotic patients[71]. However, when HVPG values exceed 10-12 mmHg, which are the threshold of clinically significant PH and the development of varices, portal pressure becomes largely independent from liver fibrosis. Accordingly, the ability of liver stiffness to predict the presence and grade of EV is not optimal[70].

Indeed, on considering the physiopathology of PH, in its earlier phases PH depends on the accumulation of fibrillar extracellular matrix, whereas in the later phases the dynamic component related to hyperdinamic circulation and splanchnic vasodilatation become predominant. Splenomegaly plays an important role in the pathophysiology of PH even if it is not clear whether splenomegaly occurs only because of spleen congestion caused by the PH or, according to more recent theories, because of an increase of splanchnic inflow. Indeed, a proliferation of fibrotic hyperplastic components, a hyperactivation of the splenic lymphoid tissue and an increased angiogenesis have been found in the enlarged spleen of rat models with PH induced by portal vein ligation[72].

According to these physiopathogenetic considerations and the anatomic changes that occur at the level of the spleen in cirrhotic patients, spleen stiffness has recently received considerable attention as a potential indicator of PH.

In an animal model Nedredal *et al*[73] performed MRE to assess SS and direct HVPG measurements and showed a positive correlation between SS and direct portal vein pressure gradient (r^2 = 0.86, P < 0.01). In the study by Hirooka *et al*[74] 60 patients with chronic liver disease of mixed etiology underwent LS and SS measurements by real-time tissue elastography, HVPG determination and upper endoscopy. Among the parameters associated with HVPG, the correlation was closer with SS (r = 0.854) than with LS (r = 0.51) (P < 0.0001). At multivariate analysis SS was the only independent predictor of HVPG ⩾ 12 mmHg (OR = 17.7; 95%CI: 2.6-765; P = 0.040)[74]. Similarly, Stefanescu *et al*[75] found a good correlation between SS and EV using a SS cut-off value of 52 kPa (AUROC 0.74) and showed that using both LS and SS the presence of EV is correctly predicted with an overall diagnostic accuracy of about 90%.

In the study by Colecchia *et al*[64], 100 patients with HCV-related cirrhosis were consecutively investigated by transient elastography and HVPG: LS and SS significantly correlated with HVPG values and accurately predicted both the degree of PH assessed by HVPG (r^2 = 0.85) and the presence of esophageal varices. Moreover, LS and SS at multivariate analysis resulted as the only independent predictors of esophageal varices. Using a SS cut-off value of 41.3 kPa the LR- was 0.029, showing that the test accurately ruled out the presence of esophageal varices and therefore can be efficiently used in a screening strategy.

In a study carried out by our own group we have confirmed these data[20]. In our study SS used as a single test or in combination with LS, accurately has ruled out the presence of esophageal varices (NPV of 100%) using a SS cut-off value < 48 kPa. Also the study by Sharma *et al*[76], using TE, found that HVPG was significantly correlated with SS (r = 0.433, P = 0.001) but not with LS (r = 0.178, P = 0.20) and using a cut-off value of 40.8 kPa the technique showed a sensitivity of 94%, a specificity of 76%, LR+ and LR- and PPV of 91%, NPV of 84% and a diagnostic accuracy of 89%: Furthermore, the authors found that SS was significantly higher in patients who had large varices (P = 0.001) and variceal bleeding (P = 0.001).

Later on, in order to better predict the degree of PH, Calvaruso *et al*[21] used a modified software version for TE, with a range between 1.5 and 150 kPa. As expected, patients with values of 75 kPa by standard TE had mean values of modified spleen TE of 117 kPa (range 81.7-149.5 kPa); linear regression revealed a significant correlation between modified spleen TE and esophageal varix size (r = 0.501; β: 0.763; SE 0.1444; P < 0.001). At multivariate analysis only modified SS and AST/ALT ratio resulted independently associated with grade 2/grade 3 EV and, compared to other non-invasive tools in diagnosis of the presence of large EV, modified SS performed better. The best cut-off value of modified SS for predicting the presence of large EV, identified on the ROC curve as the point that maximizes sensitivity and specificity, was 54 kPa (AUROC 0.82; NPV of 90%)[21]. Using modified spleen stiffness measurements, Stefanescu *et al*[22] reported similar results: a cut-off value of 75 kPa predicted the presence of large EV with an AUROC 0.90; NPV 100%. Despite their interesting results the authors stressed the concept that the correction of SS values using a calculation algorithm in the post-processing of each elastogram improves the SS measurement but better results would have to be obtained using a dedicated, modified probe and software installed on the Fibroscan device.

According to the results of the above mentioned studies, the diagnostic accuracy of spleen stiffness measurement to rule out the presence of PH was higher than that of liver stiffness and other non-invasive ultrasound signs. In particular in their study Colecchia *et al*[64] observed a better diagnostic accuracy of SS in diagnosing the presence of EV (AUROC 0.94) as compared to PSR (platelet spleen diameter ratio) (AUROC 0.86) (P = 0.05). For the detection of PH again SS showed a better accuracy in diagnosing HVPG >10 mmHg [AUROC 0.96 *vs* LSPS (LS × spleen diameter/platelet count) = 0.91 (P = 0.05) and *vs* PSR = 0.84 (P = 0.007)] and in diagnosing a HVPG > 12 mmHg [AUROC 0.96 *vs* LSPS = 0.90 (P = 0.048) and *vs* PSR = 0.82 (P = 0.003)]. Greater performance of SS compared to PSR and LSPS emerged also in the study by Sharma *et al*[76], especially in differentiating patients with large *vs* small varices. No US Doppler signs were considered in these studies among the non-invasive tests probably because they are highly specific to the diagnosis of cirrhosis and PH, but their sensitivity is relatively low, especially in compensated cirrhotic patients[65].

Singh *et al*[77] recently carried out a meta-analysis that evaluated the diagnostic accuracy of SS in predicting EV and included a total of 12 studies including 1497 patients with chronic liver disease. The analysis found a moderate SS accuracy in detecting the presence of EV (sensitivity 78%, specificity 76%; LR+ 3.4, LR- 0.2, AUROC = 0.86). Similarly, the diagnostic performance of SS in detecting the presence of clinically significant EV was moderate (sensitivity 81%, specificity 66%) even if the rate of false positive and negative results was far from optimal. The heterogeneity of the results among the studies can be related to either differences in the elastographic techniques or geographical differences among the studies (*e.g.,* Asian and European populations). No significant differences were observed in the diagnostic accuracy of SS measurements according to the etiology of chronic liver disease but the different techniques used prevent any possible conclusion.

Table 3 summarizes the main studies that assessed SS measurement as non-invasive test to predict the presence of PH and/or EV.

Finally, limited data is available to date about the reproducibility of the technique and the definition of its methodological standards thus further evidence is needed in this field.

In conclusion, the current techniques for SS determination, even if very promising, are still sub-optimal to replace upper endoscopy as the screening modality of choice to detect any presence of EV.

Recently the Faculty of Baveno VI recommended the determination of LS by TE plus platelet count to identify among patients with compensated advanced chronic liver disease those who can safely avoid screening endoscopy. According to this suggestion, the patients with a LS < 20 kPa and with a platelet count > 150000 have a very low risk of having varices requiring treatment and can avoid endoscopy (level of evidence 1b; A)[78]. Several studies are presently on going with the aim of corroborating these data.

Considering the recent finding, SS too should probably be included in these recommendations, especially in the presence of possible confounders of LS determination, when LS measurement is inaccurate or a complementary test. Again further evidence is needed in this area.

Furthermore, it is reasonable to think that composite scores or diagnostic algorithms involving both LS and SS can potentially improve diagnostic accuracy in the prediction of PH and consequentially can avoid the use of other invasive and more expensive examinations.

A few preliminary studies evaluated the correlation of SS and portal pressure before and after the placement of a transjugular intrahepatic portosystemic

Table 3 Primary studies assessing the role of spleen elastography for the detection of esophageal varices

Ref.	Technique	Cut-off	*n*	Prevalence	Sens	Spec	LR+	LR-	PPV	NPV	AUROC
Stefanescu *et al*[75], 2011 (prospective)	TE	46.4	174	85%	83.5	71.0	2.90	0.23	93.8	45.5	0.780
Hirooka *et al*[74], 2011 (prospective)	RT-SWE	8.24	60	43%	96.0	85.0	6.40	0.04	83.0	97.0	0.91 (0.873-0.99)
Bota *et al*[85], 2012 (prospective)	ARFI	2.55	140	43%	96.7	21.0	18.00	0.15	47.6	89.4	0.578
Colecchia *et al*[64], 2012 (prospective)	TE	55.00	100	53%	71.7	96.0	17.00	0.29	95.3	75.2	0.941 (0.90-0.98)
Vermehren *et al*[86], 2012 (prospective)	ARFI	4.13	166	36%	35.0	83.0	2.06	0.78	54.0	69.0	0.58 (0.49-0.67)
Calvaruso *et al*[21], 2013 (prospective)	TE	50.00	96	56%	65.0	61.0	1.70	0.60	69.0	57.0	0.701
Fraquelli *et al*[20], 2013 (prospective)	TE	48.00	26	42%	100.0	60.0	2.50	0.01	33.5	98.7	0.90 (0.79-1.00)
Sharma *et al*[76], 2013 (prospective)	TE	40.80	200	71%	94.0	76.0	3.90	0.08	91.0	84.0	0.89 (0.84-0.95)
Takuma *et al*[87], 2013 (prospective)	ARFI	3.18	340	38.8%	98.5	60.1	2.46	0.02	61.0	98.4	0.93 (0.90-0.96)
Takuma *et al*[88], 2016 (prospective)	ARFI	3.36	60	40%	95.4	77.8	4.31	0.05	74.2	96.6	0.93 (0.84-0.98)

ARFI: Acoustic radiation force impulse; AUROC: Area under receiver operative curves; LR+: Likelihood ratio positive; LR-: Likelihood ratio negative; RT 2D-SWE = Real-time two-dimensional shear-wave elastography; TE: Transient elastography; PPV: Positive predictive value; NPV: Negative predictive value.

shunt (TIPS). Gao *et al*[79] using pSWE showed a statistically significant difference in mean SS values *pre* and *post* TIPS placement ($P < 0.001$) while mean LS post-TIPS value did not significantly differ from those measured *pre* TIPS placement. Other studies obtained similar results[80,81] suggesting a potential role of spleen elastography to confirm shunt effectiveness and indicate successful portal vein pressure reduction after TIPS placement. While interesting, all these studies had a very small sample size (10-12 patients), SS was measured only over a short period (< 10 d) and inter and intra-observer variability were not taken into account. Furthermore, in a recent study Novelli *et al*[82] evidenced a mean SS values reduction in only 58% of the patients who underwent TIPS even if all patients had a reduction in the portosystemic gradient to less than 12 mmHg. In addition, the analysis showed no measurable correlation between SS and portal venous pressure before and after TIPS placement. Thus, further studies are warranted to determine the feasibility and the role of SS in the surveillance of TIPS function.

Finally, one can anticipate that interesting preliminary results would occur in the use of SS to assess changes in portal pressure after liver transplantation reflecting the resolution PH[83].

The role of spleen stiffness in the assessment of the complications of cirrhosis

Considering the recent data supporting the correlation between SS measurements and HVPG levels and considering that HVPG measurement represents the best predictor of clinical decompensation in cirrhotic patients, Colecchia *et al*[84] prospectively enrolled 92 compensated HCV-related cirrhosis, investigated with laboratory tests, spleen diameter, LS and SS (by TE), HVPG and EGD and followed up for 2 years. Patients on antiviral, beta blocker or diuretic treatment with previous clinical decompensation were excluded. At multivariate analysis the only two independent predictors of complications were SS and Model for End-Stage Liver Disease score (MELD): $P = 0.0001$, $P = 0.014$. The authors elaborated predictive models to detect patients with low risk of decompensation involving SS and MELD or only SS. Considering the simplified model including only SS, the patients with SS values lower than 54 kPa are at low risk (< 3%) of events at 2 years (Se 97%, LR- 0.05, NPV 97%). Also in their study the predictive accuracy of SS (alone or with MELD) measured by the *c* index does not seem to be higher than to that of HVPG but the presence of varices or other PH non-invasive tests and even HVPG could not provide any adjunctive information: 53.3% of patients had already small varices at their enrollment but the effect of SS on the primary outcome was independent from the presence of EV (interaction SS and EV $P = 0.232$).

Similar findings were observed also in the study by Radu *et al*[23] who used modified SS (by analysis of each elastogram to obtain an increment of the scale up to 150 kPa) performed on fifty-two compensated cirrhotic patients followed-up for above 13 mo. In their study the authors also calculated a decompensation prediction score involving modified SS, albumin and bilirubin, and found the best cut-off value for predicting liver decompensation (AUROC = 0.70, 95%CI: 0.53-0.85). In that paper a SS cut-off value was not clearly expressed and patient characteristics (diuretic,

beta-blocker, antiviral treatment) were not adequately detailed.

Despite these interesting results, those studies presented some limitations, for example a small sample size in Radu's study and the short follow-up period in both studies.

CONCLUSION

In the last few years, among several non-invasive tests proposed to better evaluate liver fibrosis and PH, elastographic techniques have gained an important role and have been firstly applied on the liver.

Currently, liver stiffness is an accepted and validated method to assess liver fibrosis stages and is a complementary test to exclude clinical PH. More recently, spleen stiffness has become particularly attractive as compared to liver stiffness: it appears to better represent the dynamic changes occurring in the advanced stages of liver cirrhosis and also its diagnostic performance in detecting esophageal varices is higher than that of liver stiffness.

For the assessment of liver fibrosis SS showed a good diagnostic accuracy especially when determined by TE and pSWE (AUROC 0.84-0.91). Up to now no meta-analysis is available to confirm these data but it would seem reasonable to use SS when LS is not reliable or its measurement is flawed.

Regarding the detection of PH many single studies have showed an optimal diagnostic accuracy of SS, particularly to rule out the presence of esophageal varices. Only one meta-analysis has been carried out to date, showing an adequate accuracy of SS measurement in detecting the presence of EV. Similarly, the diagnostic performance of SS for the detection of the presence of clinically significant EV (F3) is good enough even if it is not yet clear if this non-invasive tool can replace upper endoscopy to detect the presence of EV. Most importantly, there is a great heterogeneity among the current studies, such heterogeneity being owed to differences in the elastographic technique used and to geographical differences in the study setting.

Recent studies have showed promising yet preliminary results regarding the role of SS measurement in the prediction of cirrhosis-related complications, TIPS function and in the prediction of PH resolution after liver transplantation. These interesting findings should be confirmed by further larger prospective studies.

In conclusion, SS seems to be a very promising tool to be used in the work-up and follow-up of cirrhotic patients, In fact, in addition to the staging of PH, SS determination, alone or in association with other non-invasive markers, can predict the clinical outcome of liver cirrhosis and become a useful tool to stratify patients according to the different level of risk of disease progression and to select those patients requiring further investigations.

However, further studies are needed to better define the quality criteria and the diagnostic performance of spleen elastography techniques in clinical practice.

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REVIEW

Interaction of obesity and inflammatory bowel disease

Jason W Harper, Timothy L Zisman

Jason W Harper, Department of Gastroenterology, Swedish Medical Center, Seattle, WA 98195, United States

Timothy L Zisman, Inflammatory Bowel Disease Program, Division of Gastroenterology, University of Washington Medical Center, Seattle, WA 98195, United States

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Correspondence to: Timothy L Zisman, MD, MPH, Associate Professor of Medicine, Inflammatory Bowel Disease Program, Division of Gastroenterology, University of Washington Medical Center, 1959 NE Pacific St., Box 356424, Seattle, WA 98195, United States. tzisman@medicine.washington.edu
Telephone: +1-206-6857174
Fax: +1-206-6858684

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Abstract

Inflammatory bowel disease (IBD) is a chronic inflammatory condition of unknown etiology that is thought to result from a combination of genetic, immunologic and environmental factors. The incidence of IBD has been increasing in recent decades, especially in developing and developed nations, and this is hypothesized to be in part related to the change in dietary and lifestyle factors associated with modernization. The prevalence of obesity has risen in parallel with the rise in IBD, suggesting a possible shared environmental link between these two conditions. Studies have shown that obesity impacts disease development and response to therapy in patients with IBD and other autoimmune conditions. The observation that adipose tissue produces pro-inflammatory adipokines provides a potential mechanism for the observed epidemiologic links between obesity and IBD, and this has developed into an active area of investigative inquiry. Additionally, emerging evidence highlights a role for the intestinal microbiota in the development of both obesity and IBD, representing another potential mechanistic connection between the two conditions. In this review we discuss the epidemiology of obesity and IBD, possible pathophysiologic links, and the clinical impact of obesity on IBD disease course and implications for management.

Key words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Obesity; Body mass index

Core tip: Epidemiologic studies have shown a parallel rise in the prevalence of obesity and immune-mediated conditions, including inflammatory bowel disease (IBD). This association may be related to share dietary or environmental exposures that exert their effect through changes in the intestinal microbiota. Several lines of evidence demonstrate that obesity is a pro-inflammatory condition that impacts the incidence, disease course and response to therapy in patients with IBD. Exploring the mechanisms of interaction between obesity and IBD advances our understanding of IBD

and opens up a potential role for weight loss and weight maintenance strategies in the management of IBD.

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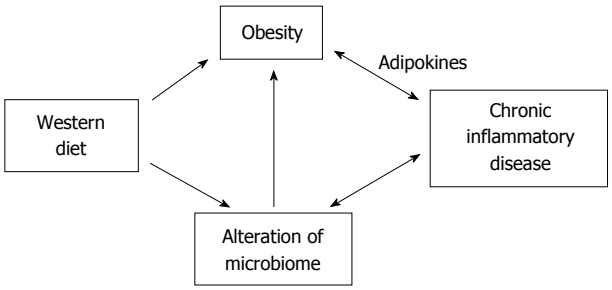

Figure 1 Proposed etiologic links between obesity and inflammatory bowel disease.

INTRODUCTION

Obesity - the chronic medical condition defined generally as excessive body fat mass leading to deleterious health outcomes - has emerged as one of the leading global public health issues of the 21st century. In the United States, according to data from the National Health and Nutrition Examination Survey (NHANES), approximately 30% of adults are obese, as defined by having a body mass index (BMI) greater than 30 kg/m^2[1]. World Health Organization estimates of the prevalence of overweight or obese individuals (BMI > 25 kg/m^2) approximate 35% of the global population, with increases over time demonstrated in both developed and developing nations[2]. The global cost of treating obesity and its resultant health complications may be as much as $2 trillion (US)[3]. Although obesity was once considered uncommon in IBD, the prevalence of obesity in IBD has risen in parallel with the general population. Several lines of evidence support a biologically plausible connection between adiposity and IBD (Figure 1). Understanding the interaction between obesity and IBD with regard to disease pathogenesis, phenotypic disease expression and response to therapy has important implications for management.

EPIDEMIOLOGY OF IBD AND OBESITY

Temporal trends have demonstrated an increase in IBD incidence across the globe over the latter half of the 20th century[4]. A number of environmental factors have been postulated to be associated with this, concordant with the long-noted observation that IBD is more prevalent in developed, as opposed to developing, nations. These disparate factors have included smoking (for Crohn's disease incidence)[5], improved levels of hygiene[6], alterations in the intestinal microbiome[7,8], and dietary changes associated with an industrialized lifestyle, including increased intake of linoleic acid, increased intake of animal fat/protein and decreased dietary fiber intake[9-11]. Given the rising incidence of both IBD and obesity - as well as the interplay between some of the aforementioned risk factors common to both conditions-an epidemiologic interaction between the two conditions has been postulated. To date there is a relative paucity of population-level studies reporting the prevalence of obesity among individuals with IBD, but reports from various IBD cohorts in both pediatric and adult populations would suggest that the current prevalence of overweight and obese IBD patients approximates that of the general population, being approximately 20%-30%[12-15]. Furthermore, when looking at the weight distribution of individuals recruited to participate in clinical trials for Crohn's disease, there has been a clear trend in increasing weight over the past two decades, from a minimum mean BMI of 20.8 kg/m^2 in 1992, to a maximum mean BMI of 27.0 in 2001, and a weakly positive linear trend (r^2 = 0.14) of increasing BMI among study participants throughout this period of time[16].

As to whether or not obesity - as reflected by the body mass index-is an intrinsic risk factor for the development of *de novo* IBD, a large prospective cohort (EPIC) of European adults demonstrated no such association, after adjustment for smoking status, physical activity and total caloric intake as assessed by food frequency questionnaire. Among those individuals with incident IBD in this cohort, BMI was not associated with phenotypic disease expression (Crohn's *vs* ulcerative colitis), nor disease location/extent[17]. An earlier retrospective cohort of incident IBD diagnoses in adults, compared to an older (age 50-70 years) non-IBD control cohort, suggested a higher rate of obesity at diagnosis in the IBD patients (OR = 2.0, P = 0.01)[18]. More recently published data from the Nurses' Health Study demonstrated a greater than two-fold increased risk of developing Crohn's disease among obese women; furthermore, an association was also observed between body morphology (as reflected by participants' waist-hip ratio as adolescents) and future development of Crohn's disease in this cohort. No such relationship between BMI or anthropometric measurements of obesity was seen with incident UC in this same study[19]. Another cohort of women recruited in Denmark also demonstrated an increased risk of Crohn's disease among obese individuals, with no such relationship seen with ulcerative colitis[20]. Perhaps accounting for the discrepancy between the data from the EPIC study and the Nurses' Health study is the fact that the EPIC cohort described above was much

Table 1 Clinical impact of obesity on inflammatory bowel disease

Medical therapy	Surgical therapy	Natural history and disease complications
Decreased clinical response to azathioprine[77]	Earlier time to 1st surgery[15]	Conflicting data:
Lower 6-thioguanine levels on treatment with azathioprine[78]	Higher rate of perioperative complications[112,114,119-121]	Higher prevalence of perianal disease[22]
Decrease likelihood of response to adalimumab[83]	Increased need for conversion from laparoscopic to open surgery[113,118]	Lower prevalence of penetrating disease[24]
Shorter time to loss of response to infliximab[92]		Increased hospitalization[22]
		Decreased IBD-related quality of life[23]
		Decreased healthcare utilization[14]

IBD: Inflammatory bowel disease.

older (median age 52) compared to the American and Danish cohorts (median age 35 and 30 respectively), and of course, the American and Danish cohorts were entirely female, as compared to a roughly equal sex distribution in the EPIC cohort. If there is in fact an increased risk of developing IBD intrinsic to obesity, this risk may interact with age and sex. Adding to the biologic plausibility of an etiologic link between adiposity and IBD, other studies have demonstrated that obesity is associated with an increased risk of developing other autoimmune conditions such as rheumatoid arthritis and psoriasis, that share similar genetic and immunologic mechanisms with IBD[21,22].

RELATIONSHIP BETWEEN OBESITY AND IBD OUTCOMES

There are mixed data regarding the impact of obesity on IBD-related health outcomes (Table 1). Co-morbid obesity has been linked to an earlier time to first surgery among patients with Crohn's disease in one retrospective registry, with no difference noted in the overall number of surgeries or the need to escalate medical therapy over time between groups[15]. In a retrospective study of French IBD patients, obese individuals with Crohn's disease were suggested to have a higher rate of perianal disease, and a more frequent need for hospitalization[23]. A more recent retrospective cohort of IBD patients, approximately 40% of whom had UC, showed no association between BMI and need for surgery, hospitalization or medication escalation across BMI categories, after adjustment for obesity-related health conditions such as hypertension or diabetes. However, increased BMI was associated with more subtle indicators of active disease, including increased C-reactive protein levels (which was controlled for in this analysis in regard to IBD outcomes) as well as a significant decrement in IBD-related quality of life measures[24]. Another recent study of a mixed IBD (CD + UC) population showed conflicting results with decreased rates of surgery or hospitalization, as well as decreased use of anti-TNF biologic therapy, among obese IBD patients[14]. In this study, however, underweight patients were grouped together with normal weight patients for the analysis, which may have obscured the association between obese and normal weight patients by including sicker patients in the comparator group. Data from a prospective registry of Crohn's patients showed a lower rate of penetrating disease activity among obese individuals with Crohn's disease, after adjustment for factors such as smoking, age, baseline inflammatory activity and genetic risk profile; this same study showed no major difference in the rate of perianal disease or surgery among obese *vs* non-obese Crohn's patients[25]. Among patients with ulcerative colitis, retrospective cohort data has been published suggesting that overweight (BMI > 25 kg/m^2) patients may have a less complicated clinical course than normal-weight or underweight (BMI 18-24 kg/m^2 and < 18 kg/m^2 respectively) patients[26]. In a prospectively recruited cohort of IBD patients in Ireland, obese patients with Crohn's disease were on average found to be older, less physically active, to have lower CDAI scores and higher C-reactive protein levels than their non-obese counterparts. Smoking status, age at diagnosis, need for surgery and corticosteroid use did not differ between groups[27].

Special mention should be made about the potential interaction between diabetes and inflammatory bowel disease independent of obesity, given the strong and well-described association between obesity and type 2 diabetes. Individuals with IBD (UC specifically) may be at higher risk of developing *de novo* diabetes according to a large UK cohort, even after adjustment for concurrent glucocorticoid usage and baseline BMI[28]. Co-morbid diabetes among IBD patients has been associated with higher rates of infection on immunomodulatory therapy, hospitalization and need for surgery over time[29-31].

All of the aforementioned studies have used body mass index as the surrogate marker of adiposity, and as yet, we have much less data about the interaction between other markers of obesity, such as volumetric analysis of visceral fat, and IBD outcomes. One small retrospective study of individuals with Crohn's disease demonstrated that individuals with higher relative amounts of mesenteric fat, as defined by the ratio between their visceral and subcutaneous fat area on cross-sectional imaging, had significantly higher

Table 2 Immunologic effect of adipokines

Adipokine	Levels in obesity	Immunologic effects	Overall effect
Adiponectin	Decreased	Decreases TNF-α, IFNγ, IL-6 Inhibits VCAM-1, ICAM-1 expression Increases IL-10, IL-1RA Promotes proliferation of Tregs Antagonizes NF-κB pathway	Anti-inflammatory
Leptin	Increased	Increased activation and proliferation of monocytes and macrophages Increased IL-6, TNF-α, IL-12 Activates NK cells Activates NF-κB pathway Promotes Th1-differentiation Inhibits proliferation of Tregs	Pro-inflammatory
Resistin	Increased	Increases IL-6, IL-12, TNF-α	Pro-inflammatory

TNF-α: Tumor necrosis factor-α; NF-κB: Nuclear factor-κB; IFNγ: Interferon γ.

rates of fibro-stenotic or penetrating disease behavior. This observation differs from the aforementioned finding of less penetrating disease among obese IBD patients when solely BMI is used to define obesity[32]. Prospective data are lacking with regard to the potential interaction between obesity and IBD complications that are independently associated with both conditions, such as venous thromboembolism; cholelithiasis and hepatic steatosis; cardiovascular disease; and, perhaps most importantly, colorectal cancer[33-36]. Also confounding the relationship between obesity and IBD outcomes is the observation that increased visceral adiposity may be associated with a higher risk of irritable bowel syndrome[37]. This interaction could confound the interpretation of studies that suggest that obese IBD patients have a less complicated clinical course, if some of their symptoms over time are in fact accounted for by a higher prevalence of co-morbid functional bowel disease, which should be associated with less need for medication escalation or surgery.

DEFINITION OF OBESITY

One of the ongoing challenges in trying to elucidate the interaction between obesity and health outcomes has been the lack of consensus regarding a standardized definition of obesity. For its ease of use, the body mass index (BMI), a simple adjusted ratio of weight to height, has been used as the operational definition of obesity when it exceeds 30 kg/m^2. As an isolated measure, the BMI does predict broad health outcomes such as all-cause mortality at higher categories of obesity (such as BMI > 35 or 40 kg/m^2), but performs poorly (and may indicate lower all-cause mortality) when it is considered among individuals presently defined as "overweight" (*i.e.*, BMI > 25 kg/m^2)[38,39]. Multiple studies have demonstrated that various anthropometric measurements of obesity - such as the waist-to-hip circumference ratio or volumetric analysis of abdominal fat *via* cross-sectional imaging-perform better than BMI as predictors of adverse health outcomes[40-42]. A glaring deficit in the current literature regarding IBD and obesity has been the general reliance on the BMI as the sole marker of obesity, and lack of validation of the standard BMI categories of overweight or obesity as clinically relevant cutoffs in IBD. As addressed in this review in later sections, it may be that adipose distribution is more clinically significant than overall body size. As such, incorporation of multiple domains of obesity to reflect the differences between health outcomes and body fat distribution, would help elucidate the interaction of obesity and IBD by minimizing the confounding effect of individuals with elevated lean body mass who happen to have BMI values that may place them in the operational categories of "overweight" or even obese, or whose adipose distribution is predominantly intra-abdominal, *vs* peripheral, fat.

OBESITY AS A PRO-INFLAMMATORY STATE

Far from being an inert repository of excess calories, adipose tissue is responsible for the secretion of a number of pro- and anti-inflammatory cytokines, some of which are unique to this tissue type and which have been labeled adipokines (Table 2). A full discussion of the complex relationship between adipokines and autoimmune conditions including IBD is beyond the scope of this discussion, but some intriguing points are worth reviewing.

Adiponectin is a 30 kD protein produced almost exclusively by adipocytes and has complex interactions with multiple inflammatory pathways; interestingly, it shares some degree of structural homology with tumor necrosis factor-alpha (TNF-α), a cytokine intrinsically involved in the pathogenesis of IBD[43]. Adiponectin appears to exert anti-inflammatory effects, with evidence of antagonism of pathways that TNF-α promotes, such as the nuclear factor-κB pathway[44]. Interestingly, increased expression of adiponectin

has been found in the so-called "creeping fat" of patients with active Crohn's disease when compared to mesenteric fat from patients with UC and with colonic adenocarcinoma; however, adiponectin concentrations were found to be lower in the mesenteric fat of CD patients with internal (*e.g.,* entero-enteral) fistulas compared to individuals with non-fistulizing disease[45]. In one study, circulating adiponectin levels appear to be increased among UC patients as compared to both CD patients and healthy controls[46]. Another study demonstrated reduced circulating levels of adiponectin in individuals with both active and inactive IBD compared to healthy controls; curiously, this same study demonstrated that hyperinsulinemia in the absence of hyperglycemia was independently associated with a higher likelihood of clinical remission in IBD patients (in tandem with decreased circulating adiponectin levels)[47]. Circulating adiponectin levels in patients with IBD treated with infliximab do not seem to be affected pre- and post-treatment[48].

Leptin was one of the originally identified adipokines, and appears to have a number of pro-inflammatory effects; it is also one of many adipokines that plays a role in appetite modulation and energy homeostasis. Increased serum leptin levels are directly associated with total body fat mass, and leptin in general has a pro-satiety effect on appetite centers in the hypothalamus[49]. Leptin appears to increase the secretion of pro-inflammatory cytokines, such as IL-1, IL-6 and TNF-α, and synergistically, pro-inflammatory cytokines promote leptin expression in inflamed tissue[50,51]. Several studies have demonstrated lower serum leptin levels, when adjusted for body weight, in patients with IBD compared to healthy controls, in both pediatric and adult populations[46,52]. Treatment with infliximab in patients with IBD does not appear to modify circulating serum leptin levels[48].

Resistin is another adipokine with complex relationships with inflammatory pathways: in humans, it is produced by adipocytes as well as by cells in the reticuloendothelial system (*e.g.,* splenic monocytes/macrophages). As with leptin, studies have demonstrated that resistin stimulates the production of pro-inflammatory cytokines such as TNF-α and IL-12, and that in turn, pro-inflammatory cytokines appear to induce resistin expression[53,54]. Multiple studies have demonstrated elevated serum resistin levels in patients with IBD (both UC and CD)[38,40,55]. Unlike with adiponectin or leptin, serum resistin levels are seen to decrease in humans with IBD treated with infliximab[42,56].

Mesenteric fat itself, along with producing both pro- and anti-inflammatory adipokines, is also a source of multiple pro-inflammatory cytokines that are integral to the inflammatory cascade involved in IBD, such as TNF-α and IL-6. In obese individuals, visceral adiposity as assessed by volumetric cross-sectional imaging analysis directly correlates with circulating IL-6 levels, and BMI is associated with C-reactive protein levels[57]. The "creeping fat" associated with Crohn's disease has received increasing attention as an intrinsic component of the inflammatory dysregulation that predisposes to transmural inflammation, rather than an epiphenomenon coincidental to this process. For instance, TNF-α gene expression can be demonstrated in the mesenteric fat of patients with active Crohn's disease, whereas this expression is not demonstrated in healthy controls[58]. Pre-adipocytes isolated from mesenteric fat from individuals with IBD also express IL-17 upon binding of substance P to the neurokinin-1 receptor, as compared to healthy controls[59]. Mesenteric adipose tissue is also a source of circulating C-reactive protein in patients with Crohn's disease[60,61]. Furthermore, when compared to healthy controls, relative to overall body size, individuals with Crohn's disease may have a predilection for accumulation of intra-abdominal fat relative to subcutaneous fat, seen in association with reduced serum growth hormone levels[62]. In a small randomized clinical trial of pediatric patients with Crohn's disease on corticosteroids, supplemental growth hormone was associated with improved PCDAI scores and quality of life indices, with an apparent corticosteroid-sparing effect as well, but with no significant effect noted on rates of mucosal healing[63]. Another small trial looking at adult patients with Crohn's disease suggested clinical benefit as measured solely by the CDAI in individuals receiving supplemental growth hormone, as compared to individuals receiving customary care[64]. To date, no studies have addressed the question as to whether changes in mesenteric fat distribution over time- or with targeted interventions designed to reduce mesenteric fat accumulation-lead to improved clinical outcomes in patients with IBD.

OBESITY AND THE MICROBIOME

The importance of the human fecal microbiome has come to the forefront in multiple fields of medicine, with IBD being a particularly robust demonstration of the link between the intestinal bacterial (and fungal/viral) population, and human health. More recent attention has been paid to the possible links between the microbiome and human obesity. The gut bacteria are capable of salvaging undigested foods to sources of calories usable by their human hosts, and the permeability of the gut mucosa to bacterial products of metabolism has been associated with multiple chronic medical conditions. Using pairs of mono- and dizygotic twins, it has been shown that obese *vs* lean individuals vary according to the diversity of their bacterial populations, with enrichment in the obese individuals of bacterial gene products associated with metabolism of carbohydrates[65]. Another study including a large number of twins demonstrated that host genetic profiles seem to interact with the microbiome, leading to the selection of a more "obesogenic" bacteriologic profile; identification of obesity-attenuating bacterial species, and colonization of germ-free mice with

those bacteria then leads to attenuated weight gain in those same mice[66]. Individuals with reduced colonic bacterial diversity-which is a state associated with IBD in general - also seem to be predisposed to obesity[67,68]. Furthermore, specific bacteria shown to exert an anti-inflammatory effect on the gut-such as *Faecalibacterium prausnitzii*-have been shown to be lacking in obese individuals without IBD[65,69].

While the specific association between mesenteric fat distribution and IBD at the level of the microbiota remains largely undescribed, it is intriguing to postulate that many of the epidemiologic associations with IBD such as diet and exposure to antibiotics could be modulated in part through interactions on the gut microbiome. This area represents a specific deficiency in our understanding of the pathogenesis of IBD, and is a ripe area for future investigation.

OBESITY AND IBD PHARMACOLOGY

Despite the increasing prevalence of obesity in IBD, and the seemingly logical differences in pharmacokinetics that may be expected in obese *vs* non-obese individuals, the influence of obesity on drug absorption, distribution, metabolism and excretion remain poorly understood, and discussion of the pharmacokinetic effects of obesity is beyond the scope of this review.

If there are inter-individual differences in drug effect that are impacted by obesity, pharmacodynamic differences likely account for the majority of these, perhaps by virtue of some of the pathophysiologic changes seen at the level of pro- and anti-inflammatory pathways described earlier that would interact with IBD-specific drugs. In fact, when considered by class, there are multiple studies that suggest that obesity does influence the efficacy of specific drugs commonly used to treat IBD. The following paragraphs will review this data by drug class.

A retrospective study of individuals with UC and CD showed a differential response to azathioprine administration and discontinuation according to BMI: individuals with UC whose BMI was > 25 kg/m^2 at initiation had a higher likelihood of exacerbation (as defined by initiation or increase in corticosteroid dosage over time) compared to individuals whose BMI was in the normal range. Among patients with CD in this study, after discontinuation of azathioprine, individuals whose BMI was > 25 kg/m^2 had a lower rate of subsequent flare than their counterparts whose BMI was in the normal range[70]. A more recently published retrospective study demonstrated that obese individuals had mean 6-thioguanine (6-TG) levels that were significantly lower than their non-obese counterparts, when adjusted for the total dose of azathiopurine or mercaptopurine they were receiving relative to total body weight. The rate of sub-therapeutic 6-TGN levels (as defined according to standard parameters) in individuals whose BMI was > 25 kg/m^2 or > 30 kg/m^2 was nearly two-fold higher than normal BMI individuals, with only about 30%-40% having levels in the therapeutic or supra-therapeutic range[71].

To date, there have been no studies addressing whether or not obesity influences the efficacy of methotrexate in treating IBD or in other similar autoimmune conditions, but there have been multiple studies that have shown that obesity and obesity-related comorbidities increase the likelihood of developing derangement in hepatic transaminases on chronic low dose methotrexate therapy in individuals with rheumatoid arthritis, sometimes requiring discontinuation of the medication[72,73]. Co-morbid diabetes has been associated with accelerated histologic fibrosis in individuals with psoriasis treated with long-term low dose methotrexate[74,75].

The effects of obesity on the response to anti-TNF therapy have also been investigated, with several studies in both the IBD and non-IBD populations that use these medications suggesting a possible interaction, although this has not been universally demonstrated. Among the subcutaneously-dosed anti-TNF agents, retrospective data have suggested that, along with prior non-response to infliximab and after adjustment for multiple co-variates, BMI is a negative predictor of response to adalimumab at initiation[76]. This has been confirmed by another retrospective analysis, showing a roughly two-fold increase in loss of response to adalimumab among individuals whose BMI is > 30 kg/m^2 compared to normal weight individuals[77]. In a post-hoc analysis of CLASSIC-II data, individuals whose BMI was less than 29 kg/m^2 were found to have higher rates of remission at week 56 than individuals whose BMI was greater than that[78]. BMI has also been demonstrated to be a negative predictor (albeit not a particularly strong one) of adalimumab trough levels at 28 wk into therapy for individuals with Crohn's disease[79]. Improved response to adalimumab in patients with psoriasis has also been demonstrated when stratified by BMI, with a roughly 20% decrease in partial response rates seen among individuals whose BMI > 30 kg/m^2 compared to their non-obese counterparts[80]. There are limited data to suggest an interaction between BMI and the other available subcutaneous anti-TNF therapies for IBD: in the PURSUIT-M trial, the relative effect of golimumab in maintaining response/remission did not appear to differ by body weight (BMI was not reported per-patient), and no interaction between BMI and maintenance response or remission rates were reported for certolizumab in the PRECISE-2 trial[81,82]. A trial of weight-based intravenous golimumab induction dosing was halted for lack of efficacy: at all weight-based doses selected, mean trough golimumab concentrations were lower than corresponding subcutaneous dosing regimens, however, limiting any inferences that could be made about a weight-based approach to dosing of this particular agent[83].

The relationship between BMI and infliximab - to date the only anti-TNF agent whose dosing is adjusted based upon total body weight - has been evaluated among both IBD and non-IBD populations. From a pharmacokinetic standpoint, infliximab primarily distributes in the vascular space, and concordant with this observation, the apparent volume of distribution of infliximab in patients with ulcerative colitis (per data from the ACT 1 and 2 trials) does increase in proportion to body weight (not BMI, as height data was not reported for those studies). This observation could explain the need for increased absolute doses given to individuals of higher body weight to maintain therapeutic drug concentrations[84]. In one of the aforementioned studies showing an attenuation of response to adalimumab over time in obese individuals, no such effect was seen with infliximab according to BMI strata[74]. Contrary to that finding, we published a retrospective analysis of individuals naïve to infliximab therapy that demonstrated a nearly three-fold increase in loss of response to infliximab - as primarily reflected by a need for dose escalation-among obese patients with Crohn's disease compared to their non-obese counterparts, after adjustment for co-variates such as smoking and corticosteroid usage at initiation of biologic therapy. A similar relationship was suggested for patients with UC, but due to small sample numbers, the effect size magnitude was likely over-stated, and the observation did not meet statistical significance. Moreover, changes in absolute weight/BMI over time (either increases or decreases) also correlated with loss of response to IFX over time in this same analysis[85]. Infliximab trough levels were not available for the majority of individuals in this study; interestingly, a more recently published study demonstrated that BMI was not associated with lower trough levels of infliximab or adalimumab (although there was a trend toward lower trough adalimumab levels in obese patients), suggesting any differential response among obese patients on anti-TNF therapy would be a pharmacodynamic, as opposed to pharmacokinetic, interaction[86]. Impaired responses to infliximab among obese patients have also been demonstrated in individuals with rheumatoid arthritis, psoriasis and with inflammatory spondyloarthropathy[87-89]. Furthermore, obese patients with rheumatoid arthritis may respond less well to a second-line anti-TNF agent than their non-obese counterparts; no such comparable data yet exist for IBD patients[90].

In individuals with psoriasis, rheumatoid arthritis and inflammatory spondyloarthropathy, weight gain is fairly consistently observed after initiation of anti-TNF therapy, driven primarily by gains in fat mass as opposed to lean body mass when this has been analyzed[91-94]. This should not be surprising, given that TNF-α is a potent anorexigen with multiple central- and peripheral effects on weight management and caloric intake/expenditure[95]. In one small open label study of infliximab in Japanese patients with Crohn's disease - all of whom were normal or underweight at enrollment according to standard BMI criteria - a mean BMI increase of about 1 kg/m^2 was seen over the course of treatment, and the magnitude of the increase in BMI was directly correlated with the likelihood of clinical response and remission[96]. Among children with IBD, early use of anti-TNF therapy has been shown to lead to more significant catch-up growth than the use of other classes of therapy[97]. While gains in weight among under- or normal-weight individuals would be a desirable outcome in individuals with IBD, we have no information about the effect of anti-TNF therapy among already overweight or obese IBD patients.

No data are available about the effects of obesity on the efficacy of anti-integrin therapies in IBD. The major IBD clinical trials of natalizumab and vedolizumab did not stratify response rates according to body weight[98-100]. In patients with multiple sclerosis, natalizumab levels have been demonstrated to be lower in individuals of larger body weight[101]. In the vedolizumab trials for both UC and CD, there was a definite dose-response relationship observed with vedolizumab trough levels and likelihood of response and remission in the induction phase; a similar dose-response relationship was not demonstrated in the maintenance phases. Further research is necessary to determine whether individuals with sub-optimal early response to anti-integrin therapy-perhaps driven in part by sub-optimal drug levels as a function of differences in body size and composition-would benefit from a weight-based induction dosing schedule.

Finally, special mention should be made concerning the glucocorticoids (GCs) and obesity. Although the pharmacologic use of glucocorticoids is often blamed for unintended weight gain, the true obesogenic effect of glucocorticoid therapy is difficult to determine, due to substantial heterogeneity in the studies that have objectively assessed this question. The studies vary in terms of the indication for GC therapy, the duration and dose of therapy, and the definition of obesity (*e.g.,* changes in body weight or anthropometric changes) as an outcome measure. Looking at multiple short- and long-term (defined generally as < 8 wk *vs* > 8 wk of continuous use) studies, variable effects of GC use on caloric intake, energy expenditure, changes in body weight and changes in fat- and fat-free mass have been demonstrated[102]. Subjectively, weight gain is reported in about 70% of individuals on chronic GC therapy, and is the most frequent self-reported complication of chronic GC therapy among patients[103]. To the extent that they exist, the short term metabolic effects of GCs may be mitigated by the use of GCs with high first pass metabolism: over 8 wk of use, the mean weight gain among patients on once daily budesonide at a dose of 9 mg was 1 kg compared to 2 kg mean weight gain among individuals on 40 mg prednisolone. The rate of developing abnormal fat distribution as reflected by a Cushingoid facial appearance was also about three-fold higher among prednisolone-treated

patients compared to patients treated with ileal-release budesonide[104]. Whether obesity alters the efficacy or risks of GC therapy remains largely unknown; furthermore, whether accumulation of intra-abdominal fat in IBD patients on chronic GC therapy would lead to worsened long-term outcomes is also unknown, but would be an intriguing question to investigate.

OBESITY AND IBD-RELATED SURGERY

Multiple retrospective studies have demonstrated a higher rate of post-operative morbidity in obese patients undergoing abdominal and non-abdominal operations, particularly driven by a higher rate of post-operative infectious complications. Post-operative mortality, however, does not appear to be significantly affected by obesity after adjustment for co-morbid diseases[105]. Obesity has also been shown to be a risk factor for conversion from laparoscopic to open surgery in patients undergoing colorectal surgery in general[106].

Specific to the IBD population, multiple lines of evidence suggest that obesity may negatively influence surgical outcomes, specifically when obesity is defined according to volumetric analysis of fat distribution, rather than solely BMI. When looking solely at BMI categories to define obesity, a large retrospective surgical registry demonstrated a 10% higher rate of post-operative morbidity among obese Crohn's patients, driven by a nearly two-fold higher rate of post-operative infection (nearly seven-fold higher in individuals whose BMI was greater than 40 kg/m^2)[107]. This same registry also demonstrated similar findings for obese individuals-not specific to the IBD population-undergoing laparoscopic colectomy[108]. Two smaller single-center retrospective series did not demonstrate a higher risk of peri-operative morbidity among obese Crohn's patients when stratified by BMI criteria[109,110]. Another single center study using BMI strata demonstrated longer operative times, blood loss and conversion from laparoscopic to open surgery among obese individuals with IBD, with no significant differences in overall post-operative morbidity and mortality[111].

Several more recently published retrospective studies described a higher rate of post-operative complications among obese Crohn's patients when obesity was defined by volumetric analysis, and in some cases, specifically noted that BMI stratification did not predict post-operative outcomes when morphologic assessment of fat distribution was taken into account[112-114]. Looking solely at visceral fat area as defined by cross-sectional imaging, one study has also demonstrated a higher rate of endoscopic disease recurrence in individuals with Crohn's disease with higher amounts of visceral/mesenteric fat at 6 mo post-operatively[115].

To date, although benefits have been seen in nutritional optimization of malnourished IBD patients before elective surgery[116], there are no studies addressing the role of weight loss in obese IBD patients in advance of elective non-bariatric surgery. Until such data become available, it would be premature to recommend any such approach given the nutritional stresses that occur after major abdominal surgery, and given that the most common metric to define obesity (*i.e.,* BMI) does not reliably differentiate lean body mass from fat mass, and that losses in the former would not be desirable before or after major IBD-related surgery.

MEDICAL AND SURGICAL WEIGHT LOSS AND IBD

Although multiple studies have investigated the interaction of diet with IBD at the level of relative macronutrient content (*e.g.,* high fat *vs* low fat diets), to date, no data exist regarding the effects of overall caloric intake or supervised dietary weight loss on outcomes in IBD patients[117]. Epidemiologic studies have suggested that regular physical activity may exert a protective effect on the development of IBD, although the confounding effects of decreased physical activity as a result of as-yet undiagnosed inflammatory bowel disease could confound this observation. Non-controlled studies, as well as a single randomized controlled trial, have demonstrated that physical activity generally leads to improved health-related quality of life in IBD patients, with no significant changes noted in markers of inflammation in the few studies that specifically report those measures pre- and post-intervention[118,119]. To date, no prospective or retrospective data are available to address the question of whether or not targeted medical weight loss in obese patients with IBD affects clinical outcomes or response to therapy. In patients with chronic plaque psoriasis-a condition in which an association with obesity is consistently demonstrated-multiple randomized controlled trials have shown a beneficial effect of medical weight loss in obese individuals. This effect has been specifically demonstrated among individuals on cyclosporine and biologic (*e.g.,* anti-TNF and anti-IL 12/23) therapy[120-123].

For patients with morbid obesity (*e.g.,* BMI > 40 kg/m^2), particularly in those who have significant co-morbidities such as diabetes, bariatric surgery has been shown to lead to reductions in all-cause mortality, and is more effective than routine medical care in the treatment of obesity-related complications such as diabetes[124-126]. There is a marked paucity of data in the literature about the safety or efficacy of bariatric surgery among patients with IBD patients. Due to concerns about the potential for increased complications among patients with Crohn's disease undergoing any elective form of bowel surgery, IBD has been considered a relative contraindication to bariatric surgery. However, small case series-each containing fewer than 10 patients - have been

published suggesting that weight loss surgery in carefully selected patients with IBD is technically feasible and theoretically safe[127-130]. In these limited series, the weight loss achieved post-surgery was comparable to what would be expected for non-IBD patients undergoing these operations, and in some cases, improvements were also noted in the underlying inflammatory bowel disease post-operatively. Case reports have described *de novo* IBD diagnosed after bariatric surgery, but the significance of this remains unclear given the extremely small number of such cases reported[131-134].

CONCLUSION

The prevalence of both obesity and IBD are increasing worldwide, and several lines of evidence suggest that these conditions may be linked through shared environmental risk factors, and mediated through alterations in the intestinal microbiome. Adipose tissue represents a metabolically and hormonally active organ, producing adipokines that exert a pro-inflammatory effect that drives disease activity in patients with immune-mediated diseases, including IBD. Studies reporting the influence of obesity on IBD disease course and response to medical therapy have described a mixed but largely detrimental impact. Lack of a standard definition of obesity hampers interpretation of the current literature, and establishing a clinically relevant measure of adiposity is essential to advancing our understating of the interplay between obesity and IBD. Future studies are required to establish whether weight loss, either medical or surgical, is safe or effective in IBD patients and whether this may have a beneficial impact on IBD or general health outcomes. Additionally, future investigations should address the value of individualizing drug dosing in IBD patients based on BMI or other measures of adiposity, but such an approach would be premature currently.

In summary, obesity has emerged as yet another important piece in the intricate puzzle of autoimmune diseases, such as IBD. Future studies that advance our understanding of the complex interactions between IBD and obesity are required to optimize patients' health outcomes.

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REVIEW

Biomarkers for colitis-associated colorectal cancer

Ru Chen, Lisa A Lai, Teresa A Brentnall, Sheng Pan

Ru Chen, Lisa A Lai, Teresa A Brentnall, Sheng Pan, Department of Medicine, University of Washington, Seattle, WA 98195, United States

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Correspondence to: Ru Chen, PhD, Department of Medicine, University of Washington, 1959 NE Pacific St, Seattle, WA 98195, United States. ruc@medicine.washington.edu
Telephone: +1-206-2214109
Fax: +1-206-6859478

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Abstract

Patients with extensive ulcerative colitis (UC) of more than eight years duration have an increased risk of colorectal cancer. Molecular biomarkers for dysplasia and cancer could have a great clinical value in managing cancer risk in these UC patients. Using a wide range of molecular techniques - including cutting-edge OMICS technologies - recent studies have identified clinically relevant biomarker candidates from a variety of biosamples, including colonic biopsies, blood, stool, and urine. While the challenge remains to validate these candidate biomarkers in multi-center studies and with larger patient cohorts, it is certain that accurate biomarkers of colitis-associated neoplasia would improve clinical management of neoplastic risk in UC patients. This review highlights the ongoing avenues of research in biomarker development for colitis-associated colorectal cancer.

Key words: Biomarker; Colitis; Dysplasia; Colorectal cancer; Surveillance; Progressor; Non-progressor

Core tip: With the incidence of ulcerative colitis on the rise, there is a need to develop clinically useful biomarkers capable of identifying and monitoring the subset of ulcerative colitis (UC) patients at highest risk for developing colon cancer. Recent studies have reported the efforts in developing clinically relevant biomarkers, laying a foundation for further clinical biomarker development. While the challenge remains to validate these candidate biomarkers in multi-center studies using larger patient cohorts, it is certain that accurate biomarkers of colitis-associated neoplasia would improve current clinical management of neoplastic risk in UC patients.

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INTRODUCTION

Ulcerative colitis (UC) is an inflammatory bowel disease, characterized by chronic, recurrent inflammation of the

colon. Patients with extensive UC of more than eight years duration have an increased risk of colorectal cancer. These patients are usually advised to undergo lifelong colonoscopic surveillance to detect the presence of dysplasia (pre-cancer) or cancer in the colon. Colitis-associated cancer (CAC) is distinct from sporadic colorectal cancer, both in disease mechanism and presentation. Progression in UC is usually dependent upon several factors. For patients with disease duration in excess of 8-10 years, the risk of developing cancer increases at a rate of 0.5%-1% per year. For example, a patient with 20 years of UC will have a neoplastic risk of approximately 10%-20%[1,2]. While the patient's age at disease onset factors into cancer risk[3], disease duration has a greater impact on cancer risk than chronological age. In addition, risk for cancer development increases with severity of inflammation[4] and extent of disease[5]. Patients with pancolitis are at higher risk compared to those with distal disease.

While current colonoscopic surveillance programs are cost-effective and able to reduce cancer incidence and mortality rate in UC patients[2,6], current methods of surveillance are expensive and invasive. Moreover, pathologic evaluation of colitis-associated neoplasia is subjective; even experienced gastrointestinal pathologists who developed the histologic standards for UC neoplasia could only agree on the diagnosis of indefinite for dysplasia half of the time[7]. Clearly, an objective molecular biomarker for dysplasia and cancer would have great clinical value in the management of cancer risk in UC patients.

NEOPLASTIC PROGRESSION IN UC

In UC, the colon epithelium undergoes repeated cycles of inflammation and tissue repair, resulting in oxidative stress and accumulation of reactive oxidative species (ROS)[8-11]. Excessive ROS causes oxidative stress and damage to DNA, proteins and lipids, leading to tumor initiation. Colitis-associated colorectal cancer progresses in a step-wise fashion from negative for dysplasia (NEG) → indefinite for dysplasia (IND) → low-grade dysplasia (LGD) → high-grade dysplasia (HGD) → cancer[12]. Neoplasia in UC is facilitated by several molecular alterations, some of which are detectable prior to dysplasia. These alterations include: telomere shortening, chromosomal and microsatellite instability, aneuploidy, and loss of p53[13-21]. In fact, alterations to the p53 tumor suppressor gene are important early events and occur in 47%-85% of colitis-associated cancers[22,23]. The second mechanism by which inflammation could contribute to carcinogenesis is through the release of proinflammatory cytokines[24-26]. Cytokines released from chronic inflammation participate in all stages of cancer development, including tumor initiation, tumor promotion, angiogenesis and metastasis. NF-κB is thought to be a key molecular link between inflammation and carcinogenesis[23]. When activated, it upregulates the expression of many pro-inflammatory mediators, such as adhesion molecules, cytokines, TNF-α and IL-6, which play a critical role in tumor development and progression of colitis-associated cancer[23].

The molecular alterations which occur in the pathogenesis of CAC are distinct from those in sporadic colon carcinogenesis. For example, the loss of APC occurs early in development of sporadic colorectal cancer, whereas it is usually a late event in UC associated disease progression if it occurs at all. In addition, p53 mutations appear as an early event in CAC, even prior to dysplasia yet p53 mutations are a late event in the sporadic disease. This evidence suggests that there are some unique pathways associated with progression of colitis-associated cancer. Studies show that the non-dysplastic mucosa is genomically abnormal in UC patients who have neoplasia elsewhere in their colon (Progressors)[15,27], *i.e.,* there is a colon-wide field defect in UC Progressors. This field defect involves the abnormal expression of proteins, genomic instability in repetitive DNA, and mitochondrial dysfunction in both the non-dysplastic and dysplastic mucosa from UC Progressors[15,16,28-30]. Such molecular changes tend to be absent in UC patients who are dysplasia-free (UC Non-progressors). These molecular alterations present in UC Progressors provide targets for the development of biomarkers for diagnostics and therapeutic applications in CAC.

CURRENT CLINICAL SURVEILLANCE OF CHRONIC UC PATIENTS

The current standard of care for surveillance of chronic UC patients involves colonoscopy starting after 8-10 years of disease duration. Surveillance is recommended every 1-2 years with frequency increasing over time to match the elevating risk with years of disease duration. Surveillance involves chromoendoscopy, where a solution is sprayed throughout the colon to highlight areas of dysplasia or, alternatively, high-definition colonoscopy with 4 quadrant random biopsy sampling at 10 cm intervals throughout the colon for subsequent histological determination of neoplasia. Moreover, UC-associated neoplastic lesions can be multi-focal and can appear either flat or raised[12] and may be difficult to diagnose amongst chronically inflamed epithelium. Overall, this translates to collection of roughly 30 to 45 biopsies in order to get a reasonable sampling of the colon. The histologic diagnosis of dysplasia[12], especially at early stages, can be complicated by the presence of inflammation in the biopsy. Patients who receive a diagnosis of dysplasia are deemed high risk for developing cancer and subsequently undergo a more frequent surveillance regimen or a colectomy. However, the proportion of UC patients who develop cancer is low, such that identification of low risk patients and subsequent use of alternate

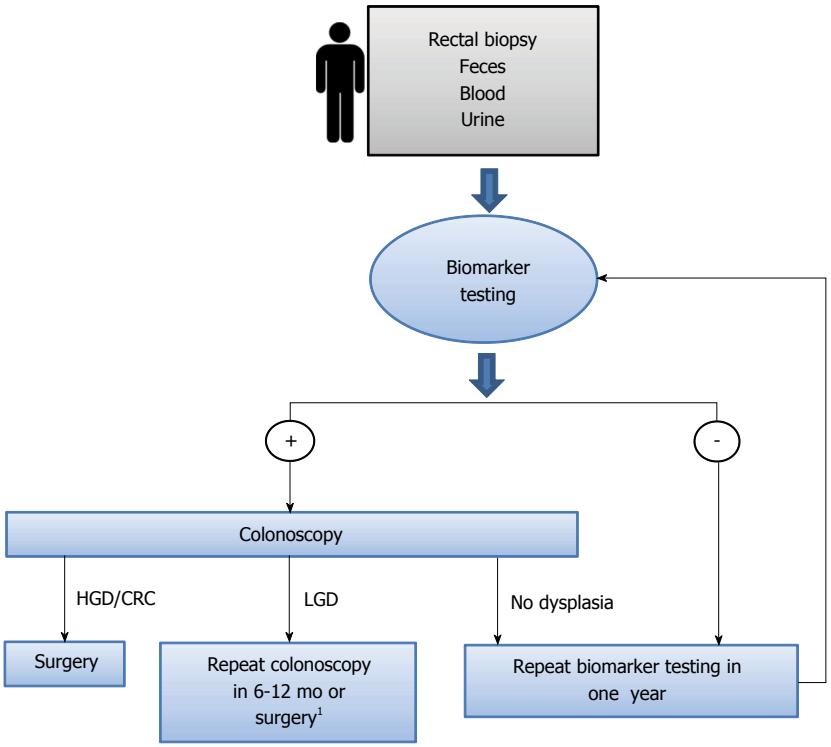

Figure 1 Application of biomarkers in ulcerative colitis surveillance protocol. [1]Management of LGD is dependent on the clinician and patient choices. HGD: High-grade dysplasia; CRC: Colorectal cancer; LGD: Low-grade dysplasia.

non-colonoscopy based surveillance could ultimately reduce health care costs associated with unnecessary colonoscopy.

It is therefore important to identify and develop biomarkers with high sensitivity that could be obtained through non- or less invasive methods (*i.e.,* blood sample, single rectal biopsy, urine) in order (1) to stratify the UC patients which require a more intense colonoscopic surveillance regimen; and (2) to provide earlier diagnostic and better prognostic information. Diagnostic biomarkers could be integrated with existing endoscopy-based UC cancer surveillance to improve the current clinical paradigm, as illustrated in Figure 1. Chronic UC Patients could be screened using a biomarker first using biosamples such as blood, urine or rectal biopsy. Only patients with a positive biomarker result would undergo further evaluation with a full colonoscopy. Patients with a negative biomarker result would be followed by biomarker testing on an annual basis. Therefore, biomarker testing could improve clinical management by identifying those UC patients who have a very low risk of dysplasia and thus reducing the need for colonoscopy.

RESEARCH AND BIOMARKERS IN DEVELOPMENT

Unlike sporadic colorectal cancer, colitis-associated cancer does not usually follow a linear pathway of gene loss or disruption but rather appears to be driven largely by inflammation-associated damage. Combined with the unclear etiology of this disease, researchers are pursuing multiple independent avenues to identify sensitive and specific biomarkers for early UC cancer. While there are currently many biomarkers in development to discriminate subtypes of patients with inflammatory bowel disease (IBD-ulcerative colitis *vs* Crohn's disease), there are few biomarkers for differentiating UC non-Progressors (patients who do not develop dysplasia) from UC Progressors (patients who develop dysplasia or cancer). We will discuss some of the major efforts here. Due to limited space of the review, there will be a primary focus on markers with the most promising clinical potential. Table 1 summarizes the biomarker candidates from recent studies according to the types of biospecimens that have been used including colonic tissue, blood, urine and stool. Since many of these studies were performed on limited numbers of patient samples, they will need to be independently validated in multi-center larger cohort studies.

Colonic biopsy

Biomarker development for UC-associated cancer has, for the most part, focused on the use of colonic biopsies obtained during colonoscopy or colectomy due to (1) the known pancolonic abnormalities that precede CAC; (2) colonic biopsies are a readily available source of material; and (3) retrospective material can be acquired. Immunohistochemical staining of formalin-fixed paraffin embedded (FFPE) material has provided information about disease etiology and

Table 1 Summary of recent studies on biomarker developments for ulcerative colitis-associated cancer

Type of specimen	Analyte	Biomarker	Ref.
Blood	Protein	p53	[58]
Colon tissue	DNA	Chromosomal instability	[15,16,18-20,30-35]
		IBD specific mutation: SOX9, EP300, NRG1, and IL16	[36]
	DNA	ER, MYOD, p16, and CSPG2	[40]
	methylation	FOXE1, SYNE1	[41]
		EYA4	[27,42]
		RUNX3, MINT1, and COX-2	[43]
		p14	[44]
		BVES	[45]
	microRNA	miR-143 and miR-145	[46]
		Decrease of 3 microRNAs (miR-192, miR-375, and miR-422b), upregulation of 8 microRNAs (miR-16, miR-21, miR-23a, miR-24, miR-29a, miR-126, miR-195, and Let-7f)	[47]
		miR-21 and miR-155	[48]
	Protein	miR-26b	[50]
		p53 and CHGA	[51]
		p53 and AMACR	[52]
		Bcl-xl	[53]
		PDCD4	[54]
		COX	[55]
		8-NG and 8-oxodG	[56]
	RNA	TRAP1	[29,57]
		CCND1, SERPINB6, PAP, IL8, and NOS2A	[37]
Stool		ACSL1, BIRC3, CLC, CREM, ELTD1, FGG, S100A9, THBD, and TPD52L1	[38]
		A panel of 20 genes (including cancer genes CYP27B1, RUNX3, SAMSN1, EDIL3, NOL3, CXCL9, ITGB2, and LYN)	[39]
	DNA methylation	VIM, EYA4, BMP3, and NDRG4	[63]
	Metabolites	Buryrate, acetate, methylamine, and trimethylamine	[62]
	Protein	Calprotectin and lactoferrin	[61]
Urine	Metabolites	Prostaglandin-E	[59]
	Protein	MMP-2 and MMP-2/NGAL	[60]

clues to possible biomarker candidates. Isolation of genetic material (*i.e.,* DNA, RNA, miRNA) as well as proteins for proteomic analysis from FFPE sections or frozen material has taken this to the next level. The hope is to identify alterations associated with UC cancerous or dysplastic regions that are also present in non-neoplastic and remote regions, ideally rectum. Rectal biopsies could be obtained with any laxative preparation, without sedation, and using a quick and minimally invasive procedure (proctoscopy) that could be performed in any clinic.

DNA: DNA-based assays using genetic material isolated from purified colonic epithelium has shown promise as a biomarker source. However, since UC doesn't follow the hallmark loss of gene pathways like sporadic colorectal cancer, several studies have focused on more general genome-wide (rather than locus specific) instability. Pan-colonic chromosomal instability has been shown to be detectable relatively early in UC disease progression by BAC array[18,31], fluorescence in-situ hybridization (FISH)[19,20] and arbitrarily primed PCR (AP-PCR)[15,16], yet none of these assays are amenable to high throughput analysis that would be required for a clinical UC neoplastic biomarker. Clonal expansions of poly glutamine repeats (PolyG) are detectable in DNA isolated from colonic epithelium relative to adjacent stromal or muscle DNA controls. The poly G assay was validated in two independent cohorts which confirmed discrimination between UC Progressors and Non-progressors, and demonstrated its utility for UC patients with early disease onset (*i.e.,* diagnosis before 50 years of age)[30,32]. A more recent study examined chromosomal instability in UC neoplastic progression using copy number variation microarrays, and found increased numbers of copy number variations with progression of UC to UC-associated colorectal cancer[33].

UC can also be thought of as an aging disease of the colon; qFISH and qPCR of colonic epithelium from UC patients showed accelerated telomere shortening compared to leukocyte or adjacent stromal controls[34,35]. Shortened telomeres result in chromosomal instability due to higher frequencies of anaphase bridges and subsequent chromosomal breakage, losses, and gains[19]. These techniques lack either the very high specificity demanded by a CAC biomarker (qPCR) or capability for high throughput (qFISH) to create a clinically useable test.

With the development of whole-genome sequencing, comprehensive investigation of the genomic landscape has identified an increasing array of mutational signatures associated with specific diseases. In CAC, the chronic oxidative injury can cause

mounting epithelial cell DNA damage which over time, overwhelms the G1 cell cycle checkpoint and results in p53 mutation. A recent study investigated somatic mutation patterns from inflammatory bowel disease (IBD, including ulcerative colitis and Crohn's disease) associated with colorectal tumor using whole-exome sequencing[36]. Not surprisingly, TP53 was the most commonly mutated gene, with mutational prevalence similar to that of sporadic colorectal tumors (63% of cases). However, APC and KRAS were mutated at significantly lower rates in tumors from patients with IBD than in sporadic colorectal tumors (13% and 20% of cases, respectively). Several IBD-specific gene mutations, including SOX9, EP300, NRG1, and IL16 were identified, confirming the notion that IBD association colorectal cancer (CRC) has its unique genetic composition compared to sporadic CRC.

RNA: Gene expression analysis using RT-PCR of materials isolated from colonic biopsy has resulted in identification of genes which are involved in or associated with UC neoplasia. Microarrays have been used to investigate gene expressional changes in UC neoplastic progression by comparing the mucosa of non-dysplastic, dysplastic and cancerous colonic tissues[37]. The study identified genes associated with UC dysplasia and UC cancer. Five genes were further verified to be common to UC dysplasia and adenocarcinoma relative to non-dysplastic UC (CCND1, SERPINB6, PAP, IL8, and NOS2A).

Because the dysplasia or cancer in UC patients can appear as flat mucosa and/or is multi-foci, there is an interest in identifying neoplastic biomarkers that are present in both dysplastic and non-dysplastic tissues. Such biomarkers could then be tested in random biopsies, including rectal which could be obtained with a minimally invasive procedure. Numerous studies have identified gene expression changes in non-dysplastic tissues from UC Progressors. In one study, a gene signature which included *ACSL1, BIRC3, CLC, CREM, ELTD1, FGG, S100A9, THBD*, and *TPD52L1* were progressively increased with neoplastic progression[38]. These findings support the concept of a field defect phenomena in CAC. Two markers (S100A9 and REG1α) were further validated by IHC showing increased staining in the dysplastic and non-dysplastic tissues from UC Progressors compared to UC Non-progressors and normal controls[38]. In a separate study, by surveying the expressions of 189 carcinogenesis related genes in non-dysplastic rectal mucosa from UC Progressors and Non-progressors, researchers identified a panel of 20 genes (including cancer genes such as *CYP27B1, RUNX3, SAMSN1*, *EDIL3, NOL3, CXCL9, ITGB2*, and *LYN*) in rectal tissues as Progressor associated genes[39]. Using non-dysplastic rectal tissues, the 20-gene panel was able to predict UC-cancer patients from UC-non cancer patients with 83% accuracy and a negative predictive value of 100%.

DNA methylation: Promotor methylation plays an important role in tumorigenesis though transcriptional silencing of critical genes. The early study of DNA methylation in UC dysplasia could be traced back to a study investigating methylation status of five age or cancer related genes in UC dysplasia[40]. Hypermethylation of ER, MYOD, p16, and CSPG2 were detectable in the high-grade dysplasia and cancer tissues from UC Progressors. Moreover, hypermethylation of ER, MYOD and p16 could be detected in the non-dysplastic tissues from Progressors compared to Non-progressors[40]. In another study using methylation-specific PCR, hypermethylation of the tumor growth genes, *FOXE1* and *SYNE1*, was detected in approximately 60%-80% of cancer samples (whereas it was undetectable in controls), suggesting an increase in methylation with disease progression[41]. Methylation of the eyes absent homolog 4 (*EYA4*) gene was present both in neoplastic and remote non-neoplastic tissue of UC patients with cancer but absent in UC control patients without neoplasia[27,42]. Another study found altered methylation status of RUNX3, MINT1, and COX-2 in both the non-neoplastic regions and neoplastic regions of UC -CRC colons as compared with that in the UC controls[43]. Either RUNX3 or MINT1 showed interaction with COX-2 with an additive effect[43]. Further study is needed to evaluate whether this three-gene panel can predict the likelihood of patients to progress to CRC and/or to identify patients with dysplasia. While CpG island hypermethylation of p14 (ARF) but not p16 (INK4) was detectable in 100% of UC dysplasia tested, only 20% (2/10) of patients with dysplasia showed hypermethylation in DNA extracted from non-dysplastic rectum[44]. In a recent study, the reduced expression of a tight junction-associated protein, BVES in UC neoplasia was shown to be the result of promotor hypermethylation of this gene[45]. The BVES promoter hypermethylation could be detected in dysplastic colonic tissues as well as distant non-malignant-appearing mucosa from UC Progressors in comparison to UC Non-progressor and non-UC controls. The study further suggests that BVES interacts with PR61α to promote inflammatory tumorigenesis through c-Myc destruction. Based on the results, BVES promoter hypermethylation status could be a potential biomarker to identify patients with UC at risk of cancer[45].

MicroRNA: MicroRNAs are small non-coding 20-25 nucleotide single-stranded RNA molecules which usually bind to the 3' untranslated region of target mRNA transcripts, effectively silencing them by inhibition of translation or through degradation. MicroRNAs can function as oncogenes to enhance cellular proliferation and survival or as tumor suppressors. Downregulation of miR-143 and miR-145, as well as concomitant upregulation of their predicted targets, IRS-1, K-Ras, API5, and MEK-2 was found in colon biopsies of UC patients, suggesting that some

microRNAs could contribute directly to transformation of UC colonic epithelium[46]. In colitis, the inflammatory microenvironment can modulate microRNA expression and further influence target gene expressions. Currently, there is much focus on the investigation of microRNAs that affect immune response in order to maintain intestinal homeostasis. MicroRNA analysis of colonic biopsies revealed decreased expression of 3 microRNAs (including miR-192, miR-375, and miR-422b) in active UC whereas upregulation of 8 microRNAs (miR-16, miR-21, miR-23a, miR-24, miR-29a, miR-126, miR-195, and Let-7f) was noted in active UC compared to normal controls[47]. Upregulation of miR-21 and miR-155 in inflamed colon from UC patients compared to controls was confirmed in an independent study[48]. Mouse studies suggest that miR-155 is involved in the proinflammatory cellular response due to decreased numbers of CD4+T, Th1/Th17, CD11b+, and CD11c+ cells in miR-155$^{-/-}$ mice[49]. Dysregulation of some microRNAs could be related to inflammation and thus could be used to discriminate colitis associated cancers from sporadic colorectal cancers. For example, miR-26b was shown upregulated in CAC, and down-regulated in sporadic colon cancer[50].

Protein: Protein-based research has revealed some interesting CAC biomarker candidates. Protein participants in major known pathways involved in UC tumorigenesis have been further scrutinized to evaluate their potential as biomarkers. While p53 mutations have been shown to occur as an early event in UC neoplasia[13], the value of the mutated p53 protein as a clinical marker by IHC is controversial. The results of IHC studies are variable and limited due to the use of different antibodies and small sample sizes per study. Protein expression of p53 and chromogranin A provides moderate sensitivity (66.7%) and specificity (80%) for HGD detection[51]. Other protein expression data shows that co-detection of p53 and α-methylacyl coenzyme A racemase (AMACR), using IHC, could discriminate UC patients who had dysplasia or cancer from those who did not; and further, the co-expression of p53 and AMACR was detectable in biopsies taken as early as 10-14 months prior to dysplasia[52]. In a different study, expression of the apoptosis gene, Bcl-xl, is absent in non-dysplastic UC epithelium, but highly positive in dysplasia or cancer samples by IHC[53]. Nuclear IHC staining of the programmed cell death 4 (PDCD4) tumor suppressor showed a reduction in colonic UC dysplasia and cancer tissue samples, and could be useful as a biomarker in the histological assessment of IBD-associated dysplastic lesions[54]. Mitochondrial alterations as defined by patchy loss of cytochrome C oxidase (COX) IHC staining within colonic crypts preceded dysplasia and the staining was lowest in regions of dysplasia and adjacent regions[55]. Chronic inflammation in UC results in the generation of reactive oxygen and nitrogen species, leading to the accumulation of DNA damage, which can be measured by 8-nitroguanine (8-NG) and 8-oxo-7,8-dyhydro-2′-deoxyguanosine (8-oxodG). IHC of rectal mucosa from patients with UC-associated dysplasia showed statistically significant increases in 8-NG and decreases in 8-oxodG compared to UC non-neoplastic controls[56].

Proteomics has also been applied in colitis-associated colon cancer studies with research interests ranging from investigation of disease mechanism to biomarker discovery. To investigate proteomic alterations linked to UC-associated dysplasia and invasive cancer, one study applied stable isotope label based quantitative proteomics to examine the protein expression in the colonic mucosa[28]. The study identified a roster of proteins that displayed at least a 2-fold expression change in random non-dysplastic colon biopsies from Progressors compared to random biopsies from Non-progressors. Among the differentially expressed proteins in the random non-dysplastic biopsies, almost 60% of them were also concurrently expressed in the dysplastic tissues from the same Progressors. These findings suggest that changes in protein expression occur very early in the neoplastic process, before the histologic changes become evident in epithelial cells[28]. Protein activities associated with neoplastic progression included proteins related to mitochondrial function, oxidative activity, and calcium-binding. Protein network analysis suggested that SP1 and c-MYC may play key roles in UC early and late stages of neoplastic progression, respectively.

In a follow-up quantitative proteomics study, individual random rectal samples from UC Progressors were profiled compared to random rectal samples from UC Non-progressors[29]. The study identified over 60 proteins that were differentially expressed in both non-dysplastic rectal tissue and the corresponding dysplastic colonic tissue from Progressors. Mitochondrial proteins, cytoskeletal proteins, RAS superfamily and proteins related to apoptosis were the important protein classes differentially associated with Progressors. One of the mitochondrial proteins, TRAP1, was further validated by IHC in an independent UC cohort, and showed up-regulation in the colon tissues of UC Progressors, but not in the colon tissues of UC Non-progressors[57]. Moreover, up-regulation of TRAP1 preceded the neoplastic changes: it was present in both the dysplastic and non-dysplastic tissue of UC Progressors. TRAP1 staining in dysplastic tissue could achieve 94% sensitivity and 80% specificity in separating Progressors from Non-progressors. In random non-dysplastic rectal biopsies, TRAP1 staining could separate Progressors from Non-progressors with 59% sensitivity and 80% specificity[57].

Blood biomarkers

Blood represents an ideal diagnostic specimen for clinical tests due to its low cost and easy accessibility. It provides abundant material (*e.g.*, DNAs, RNAs,

proteins, cells, exosomes *etc.*) for analysis by a variety of molecular approaches. The gold standard for blood testing of protein levels has traditionally been enzyme linked immunosorbent assays (ELISA) while new technologies such as modified ELISAs, targeted proteomics, and nanotechnology are emerging, making it possible to more thoroughly investigate cancer associated protein abnormalities, particularly for proteins with low abundance. One study reported serum p53 levels detected by ELISA was higher in UC patients compared to normal controls, however elevated levels were detectable in only 8/13 of UC patients with CRC or p53[58]. While p53 is an early event in UC-related tumorigenesis, detection of p53 protein in serum showed only moderate sensitivity-limiting its utility as a neoplastic biomarker for high-risk patients.

Urine

Urine is one of the most convenient sources of material for UC biomarker discovery due to the ease of collection. Most of the current urine-based biomarker studies are focused on discriminating UC patients from those with other types of inflammatory bowel disease (*i.e.,* Crohn's Disease) or to monitor treatment non-adherence. Only a few urine studies reported data that is relevant to neoplastic biomarker discovery in CAC. In a small cross-sectional study, the urinary metabolite, prostaglandin-E, (PGE-MUM assay) was reported to have some potential as a biomarker for UC activity (compared to C-reactive protein), which may assist in staging disease inflammatory activity[59]. Another report suggested that urine MMP-2 and MMP-2/NGAL levels could discriminate UC patients from normal controls[60]. The urinary biomarkers from the current studies may be useful as non-invasive biomarkers for disease activity measurements in IBD patients. However, it is unclear whether these markers would be of value in discriminating UC with and without dysplasia.

Stool

Due to the close proximity to intestinal mucosa, stool samples are a rich resource of material for UC biomarker development. It is estimated that approximately one half of stool is comprised of gut microflora and that upwards of one million colon epithelial cells can be isolated from one gram of stool, which would allow for the study of changes in the genetic material or proteome of a patient's colonocytes, as well as their gut microbiome. Given the complexity of stool samples, it is critical to distinguish changes caused by dysplasia or disease progression, chronic inflammation or consequences of inflammation (*i.e.,* DNA damage, reactive oxygen species, *etc.*), microbial changes, and/or diet. While biomarkers for UC-associated cancer from stool have not yet been adapted for clinical use, fecal biomarkers are being used to diagnose IBD and irritable bowel syndrome (IBS) patients, to distinguish between UC and CD patients, as well as to assess active inflammation. Currently, the two most widely used stool biomarkers, calprotectin and lactoferrin, are derived from neutrophils, which penetrate the intestinal mucosa in areas of active inflammation and are subsequently shed into the lumen. Both biomarkers may be valuable in assessing the active inflammation as a way to gauge therapeutic response in UC patients and as a non-invasive method to monitor relapses[61]. Use of NMR-based spectroscopy to analyze stool is also gaining appeal due to minimal sample preparation, the ability to detect multiple metabolites at once, and the high reproducibility. Mass resonance metabolomics studies have revealed reductions in buryrate, acetate, methylamine, and trimethylamine as well as differences in the levels of several amino acids when stool samples of UC patients are compared to controls[62]. In addition to protein and metabolite markers, DNA isolated from stool offers a promise as a UC biomarker source. Although mutational analysis failed to discriminate UC-associated cancer samples from cancer-free patients, the methylation changes of four genes - including vimentin, eyes absent homolog 4 (*EYA4*), bone morphogenetic protein 3 (*BMP3*), and N-myc downstream regulated gene 4 (*NDRG4*) - showed AUC (areas under the ROC curve) ranging from 0.84-0.91 in distinguishing UC patients with or without neoplasia, suggesting both high specificity and sensitivity for dysplasia detection[63].

CONCLUSION

With the incidence of ulcerative colitis on the rise, there is a need to develop clinically useful biomarkers capable of identifying and monitoring the subset of UC patients at highest risk for developing colon cancer. Cologuard is a newly FDA approved stool biomarker test for sporadic colorectal cancer screening. The most recent study suggests that it can detect 100% colorectal cancer in people between the ages of 40-85[64]. However, this test has about 52% sensitivity in detecting premalignant lesions, such as adenomas, and its utility for screening dysplasia in UC patients has not been tested. Therefore, a new objective molecular test for detection of dysplasia and cancer could be helpful for the clinical management of cancer risk in UC patients. Recent studies have reported the efforts in developing clinically relevant biomarkers for early detection of UC associated cancer using a variety of biosamples, including colonic biopsies, blood, stool, and urine. These studies have revealed a roster of biomarker candidates and laid a foundation for further clinical biomarker development. While the challenge remains to validate these candidate biomarkers in multi-center studies and with larger patient cohorts, it is certain that accurate biomarkers of colitis-associated

neoplasia would improve clinical management of neoplastic risk in UC patients.

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REVIEW

New insights into the pathophysiology of achalasia and implications for future treatment

Janette Furuzawa-Carballeda, Samuel Torres-Landa, Miguel Ángel Valdovinos, Enrique Coss-Adame, Luis A Martín del Campo, Gonzalo Torres-Villalobos

Janette Furuzawa-Carballeda, Department of Immunology and Rheumatology, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico City, CP 14080, Mexico

Samuel Torres-Landa, Luis A Martín del Campo, Gonzalo Torres-Villalobos, Department of Experimental Surgery, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico City, CP 14080, Mexico

Miguel Ángel Valdovinos, Enrique Coss-Adame, Department of Gastroenterology, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico City, CP 14080, Mexico

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Correspondence to: Gonzalo Torres-Villalobos, MD, PhD, Department of Experimental Surgery, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Vasco de Quiroga No. 15, Col. Belisario Domínguez Sección XVI, Mexico City, CP 14080, Mexico. torresvgm@yahoo.com.mx
Telephone: +52-55-54070900-2501
Fax: +52-55-55732096

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Abstract

Idiopathic achalasia is an archetype esophageal motor disorder, causing significant impairment of eating ability and reducing quality of life. The pathophysiological underpinnings of this condition are loss of esophageal peristalsis and insufficient relaxation of the lower esophageal sphincter (LES). The clinical manifestations include dysphagia for both solids and liquids, regurgitation of esophageal contents, retrosternal chest pain, cough, aspiration, weight loss and heartburn. Even though idiopathic achalasia was first described more than 300 years ago, researchers are only now beginning to unravel its complex etiology and molecular pathology. The most recent findings indicate an autoimmune component, as suggested by the presence of circulating anti-myenteric plexus autoantibodies, and a genetic predisposition, as suggested by observed correlations with other well-defined genetic syndromes such as Allgrove syndrome and multiple endocrine neoplasia type 2 B syndrome. Viral agents (herpes, varicella zoster) have also been proposed as causative and promoting factors. Unfortunately, the therapeutic approaches available today do not resolve the causes of the disease, and only target the consequential changes to the involved tissues, such as destruction of the LES, rather than restoring or modifying the underlying pathology. New therapies should aim to stop the disease at early stages, thereby preventing the consequential changes from developing and inhibiting permanent damage. This review focuses on the known characteristics of idiopathic achalasia that will help promote understanding its pathogenesis and improve therapeutic management to positively impact the

patient's quality of life.

Key words: Achalasia; Treatment; Autoimmune disease; Pathophysiology

Core tip: Primary achalasia is associated with loss of ganglion cells in the esophagus and in the lower esophageal sphincter. In the last decade, achalasia pathophysiology has been widely studied, and investigations have aimed to expand clinical management beyond mere treatment of symptoms and towards attacking the specific cause of the disease. While the etiologies remain unclear and no cure exists, the most recent findings suggest an interaction between autoimmune and inflammatory responses, possibly triggered by viral infection, in genetically susceptible individuals. This article reviews the recent advances in understanding the disease pathogenesis with implications for improving therapeutic management.

Furuzawa-Carballeda J, Torres-Landa S, Valdovinos MA, Coss-Adame E, Martín del Campo LA, Torres-Villalobos G. New insights into the pathophysiology of achalasia and implications for future treatment. *World J Gastroenterol* 2016; 22(35): 7892-7907 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i35/7892.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i35.7892

INTRODUCTION

Idiopathic achalasia is a primary motility disease of the esophagus that has a worldwide prevalence of 10 individuals in every 100000 and 1 new case reported per year for every 100000[1,2]. Clinically, it is characterized by two principal findings from esophageal manometry: (1) loss of esophageal peristalsis; and (2) incomplete relaxation of the lower esophageal sphincter (LES)[3,4]. Although its etiology has not yet been completely elucidated, autoimmune, viral and neurodegenerative factors have been proposed as potentially causative or promoting[3,5]. What has been studied and established is that there is a degenerative process, of unknown origin, that involves loss of ganglion cells in the myenteric (Auerbach's) plexus of the esophagus and is accompanied by local and systemic inflammation[6] and subsequent loss of important neurotransmitters such as the vasoactive intestinal peptide (VIP) and nitric oxide (NO)[7].

The principal (presenting) symptoms of the disease arise secondary to the partial and progressive obstruction of the gastroesophageal junction (GEJ) and aperistalsis, and include dysphagia, regurgitation, weight loss, retrosternal chest pain and heartburn, all of which represent the current therapeutic targets[8]. However, the low prevalence of idiopathic achalasia can delay diagnosis and the similarity of its presenting symptomology to that of gastroesophageal reflux disease (GERD) can lead to misdiagnosis[9]. In general, patients presenting with progressive dysphagia and regurgitation are treated with proton pump inhibitors, the usual treatment for GERD, and non-response to treatment is considered an indicator for idiopathic achalasia suspicion.

The routine work-up for clinical suspicion of idiopathic achalasia includes upper endoscopy, which helps to exclude other differential diagnosis such as tumors and will show a dilated esophagus retaining undigested food and saliva, a barium esophagram, which will show esophageal dilatation, poor emptying, aperistalsis and a narrowed GEJ "bird-beak sign", and lastly a high-resolution manometry (HRM) that will confirm the diagnosis and facilitate disease subtype classification[3,8]. The following manometrical characteristics correspond to the 3 different subtypes, as outlined by the Chicago Classification criteria: achalasia with absence of peristalsis ("classic"), type Ⅰ; classic achalasia with esophageal compression, type Ⅱ; classic achalasia with spastic contractions, type Ⅲ[10]. This classification system also represents an important tool regarding outcomes after treatments for the disease[11].

In the last decade, researchers have turned their attention towards determining the pathophysiological underpinnings of achalasia in order to not just treat the symptoms but to target the specific cause(s) of the disease that remain unclear and currently do not have a cure.

ETIOLOGY AND PHYSIOPATHOLOGY

Primary achalasia is associated with loss of ganglion cells in the esophagus and in the LES. The loss of ganglion cells has been associated with both inflammation and deposition of collagen. Findings from the collective research suggest that the disease process involves an interaction between autoimmune and inflammatory responses, possibly triggered by viral infection, in genetically susceptible individuals (Figure 1).

Ganglion cell loss

The enteric nervous system is distributed along the gastrointestinal tract, including the esophagus. The myenteric plexus is situated between the circular and longitudinal smooth muscle layers of the gut and consists of postganglionic neurons that differentiate into excitatory cholinergic neurons and inhibitory nitrergic neurons. While the excitatory neurons release acetylcholine, the inhibitory neurons release the free radical NO and the neurotransmitter/anti-inflammatory cytokine VIP; the coordinated release creates the balance of relaxation and contraction that is vital for

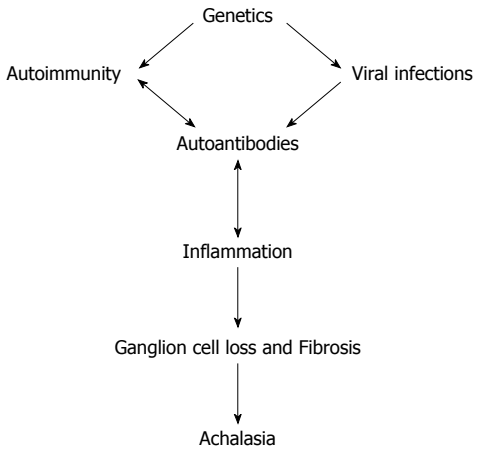

Figure 1 Main mechanisms in the etiopathogenesis of achalasia.

normal esophageal peristalsis[12].

Achalasia is known to be caused by the reduction of interstitial cells of Cajal, but most importantly to a selective loss of inhibitory ganglion in the myenteric plexus of the esophagus, which is associated with a decrease of NO and VIP. These pathophysiological and morphologic features have been demonstrated in a benzyldimethyltetradecylammonium chloride-induced amyenteric rat model and in human tissues[5,13-20]. In addition, the ability of nitrergic nerves to mediate esophageal neuromuscular functions, such as LES relaxation and normal peristalsis, was demonstrated in experimental animal systems.

Animal studies have also provided the first clues about the genetic underpinnings of achalasia. Mice with neuronal NO synthase 1 gene disruption (*nNOS*$^{-/-}$) presented with significantly increased hypertensive LES, and consequently markedly impaired relaxation that developed into an achalasia-like LES dysfunction and gastroparesis; these findings contrast with the hypotensive LES present in the W/W^{v} mice that lack the interstitial cells of Cajal as intermediary of LES relaxation[21,22].

Human studies have also suggested a significantly decreased or absent NO innervation in the myenteric plexus of patients with achalasia[23]. Immunohistochemical studies of biopsies from patients with achalasia who underwent surgical treatment showed that levels of the VIP, nNOS, neural proteins, S-100, substance P and protein gene product 9.5 (PGP9.5) were significantly lower than in healthy individuals[5,13,24]. Thus, impaired production of NO and VIP are involved in the pathophysiology of achalasia, and - as discussed later in this review - may be genetic components and therapeutic targets.

An underlying pathogenic mechanism in the early stage of achalasia is the esophageal myenteric immune-mediated response and inflammatory state accompanied by T cell infiltration, triggered by a yet unknown etiologic factor. This pathologic condition may cause neuritis and ganglionitis, with no initial ganglion cell loss or with only mild to severe fibrosis in the smooth muscle layer. A proteomic analysis of serum from patients with achalasia and from healthy individuals showed disease-related up-regulation of transthyretin (TTR); TTR is a serum and cerebrospinal fluid carrier of the thyroid hormone thyroxine (T4) and a retinol-binding protein that is associated with familial amyloid polyneuropathy, and its observed up-regulation corroborates with the consequent neural degeneration observed in patients[25]. Moreover, other studies of achalasia patients have shown increased deposits of the complement complex C5b-C9 and of IgM within or proximal to ganglion cells of myenteric plexus[26]. The imbalance produced by loss of VIP- and NO-secreting neurons can lead to irreversible esophageal motor dysfunction. Furthermore, the progressive destruction of myenteric ganglion cells and the occurrence of neural fibrosis would lead to the classic subtype of achalasia[19,27].

In addition to the destruction of inhibitory ganglionic cells (due to the abundant inflammatory infiltrates and autoimmunity), hypertrophy and neuronal fibrosis accompany the disease progression[28]. Most reports of pathological findings for human cases show the presence of neuronal autoantibodies[29,30]. These autoimmune constituents have been shown to directly contribute to the destruction of the myenteric plexus[31,32]. In particular, Bruley des Varannes *et al*[23] showed that serum from patients with achalasia, and not from patients with GERD, can induce phenotypic and functional changes in myenteric neurons that reproduce the disease characteristics, thereby refuting the theory that these autoantibodies could be an epiphenomenon.

Infections

In order to explain the loss of ganglion cells in achalasia, several studies have explored the potential of pathogenic factors as the disease-initiating agents, in particular those related to chronic latent or active neurotrophic viral or bacterial infections. The proposed virus candidates have included herpes simplex virus (HSV) (a neurotropic virus with predilection for squamous epithelium), John Cunningham (commonly known as "JC") virus, bornavirus, varicella zoster, measles, and human papilloma virus[33-36]. In fact, Boeckxstaens[35] proposed that during latent HSV-1 infection, the virus persists in the neurons of the LES and esophagus, giving rise to a persistent immune activation that in genetically predisposed individuals can elicit neuronal destruction. Moreover, an epidemiological study and genotype-phenotype analysis carried out by Becker *et al*[37] demonstrated that patients were frequently affected by viral infections, especially varicella zoster virus infections, before achalasia onset, and that pregnancy may stimulate the disease in females who are carriers of the HLA-DQβ1 insertion[37].

Several studies of esophageal biopsy specimens

from patients with idiopathic achalasia have used the polymerase chain reaction to investigate the presence of various virus strains. Infection with and active replication of HSV-1, but not cytomegalovirus or Epstein-Barr virus, were detected along the myenteric plexus. The preferential ports of entry for HSV-1 are the perioral and esophageal mucosa. Furthermore, herpes viruses exhibit a strong tropism for nerve fibers and following the primary exposure the viruses can remain in a latent form in neuronal nuclei[6,34]. T lymphocytes are known to specifically respond to HSV-1 antigens, and Facco *et al*[34] demonstrated that achalasia patients have a significantly higher rate of oligoclonal $CD3^+/CD8^+$ lymphocytic infiltrates in LES, as compared with healthy controls.

Since not all patients with viral infections develop achalasia, it has been proposed that specific genetic changes affecting the immune system may create susceptibility to this disease. Chronic viral infection could trigger an aberrant immune response that under an appropriate genetic and environmental background would facilitate the loss of esophageal neurons. In support of this theory, detailed examinations of the myenteric plexus of patients with achalasia have shown infiltration of $CD3^+/CD45RO^+$ T cells, predominantly $CD8^+$ T cytotoxic lymphocytes expressing activation markers[5,17,18,38].

Autoimmunity

The proposed autoimmune etiology of achalasia is supported by the presence of anti-myenteric antibodies in the circulation and inflammatory T cell infiltrates in the myenteric plexus, as well as demonstrated statistical correlations between the disease and particular HLA class Ⅱ antigens. Autoimmune diseases often occur in association with one another, either involving a single individual or within a family. It has been proposed that the etiology of achalasia includes an autoimmune component. Findings from a recent study, and numerous case reports, have characterized patients with achalasia as being 3.6-times more likely to have autoimmune diseases, including uveitis (RR = 259), Sjögren's syndrome (RR = 37), systemic lupus erythematosus (RR = 43), type Ⅰ diabetes (RR = 5.4), hypothyroidism (RR = 8.5), rheumatoid arthritis (RR = 2.4), scleroderma, ankylosing spondylitis, myasthenia gravis, Guillain-Barre syndrome, autoimmune acquired hemophilia A, polyglandular autoimmune syndrome type Ⅱ, psoriasis and asthma[29,39-45]. Intriguingly, the younger population of patients with achalasia was shown to have a higher prevalence of autoimmune comorbidities (RR = 3.3)[43], compared with an older population of patients with achalasia. Finally, there are some reports of patients with achalasia responding to immunosuppressive drugs, lending further credence to the notion that this disease has an autoimmune component[42].

It is most likely that the etiology of achalasia is multifactorial, involving genetic and immune-related factors, possibly both pathogen-/environmental- and host-derived[15]. Such an etiological profile may trigger damaging autoimmune mechanisms or chronic inflammation.

Inflammation

Histological and immunohistochemical studies of the esophageal tissues of patients with achalasia have also found inflammatory infiltrates of varying intensity around myenteric neurons, contrasting with the non-infiltrate findings of control groups with normal myenteric plexus[6,18,38,46]. $CD3^+$, $CD4^+$, $CD25^+$ and $CD8^+$ T lymphocytes predominated in all the diseased tissues, as well as $CD20^+$ B lymphocytes and eosinophilic granulocytes but to a lesser degree, with detection of occasional plasma and mast cells along the nerve fascicles and around the ganglion cells[5,6,18,46]. Moreover, T and B inflammatory infiltrates predominated in tissues with advanced stage disease (> 10-year symptom history)[5].

A serum proteomic analysis has demonstrated that C4B5, C3, cyclin-dependent kinase 5, and α_2-macroglobulin are up-regulated in achalasia patients, as compared with controls, corroborating the theory of immune-mediated response and/or neural degeneration components of the disease pathogenesis[25]. Moreover, another study of achalasia patients had shown that the complement complex C5b-C9 (membrane attack complex) and IgM are deposited within or at the ganglion cells of the myenteric plexus[26].

Turnover of extracellular matrix and some $CD4^+$ T cell subsets and cytokines have been also described in achalasia patients. In particular, a significant increase has been observed in the expression of matrix metalloproteinase-9 (MMP-9, also known as 92 kDa gelatinase) and its tissue inhibitor, TIMP1, in LES from patients with achalasia, as compared to controls[6]. Some tissue and circulating $CD4^+$ T cell subsets have been characterized in patients with achalasia as well (Figure 2).

Up-regulated expression of interleukin-22 (IL-22) has been detected in tissue (especially in myenteric plexus)[6] and circulating cells of patients with achalasia (Figure 3). IL-22 belongs to the IL-10 superfamily, and acts as an initiator of the innate immune response against pathogens in gut epithelial and respiratory cells, as a modulator of tissue repair/regeneration processes, and as a regulator of antibody production. IL-22 is synthesized by the T helper (Th) cell subsets of Th22 and Th17. The Th22 cells differentiate from naïve T cells in response to TNF-α and IL-6 signals, and subsequently synthesize and secrete IL-26, IL-13 and IL-22; IL-26 plays important roles in cellular proliferation and survival, antimicrobial peptide production, epithelial renewal and immunity[47].

The cytokine IL-17A is produced by the Th17 subset and is a key mediator of auto-inflammatory

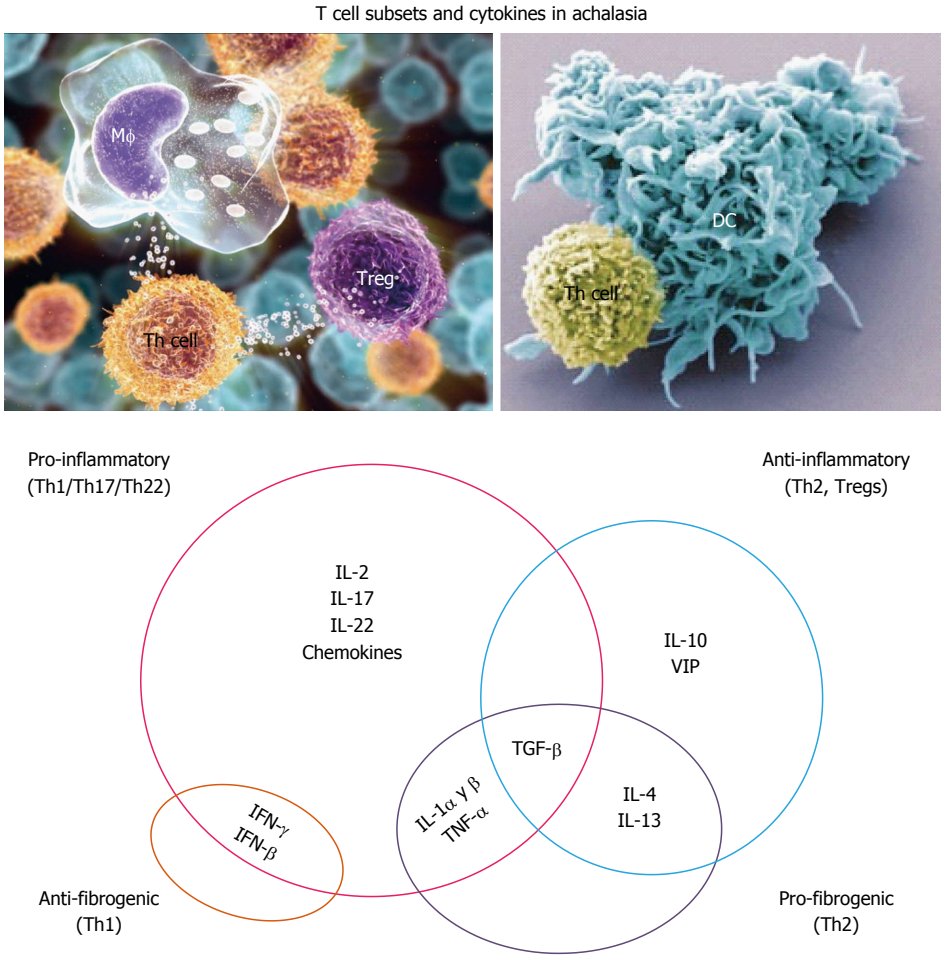

Figure 2 Hypothetical interplay of cytokines in the pathophysiology of achalasia. IL: Interleukin; IFN: Interferon.

diseases. Under both physiological and pathological conditions, IL-17A acts to stimulate T cells, increase the production of autoantibodies and inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8, IL-17, IL-22, *etc*) and chemokines (CCL2, CCL7, CCL20, CXCL1, CXCL5), induce neutrophil recruitment through chemokine regulation, activate innate immune cells and enhance B cell functions[48]. The frequency of IL-17A-secreting cells is reportedly higher in the peripheral cells and myenteric plexus of esophageal tissue of patients with achalasia, as compared to controls (Figure 3)[6].

Another important cytokine, interferon-gamma (IFN-γ), may play a role in achalasia pathogenesis. Produced by activated T cells and natural killer cells (NKs), IFN-γ regulates various immune and inflammatory responses. Specifically, this cytokine potentiates the effects of the type Ⅰ IFNs, recruits leukocytes to infected tissue in order to potentiate beneficial inflammation, and stimulates macrophages to engulf and kill bacteria. IFN-γ released by Th1 cells is also important in regulating the Th2 response. As IFN-γ is vitally implicated in the regulation of immune response, aberrant regulation of its production can lead to autoimmune diseases. Moreover, IFN-γ inhibits collagen synthesis and induces the production of chemokines including CXCR3, CXCL9, CXCL10 and CXCL11[48]. Studies have found that patients with achalasia have a significantly higher percentage of circulating and tissue IFN-γ^+/CD4$^+$ T cells, as compared to controls (Figure 3)[6].

Increased expression of IL-1β, IL-2 and TNF-β has also been detected in tissue from achalasia patients, as compared with controls[34]. Other cytokines with dual anti-inflammatory/pro-fibrogenic functions have been evaluated in patients with achalasia. Under normal physiologic conditions, TGF-β1 controls cellular growth, proliferation, differentiation, negative regulation of inflammation, collagen synthesis and apoptosis; patients with achalasia have shown significantly increased TGF-β1$^+$ cell expression in the myenteric plexus of esophageal biopsies, as compared to tissues from controls[6].

IL-4 is an anti-inflammatory cytokine that inhibits the synthesis of several important cytokines (IL-1β, TNF-α, IL-6, IL-17A, *etc*), regulates B cell proliferation and differentiation, and functions as a potent inhibitor of apoptosis. IL-4 is synthesized primarily by Th2 cells and is required for the initiation and maintenance of fibrosis. The IL-4-expressing CD4$^+$ Th2 subset is defined by production of IL-4, IL-5, IL-9 and IL-13 and

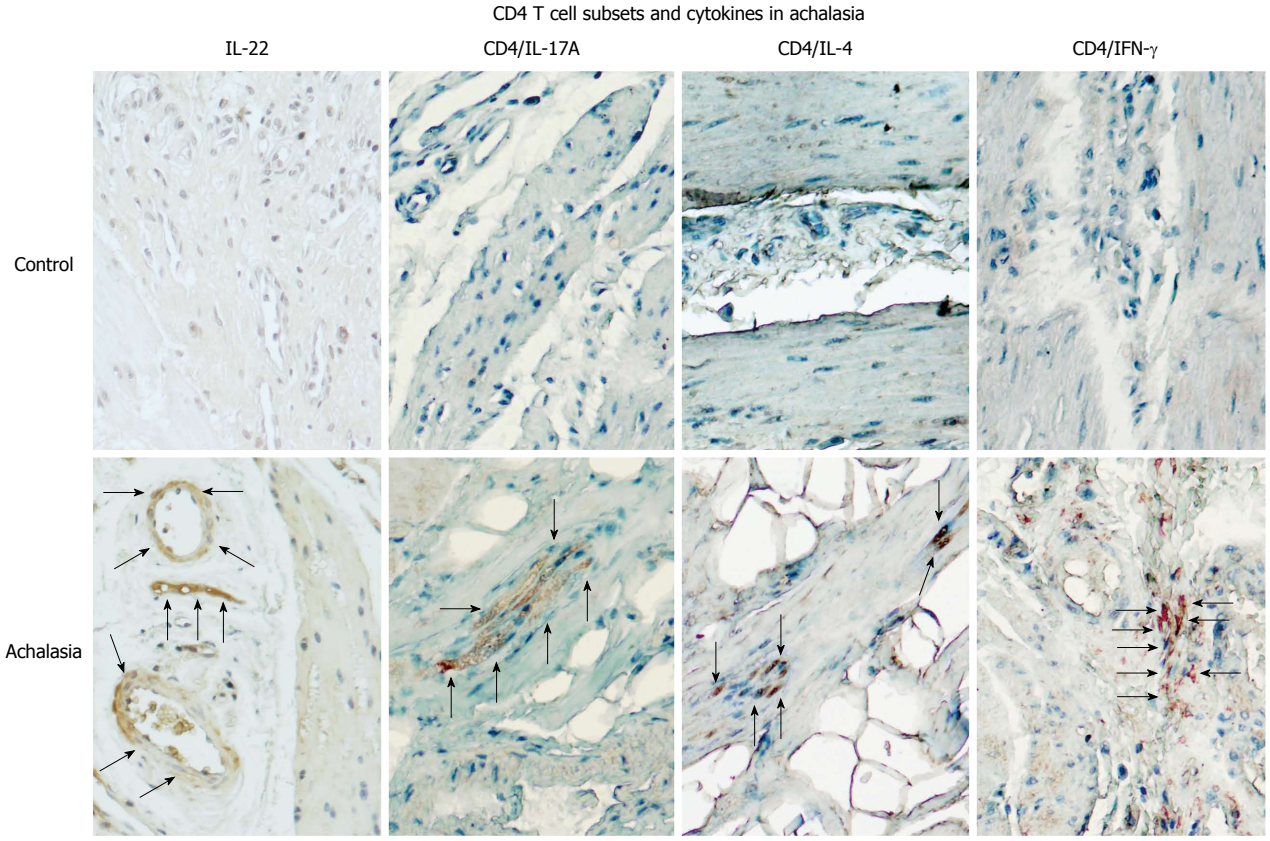

Figure 3 CD4$^+$ T cell subsets and their expression of pro-inflammatory/anti-fibrogenic cytokines in achalasia. Immunohistochemical analysis of IL-22, CD4/IL-17 (Th17 cells), CD4/IL-4 (Th2 cells), and CD4/IFN-γ (Th1 cells) in specimens of esophagus from healthy control donors and achalasia patients. Arrows denote the immunoreactive cells. Original magnification × 320.

functions in type 2 immunity for fighting off infectious disease, the process of which involves neutralization of toxins, maintenance of metabolic homeostasis, regulation of wound healing, regeneration of tissue and production and deposition of fibrosis-enhancing collagen; in addition, these cells suppress autoimmune disease mediated by Th1 cells[49]. Studies have shown that patients with achalasia have significantly higher circulating and tissue IL-4$^+$ cell percentage, as compared to controls (Figure 3)[6].

IL-13 has functions similar to IL-4; in contrast, however, it regulates the type Ⅰ collagen gene, thereby participating in fibrosis. Its expression pattern in patients with achalasia is also similar to that of IL-4[6].

Immune "regulatory cells" (such as the T regulatory cells commonly known as Tregs) include a variety of cell subpopulations with specialized functions that allow them to exert cell extrinsic immunosuppression and tolerance to self as well to foreign antigens. Tregs modulate the natural course of protective immune responses in order to limit tissue damage and autoimmunity. In addition, they suppress immunologic responses by producing granzymes and perforins, depleting IL-2, secreting suppressor molecules such as IL-19 and TGF-β1, and diminishing the functions of antigen presenting cells (APCs) that otherwise promote anergy or apoptosis of effector T cells. As such, the Tregs play important roles in tissue repair and homeostasis[50]. Patients with achalasia show a higher Treg frequency in the myenteric plexus of esophageal tissue, as compared to controls[6]. Nonetheless, Sodikoff *et al*[46] reported that Foxp3 (the distinguishing marker of Tregs) was not detectable in achalasia biopsies of their cohort, contrasting our findings in which a higher percentage of CD25$^+$/Foxp3$^+$ cells were detected in esophageal smooth muscle tissue from patients with type Ⅲ achalasia, followed by types Ⅰ and Ⅱ, as compared to controls. These discrepant findings may be explained by different techniques used to conserve the samples prior to examination or to perform the immunohistochemical evaluation, or the findings may reflect important differences in disease evolution among the two studies' cohorts (Figure 4)[46].

In addition to Tregs, a newly described population of regulatory B cells (termed Bregs) has also been shown to contribute to immunosuppression, not only in autoimmune diseases but also in inflammatory and organ/tissue transplant conditions; regardless, however, the effects are direct and occur *via* enhancement of Treg function. This CD19$^+$CD24^{hi}CD38^{hi} immature/transitional T1 B cell subset suppresses the differentiation of Th1 cells in an IL-10-dependent manner[51]. Intriguingly, biopsies of myenteric plexus obtained from patients with achalasia showed a higher

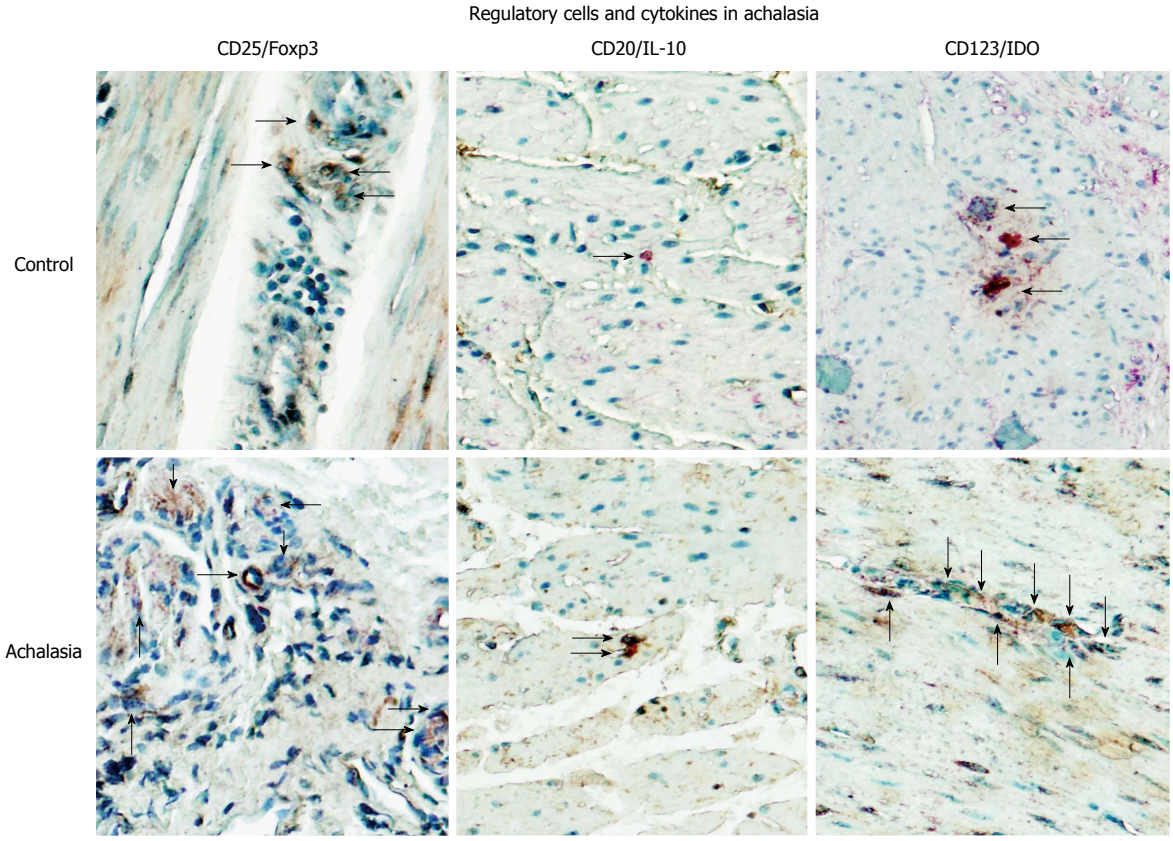

Figure 4 Regulatory cells in achalasia. Immunohistochemical analysis of CD25/Foxp3 (T regulatory cells), CD20/IL-10 (B regulatory cells) and CD123/IDO (plasmacytoid dendritic regulatory cells) in specimens of esophagus from healthy control donors and achalasia patients. Arrows denote immunoreactive cells. Original magnification × 320.

relative IL-10-producing B cell percentage than tissues from a control group (Figure 4)[6].

Lastly, it is known that dendritic plasmacytoid regulatory cells (termed pDCregs) are a sub-population of immune cells that express the indoleamine 2,3-dioxygenase (IDO) enzyme that is responsible for mediating tryptophan metabolism, which suppresses T effector cell activity and induces $CD4^+/CD25^{hi}$ regulatory T cell polarization. IDO-mediated deprivation of tryptophan halts the proliferation of T cells at mid-G1 phase, which in concert with the pro-apoptotic activity of kynurenine leads to immune tolerance. IDO has a selective role in Th2 differentiation and is regulated positively during antigenic presentation and the functional complexing of CTLA-4/B7-1/B7-2 in lymphocytes and dendritic cells. In addition IDO contributes to the immune responses to pathogens, being up-regulated by circulating nucleic acids (from host and non-host genomes) through the activation of TLR4 and TLR9, and it contributes to adaptive immunity processes that subsequently modulate the inflammatory process[52]. Patients with achalasia have shown a higher frequency of pDCregs in the myenteric plexus of esophageal tissue, as compared to control tissues (Figure 4).

Autoantibodies

The observation of increased prevalence of circulating IgG antibodies against myenteric plexus in most patients with achalasia has led to the suggestion of a role for autoantibodies in the pathogenesis of this disease. Studies have also demonstrated a notable absence of anti-myenteric autoantibody in achalasia-free controls, patients with Hirschsprung's disease, esophageal cancer, peptic esophagitis, gastroesophageal reflux or myasthenia gravis[53-55]. Nonetheless, a study by Moses *et al*[32] suggested that these circulatory antibodies are more likely the result of a non-specific reaction to the disease process, rather than being the cause of the disease; this idea was supported by detection of similar antibodies in patients without achalasia. In accordance with the hypotheses, evidence of autoantibodies against myenteric neurons were detected in serum samples from patients with achalasia, especially in carriers of HLA DQA1*0103 and DQB1*0603 alleles[55]. Recently, Kallel-Sellami *et al*[30], as well as our group[6], determined the levels of circulating anti-myenteric antibodies in serum from patients with achalasia; the measurements in both studies were carried out with the commercially available kit Neurology Mosaic 1 (Euroimmun, Leubeck, Germany) that involves a

standard indirect immunofluorescence screening assay using frozen monkey nerves, cerebellum and intestinal tissue as antigenic substrates. The prevalence of nuclear or cytoplasmic circulating antibodies against myenteric plexus in the sera from idiopathic achalasia patients was 63% and 100% *vs* 12% and 0% in the sera from healthy donors, respectively; moreover, most antibodies showed positive reaction in the nuclear and nucleolar compartments of cells in the myenteric plexus[6,30]. These two studies also analyzed the target antigens of circulating anti-myenteric autoantibodies by testing sera with the Neuronal Antigens Profile Plus RST kit (Euroimmun) that involves immunoblotting for a panel of individual neuronal antigens, including amphiphysin, CV2, PNMA2 (Ma-2/Ta), onconeuronal antigens (Ri, Yo, Hu), recoverin, SOX-1 and titin. A majority (69%) of the sera samples from the idiopathic achalasia patients reacted with PNMA2 (Ma2/Ta), and a few (8%) reacted with the recoverin antigen that is related to Sjögren's syndrome[6,30].

Other autoantibodies have been detected in serum from non-diabetic patients with achalasia, including glutamic acid descarboxylase-65 (GAD65) antibody, which showed a remarkably higher frequency compared to the control subjects (21% *vs* 2.5%)[29]. The enzyme GAD65 converts glutamic acid to gamma-aminobutyric acid, and is expressed in GABAergic nerve terminals in the enteric nervous system. GAD65 antibodies are reportedly present in approximately 80% of patients with type I diabetes mellitus and in approximately 20% of patients with various organ-specific neurological disorders, including myasthenia gravis, Lambert-Eaton syndrome, autoimmune dysautonomias and encephalopathies[29]. Approximately 40% of the achalasia patients evaluated in similar studies have shown at least one other organ-specific autoantibody, namely thyroid or gastric parietal cell antibodies, and > 40% of patients have shown anti-nuclear antibodies[23,29].

Genetics

Genetic factors may play an important role in the development and progression of achalasia. The existence of familial cases suggests that achalasia may be inherited and thus have a genetic component. Furthermore, statistical correlations of achalasia with well-defined genetic syndromes have been found. For example, mutation in the *ALADIN* 12q13 gene, which is associated with Allgrove syndrome, specifically (Triple-A) in exons 1, 2, 7, 8, 10-14 and 16, and a poly(A) tract, shows clinical manifestations of achalasia, alacrima, and adrenocorticotrophic hormone-resistant adrenal insufficiency[56-58]. In addition, achalasia has been associated with the multiple endocrine neoplasia type 2 (MEN 2) B syndrome, specifically a germline mutation in exon 16 (M918T) of the RET proto-oncogene on chromosome 10q11 that leads to a methionine-threonine amino acid substitution in the tyrosine kinase domain and thus constitutive activation of the oncogene. The MEN 2 mutation can manifest as one of three types of cancer predisposition, all with an autosomal dominant mode of inheritance; these include the familial medullary thyroid carcinoma, MEN2A and MEN 2B forms[27]. Riley-Day syndrome (familial dysautonomia) and Smith-Lemli-Optiz syndrome (elevated concentrations of the 7- and 8-dehydrocholesterol due to reductase deficiency) have also been linked to achalasia[27]. The combination of achalasia with Hirschsprung's disease or aganglionic megacolon has been also described[59]. Up to 2% of children with Down's syndrome have achalasia, presumably due to a significant reduction in the number of neurons present in the esophageal plexus of this population[56,60]. Finally, congenital central hypoventilation syndrome has been also associated with achalasia in children[56].

As idiopathic achalasia is a relatively rare disorder, another approach to genetic analysis of these cases has been to identify candidate genes *via* identification of single nucleotide polymorphisms (SNPs) and classification of their clinical phenotype. The neuronal nitric oxide synthase (*NOS1*) gene is located on human chromosome 12q24.2, and a disease-related microsatellite (CA repeat) polymorphism has been found within the 3'-untranslated region (UTR) of exon 29. Additionally, an exome analysis of two siblings with infant-onset achalasia revealed homozygosity for a premature stop codon in the gene encoding *NOS1* (at residue Tyr1202, instead of at residue 1435). Kinetic analyses and molecular modeling indicated that the truncated protein product has defective folding capacity, as well as defective capabilities for NO production and binding of co-factors[15,61]. Other genetic polymorphisms of *NOS* gene isoforms that have been discovered involve the endothelial NOS4a4a, inducible NOS22GA and neuronal NOS29TT[62]. The receptor of vasoactive intestinal polypeptide (*VIPR1*) gene is located on chromosome 3p22. VIPR1 belongs to the secretin receptor family, a group of G-protein coupled receptors expressed by immune cells (T cells, macrophages and dendritic cells) and myenteric neurons of the distal esophagus and LES. It is highly polymorphic, and five SNPs have been reported in patients with late achalasia, including (rs421558) intron-1, (rs437876) intron-4, (rs417387) intron-6, and (rs896 and rs9677) 3'-UTR[63]. The IL-23 receptor (*IL-23R*) gene is located on chromosome 1p31 and its encoded protein, IL-23R, is expressed by Th17 cells and has been associated with chronic autoimmune disorders. One study showed an *IL-23R* gene polymorphism, wherein arginine replaces glutamine at codon 381, as significantly more common in patients with achalasia than in healthy controls[15,64]. The protein tyrosine phosphatase non-receptor 22 (*PTPN22*) gene is located on chromosome 1p13.3-p13, within a region known to be associated with autoimmune disease;

the encoded protein, an intracellular lymphoid-specific tyrosine phosphatase (Lyp), is a down-regulator of T cell activation. A SNP in the *PTPN22* gene at position 1858C/T, which leads to a replacement of arginine with tryptophan in codon 620, has been shown to increase risk of achalasia in females of Spanish descent[15,65]. Polymorphisms in the *IL10* gene have been associated with different autoimmune conditions, such as systemic lupus erythematosus, type 1 diabetes, ulcerative colitis and asthma; for achalasia, however, the GCC haplotype of the *IL10* promoter has been associated with a lower risk of the disease[15,66]. Finally, polymorphisms in the *IL-33* gene, which encodes the IL-1 cytokine family member IL-33 and is known to play a critical role in chronic inflammatory autoimmune diseases, have been reported as more frequent in females with achalasia, as compared to controls; specifically, the polymorphisms are the rs3939286 SNP and the rs7044343T/rs3939286A risk haplotype[67].

Human leukocyte antigen (HLA) class Ⅱ antigens have also been associated with autoimmune diseases such as systemic lupus erythematosus, Sjögren's syndrome, and other connective tissue diseases. In addition, the myenteric infiltrates are predominantly T cell lymphocytes than can recognize certain class Ⅱ antigens. Associations between achalasia and HLA-DQβ1 (HLA-DQB1*05:03 and HLA-DQB1*06:01), HLA-DQα1 (HLA-DQA1*01:03), and HLA-DQβ1 (HLA-DQB1*03:01 and HLA-DQB1*03:04) have been determined[15,45]. In fact, many studies have shown a positive association with this disease and the various class Ⅱ HLA antigens, including DQw1, DQA1*0103, DQB1*0601, DQB1*0602, DQB1*0603, DQB1*0601, DQB1*0502, and DQB1*0503 alleles, in Caucasians. Moreover, patients with the DQA1*0103 and DQB1*0603 alleles have been shown to have a significantly higher prevalence of anti-myenteric antibody[37,54,55,67-70]. In a study of 1068 cases of achalasia from central Europe, Spain and Italy, an 8-residue insertion at position 227-234 in the cytoplasmic tail of HLA-DQβ1 (encoded by HLA-DQB1*05:03 and HLA-DQB1*06:01) was characterized as conferring the strongest risk for achalasia. Two amino acid substitutions in the extracellular domain of HLA-DQα1 at position 41 (lysine encoded by HLA-DQA1*01:03) and of HLA-DQβ1 at position 45 (glutamic acid encoded by HLA-DQB1*03:01 and HLA-DQB1*03:04) were characterized as independently conferring achalasia risk[70]. Moreover, the HLA-DQβ1 insertion was characterized as a strong risk factor for achalasia and showed a particular geospatial north-south gradient among Europeans. The finding of this insertion being less common in northern European populations, as compared with those from the southern regions mirrored the differential prevalence of the disease between populations[4]. This geographic profile may reflect a genetic predisposition, putting certain individuals at increased risk of developing achalasia, possibly after exposure to some particular environmental conditions. It is important to note, here, that not all patients with achalasia carry the putative "predisposing" HLA and not all people with the HLA have the disease[37,71].

Thus, the initial event that triggers achalasia may be the result of a repetitive insult produced by neurotropic virus infection, likely HSV-1[6,34-36,72,73], which induces a conspicuous and persistent inflammation at the perineural level, in the myenteric plexus. Not all infected patients will develop achalasia, on account of a genetic predisposition to develop a chronic auto-inflammatory response that has the potential to progress to the disease[35] (Figure 5).

THERAPEUTIC APPROACHES

Currently, there is no cure for achalasia. Treatment goals are to ameliorate the patient's symptoms, improve esophageal body emptying and limit esophageal dilation, all of which can be accomplished by resolving the esophageal outflow obstruction (Table 1). The different therapeutic approaches available today, including pharmacotherapy, botulinum toxin injections, endoscopical dilatations, esophageal stents, peroral endoscopy myotomy and surgical treatment for achalasia (Figure 6), all aim to treat the symptoms but are not capable of use as preventives or address the underlying pathology of the disease[8,74,75].

Pharmacologic therapy

Calcium channel blockers, long-acting nitrates and phosphodiesterase type 5 inhibitors, to name a few, have been used for achalasia; however, none has proven an effective therapy. Use of these drugs is only recommended in the early stages of the disease, as a temporary regimen immediately prior to another definitive treatment, or in patients who are unable or unwilling to undergo any other of the therapeutic alternatives[3,8]. Nevertheless, these drugs are capable of providing a clinical response, albeit an incomplete and short-acting one. Another important feature of these drugs are the side effects, which are generally poorly tolerated and span the spectrum from inconvenient (headache) to severe (hypotension and edema).

Endoscopic therapies

Botulinum toxin is a presynaptic inhibitor of acetylcholine release from motor neurons. Endoscopic intrasphinteric injection of botulinum toxin A has been demonstrated as a safe approach to achieve short-term improvement of symptoms (85% of the patients experience improvement with a single injection)[76]; however, this therapy loses efficacy over the long-term, resulting in patients frequently requiring increasingly repeated injections (60% of patients starting at 6 mo after the first injection, and 30% at 1 year after). Although some authors advocate for

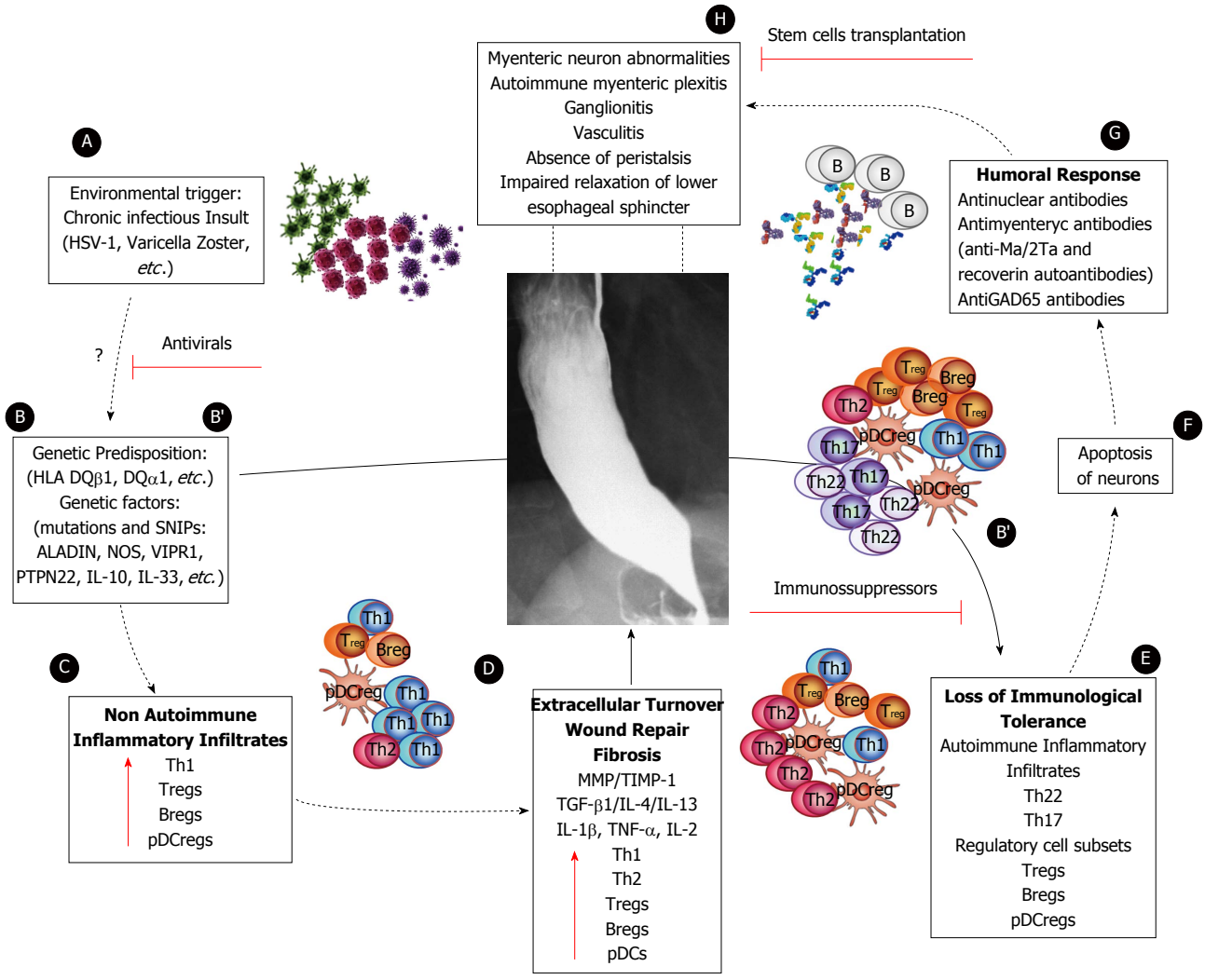

Figure 5 Proposed model of achalasia pathophysiology (modified from Furuzawa-Carballeda *et al*[6]). A: An initial active or latent infectious insult, likely involving a neurotropic virus such as the herpes family of viruses or varicella zoster, which have predilection for squamous epithelium and neurons and may cause ganglion cell damage which would be limited to the esophagus; B: Some individuals with genetic predisposition will progress with an aggressive inflammatory response; C: At a very early stage of the disease, the inflammatory infiltrates may be predominantly composed of Th1, Th2 and regulatory cell subsets [T regulatory cells (Tregs), B regulatory cells (Bregs) and plasmacytoid dendritic regulatory cells (pDCregs)]; D: Repair of tissue after injury would require orchestrated coordination of several cell types and biosynthetic processes and would be coordinated by an interacting group of pro- and anti-inflammatory cytokines, fibrous extracellular matrix (ECM) proteins to replace lost or damaged tissue, and products of metabolism such as oxygen radicals. The most prominent pro-fibrogenic cytokines are TGFβ, IL-4 and IL-13. ECM also mediates cellular crosstalk, and does so in two ways. Newly deposited ECM is then rebuilt over time to emulate normal tissue. Matrix proteinases and their inhibitors (TIMPs) also are important, during wound repair, tissue remodeling and fibrosis. B'; E: If steps A-D occur repeatedly, such as in a chronic infection condition, only those individuals with genetic predisposition to developing a long-lasting autoinflammatory response will progress to development of the disease (loss of peripheral tolerance). Thus, autoinflammatory infiltrates would be predominantly composed of Th22, Th17 and regulatory subpopulations; F: Degeneration and significant loss of nerve fibers, associated with autoinflammatory infiltrates of the myenteric plexus, provide evidence of an immune-mediated destruction of the inhibitory neurons, not only by necrosis but also apoptosis (Fas/FasL overexpression); G: Autoimmune etiology of achalasia is further supported by the presence of anti-myenteric autoantibodies in sera; H: Pathophysiologically, achalasia is caused by autoinflammation, degeneration of nerves in the esophagus, plexitis, abnormalities in microvasculature, ganglionitis, and finally by the loss of inhibitory ganglion in the myenteric plexus. Red lines: Potential therapeutic targets.

its use as a bridge therapy, to be administered while another definite treatment option is actively sought for the patient under care, they note that it should be applied with caution as it is not free of complications[77]. Perhaps botulinum toxin has a role in treating patients in whom dilation or myotomy are contraindicated[3,8,78].

Endoscopical dilatation (ED) has been demonstrated as the most effective nonsurgical treatment of achalasia, but it has the highest rates of complications. For this procedure, pneumatic dilators are preferred over rigid and using a greater diameter of the dilator produces better long-term results (*i.e.*, change from 3.0 to 4.0 mm diameter increased the 4-year follow-up success rate to 93%). Dysphagia success rates at the 5-year and 15-year follow-ups were reported as 40%-78% and 12%-58%, respectively[8]. The complications of ED are various and include esophageal perforation (0%-16%; as low as 1.9% in experienced hands), GERD (15%-33%) and intramural hematomas. Due to the risk of esophageal perforation, all patients must first be confirmed as candidates for subsequent surgical interventions that will repair the

Table 1 Current treatment options in achalasia

Treatment option	Pros	Cons	Success rate
Oral agents	Non operative patients, on demand, dose adjustment	Adverse events, low duration, not a definitive method	28%-66% reduction of LES pressure
Pneumatic dilation	Short recovery, low procedure time, best non-surgical method	Perforation, multiple procedures needed, post procedure reflux	66%-90% 1 yr and 48% 10 yr
Heller myotomy	Most durable effect	Not applicable for high risk surgical patients, post-surgical reflux, anesthesia required	93% 1 yr 69%-80% 10 yr
Self-expanding metal stent	Good palliative option, high risk surgical patients	Expensive, stent migration, reflux (single center experience)	100% 1 mo, 83% 10 yr
POEM	Non-surgical, -low and -high risk patients	Complications (pneumothorax, reflux), not widely available, expertise	5%-62% reduction of LES pressure

Modified from Krill *et al*[90] and Dobrowolsky *et al*[92].

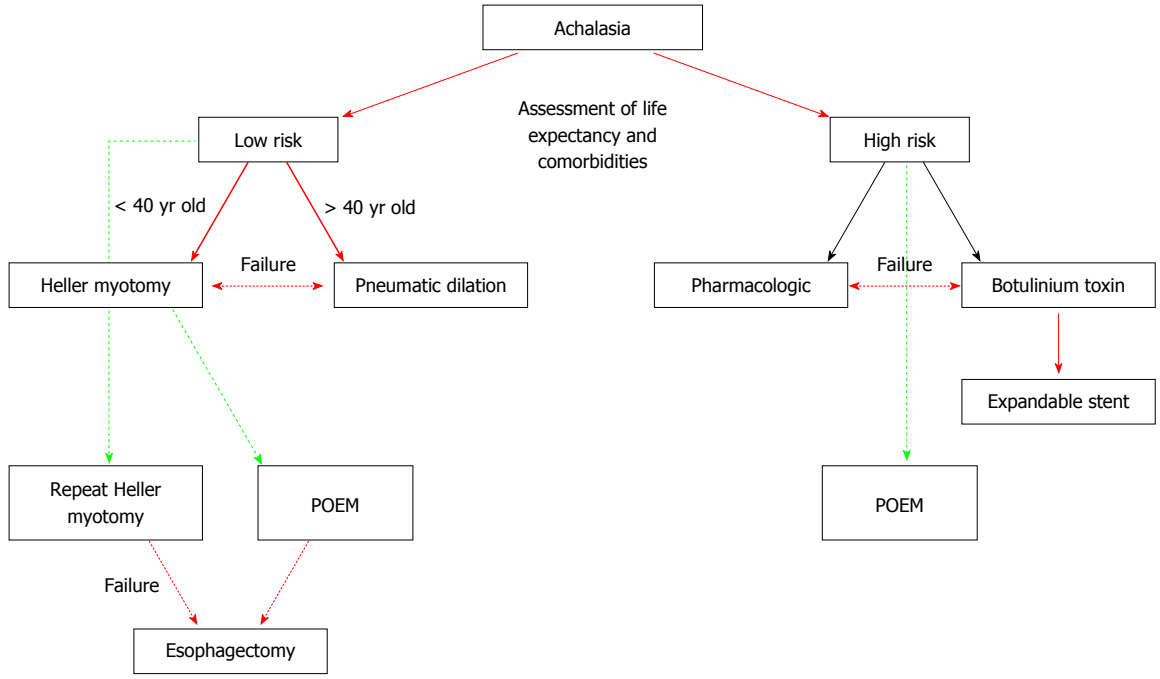

Figure 6 Proposed algorithm for the treatment of Achalasia patients (modified from Sioulas *et al*[95]). Red single arrow: recommended strategy; Red bold arrow: treatment for low-risk; Black single arrow: treatment for high-risk; Green dashed line: potential, low evidence treatment; Red dashed double arrow: alternative for treatment failure.

damage, and in cases of GERD post-dilation proton pump inhibitors are recommended[3,8].

Another treatment alternative for the treatment of achalasia includes self-expanded metal stents (SEMS). Experience is limited using this treatment option and includes a study with 75 achalasia patients in which a 30 mm SEMS was temporally placed under fluoroscopic guidance. After 4-5 d, SEMS where endoscopically removed and patients followed for up to 10 years. Success rate at 1 mo was 100% and 83% at 10 years[79]. Another study compared the efficacy of different SEMS diameters (20, 25 and 30 mm) in achalasia patients. A total of 90 patients were included and followed at 10 years and showed better success rate (83.3%) with a 30 mm SEMS compared to lower diameters[80]. SEMS have shown promising results but experience is limited to a single institution and cannot be widely recommended and more studies are needed in order to include this in the treatment algorithm for the treatment of achalasia.

Surgical therapies

The surgical approach that involves disruption of the muscle fibers of the LES (known as a Heller myotomy) has been demonstrated as very effective in treating patients with achalasia. Since its first description in 1913, this procedure has undergone multiple modifications that have optimized its application and outcome[8,74]. The current laparoscopic Heller myotomy (LHM) has reported success rates of 95% and 75% at 5-year and 15.8-year follow-ups, respectively. Comparison of the LHM approach against a combination approach of LHM + fundoplication showed that the latter reduced GERD rates substantially (48%

Table 2 Pathophysiology mechanisms and potential therapeutic targets in achalasia

Physiopathology mechanism	Therapeutic target
Incomplete relaxation of lower esophageal sphincter	[3]Botulinum toxin injection[3,8,74-77] [3]Pneumatic dilatation[3,8] [3]Heller myotomy[8,74,81-84] [3]Self-Expanding metal stent[79,80] [3]POEM[85,86,91]
Loss of inhibitory ganglion neurons and decrease of NO and VIP	[3]Nitrates administration (isosorbide dinitrate and nitroglycerin)[3,7,8] [1]Treatment with immunosuppressive drugs (prednisolone, methylprednisolone, beclomethasone, methotrexate, azathioprine or cyclophosphamide) in the early stage of the disease[39,42,87,88] [2]Transplantation of neural progenitor cells[69,89]
Immune-mediate response, inflammation, organ-specific autoimmune disease, and autoantibodies that causes neuritis and glanglionitis	[1]Treatment with immunosuppressive drugs (prednisolone, methylprednisolone, beclomethasone, methotrexate, azathioprine or cyclophosphamide) to avoid immune-mediate insult damaging the enteric innervation. Suppress the whole activated immune system, exerts antiproliferative and pro-apoptotic effects, particularly on activated T cells and suppress antibody formation by B cells[39,42,87,88] [2]Biologic therapy (monoclonal antibodies: TNF-α inhibitors, IL-6 inhibitor, anti-CD20 antibody, CTLA-4 antibody, IFN-g antibody, *etc*) in the early stage of the disease[48,93] [2]Administration of regulatory cells to downregulate inflammation and to induce immunological tolerance[50-52]
Fibrosis	[2]Extracellular matrix remodeling (Polymerized type I collagen)[94]
Neurotrophic viral infection (Herpes simplex virus, varicella zoster, measles, *etc*.), molecular mimicry, bystander activation, viral persistence and polyclonal activation	[2]Antiviral therapy in patients with recent-onset achalasia[6,34-36,72,73]
Genetics	[2]Genomic medicine for decrease the effect of certain environmental factors, such as infectious agents, on the burden of disease[61-66]

[1]Drugs or experimental procedures with therapeutic benefit; [2]Drugs or experimental procedures with potential therapeutic; [3]Therapies with marginal benefit.

vs 9%)[81]. Of note, it is recommended to perform a partial fundoplication after the Heller myotomy[82-84].

Peroral endoscopic myotomy

Peroral endoscopic myotomy (POEM) is a newly developed technique that involves creation of a submucosal tunnel to disrupt the muscular layers of the esophagus, and it is considered a promising approach for treating achalasia with minimal complications. It was developed by Inoue *et al*[85] in 2010 as a method that combines the benefits of myotomy with those of an endoscopic application; since then, this less invasive procedure has proven to be feasible, safe and effective. A meta-analysis found that POEM efficacy for dysphagia may be similar to that of laparoscopic myotomy, but with the added benefit of a lower hospital stay[86]. Like myotomy, POEM modifies the LES pressure and esophageal body motility. The rates of short-term clinical success (at 1-year follow-up) have been high, from 82% to 100%. Although long-term outcomes have not been reported yet, the 2-mo, 2-year and 3-year success rates are high, at 91.3%, 91.0% and 88.5%, respectively[86].

NEW PERSPECTIVES AND FUTURE TREATMENTS

The therapeutic approaches available today for the treatment of achalasia implies the destruction of the LES, rather than restoring or modifying the underlying pathology of the disease. In the last 10 years the immune-mediated hypothesis to explain the pathophysiology of achalasia has gained strong support based on objective evidence[6]. Considering an autoimmune etiology, it is theoretically reasonable to treat patients with immune modulatory drugs in the early stages of the disease, when there is presumably still a number of functional neurons that can be protected[69].

To our knowledge, there are only three case reports of corticosteroid use (specifically of prednisolone, methylprednisolone or beclomethasone) alone or combined with other immunosuppressive therapy (specifically of methotrexate, azathioprine or cyclophosphamide) for achalasia and the results show dramatic improvement of the clinical picture, with complete recovery of peristalsis corroborated by HRM[39,42,87,88]. This feature reinforces the concept of a cause-effect relationship of the immune-mediated insult damaging the enteric innervation. Although the studies show an important improvement, until today there is no definitive evidence that has been reported to support the widespread application of this therapeutic approach. Thus, future studies to estimate the benefit of immunosuppressive therapy are still required.

A possible alternative therapeutic approach, which still lacks strong evidential support, is transplantation of neural progenitor cells. Recently, investigators have demonstrated that stem cells with neurogenic potential can successfully engraft, survive, migrate and differentiate into neurons and glia within the

aganglionic intestine. Moreover, preliminary evidence has indicated that transplanted cell-based therapies can lead to a functional recovery of aganglionic gastrointestinal diseases, including achalasia[69,89]. This finding opens a promising avenue of scientific investigation for future studies to improve screening for early diagnosis or genetic predisposition[37,45,54,55,67-70] for idiopathic achalasia.

Last but not least, the use of antiviral therapy in patients with recent-onset achalasia is promising. By this therapeutic approach, the antigenic challenge would be eliminated and the immune response might be controlled in these patients[6,34-36,72,73] (Figure 5 and Table 2).

CONCLUSION

New research has introduced different perspectives regarding the possible etiology of achalasia. In the last 10 years, the immune-mediated hypothesis (as the primary pathophysiologic abnormality) has gained strong objective support. Yet, the therapeutic approaches available today for the treatment of achalasia still do not resolve the cause of the disease; instead, they target the consequences, focusing on the destruction of the LES rather than on restoring or modifying the underlying pathology. With better understanding of the pathophysiology of achalasia, new therapies may prevent permanent damage and stop the disease at early stages.

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REVIEW

Anemia and iron deficiency in gastrointestinal and liver conditions

Jürgen Stein, Susan Connor, Garth Virgin, David Eng Hui Ong, Lisandro Pereyra

Jürgen Stein, Interdisciplinary Crohn Colitis Centre Rhein-Main, Frankfurt am Main, Department of Gastroenterology and Clinical Nutrition, DGD Clinics Sachsenhausen, Frankfurt am Main, 60594 Frankfurt, Germany

Susan Connor, Department of Gastroenterology and Hepatology, Liverpool Hospital, South Western Clinical School, University of New South Wales, Liverpool NSW 2170, Australia

Garth Virgin, Vifor Pharma, Flughofstrasse 61, CH-8152 Zurich, Switzerland

David Eng Hui Ong, Division of Gastroenterology and Hepatology, University Medicine Cluster, National University Hospital, Singapore 119074, Singapore

Lisandro Pereyra, Servicio de Gastroenterología y Endoscopia, Hospital Alemán, Buenos Aires C1118AAT, Argentina

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Correspondence to: Jürgen Stein, MD, PhD, Professor, Interdisciplinary Crohn Colitis Centre Rhein-Main, Frankfurt am Main, Department of Gastroenterology and Clinical Nutrition, DGD Clinics, Schulstrasse 31, Frankfurt am Main, 60594 Frankfurt, Germany. j.stein@em.uni-frankfurt.de

Telephone: +49-69-905597810
Fax: +49-69-905597829

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Abstract

Iron deficiency anemia (IDA) is associated with a number of pathological gastrointestinal conditions other than inflammatory bowel disease, and also with liver disorders. Different factors such as chronic bleeding, malabsorption and inflammation may contribute to IDA. Although patients with symptoms of anemia are frequently referred to gastroenterologists, the approach to diagnosis and selection of treatment as well as follow-up measures is not standardized and suboptimal. Iron deficiency, even without anemia, can substantially impact physical and cognitive function and reduce quality of life. Therefore, regular iron status assessment and awareness of the clinical consequences of impaired iron status are critical. While the range of options for treatment of IDA is increasing due to the availability of effective and well-tolerated parenteral iron preparations, a comprehensive overview of IDA and its therapy in patients with gastrointestinal conditions is currently lacking. Furthermore, definitions and assessment of iron status lack harmonization and there is a paucity of expert guidelines on this topic. This review summarizes current thinking concerning IDA as a common co-morbidity in specific gastrointestinal and liver disorders, and thus encourages a more unified treatment approach to anemia and iron deficiency, while offering gastroenterologists guidance on treatment options for IDA in everyday clinical practice.

Key words: Iron deficiency anemia; Gastrointestinal bleeding; Nonsteroidal anti-inflammatory drugs; Gastritis; Infection; Bariatric surgery; Celiac disease; Gastrointestinal neoplasm; Chronic hepatitis; Non-alcoholic fatty liver disease

Core tip: Iron deficiency anemia (IDA) frequently originates in the gastrointestinal (GI) tract and is a common cause of patient referral to gastroenterologists. Guidelines for the management of IDA in GI conditions are lacking. Symptoms such as fatigue and impaired exercise capacity should prompt a diagnostic work-up for anemia (hemoglobin), iron status (transferrin saturation, ferritin) and inflammation (C-reactive protein). Treatment of IDA should aim to restore normal hemoglobin levels, red cell indices and iron status. Intravenous administration is the preferred iron treatment in patients with chronic GI bleeding, patients being unresponsive or intolerant to oral iron and patients requiring rapid hemoglobin correction.

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INTRODUCTION

Iron deficiency anemia (IDA) is a common complication in routine clinical practice that frequently originates in the gastrointestinal (GI) tract[1-3]. Patients with IDA are therefore often referred to gastroenterologists for further examination and/or treatment. IDA associated with GI disorders can substantially reduce quality of life, contribute to fatigue, and may even lead to hospitalization[4,5].

In contrast to the well-documented inflammatory bowel disease (IBD)-associated IDA[6,7], prevalence data for IDA associated with other pathological conditions of the GI tract are sparse (Table 1). Guidelines for the diagnosis and management of anemia and iron deficiency are available for IBD[8], but not for other GI conditions. Overall, there are three main pathological contributors to IDA, namely chronic bleeding, malabsorption and inflammation[9-13]. However, other factors, such as poor or selective diet, as well as iron malabsorption (*e.g.,* due to decreased gastric pH) should not be neglected in patients referred for IDA assessment. This applies particularly to elderly patients[14-18].

Despite the increasing availability of effective and well-tolerated parenteral iron preparations for the treatment of IDA[19,20], a comprehensive overview of treatment approaches of IDA in GI conditions is currently lacking. Furthermore, definitions of IDA are inconsistent across clinical studies and publications, with the terms "iron deficiency", "iron deficiency anemia" and "anemia" being used almost interchangeably. In addition, the diagnostic markers and cut-off levels used to define iron deficiency vary widely. While anemia is clearly defined according to the World Health Organization as a hemoglobin (Hb) level < 12 g/dL in women (< 11 g/dL in pregnant women) and < 13 g/dL in men, the situation is ambiguous for iron deficiency. Commonly, serum ferritin levels below 15-100 ng/mL (depending on the presence of concomitant inflammation) and transferrin saturation (TSAT) below 16%-20% are considered indicative of iron deficiency[1,7,8].

These aspects complicate the interpretation and comparability of data and highlight the need for standardization of definitions and proper assessment of iron status across different GI and liver diseases. Recently published reviews on IDA discuss IDA in general[21,22] but give only little attention to the fragmented yet consistent evidence that IDA is a common issue in most GI conditions. The aim of this review is therefore to illustrate how IDA represents a common co-morbidity in these disorders, and to encourage a more unified treatment approach to GI condition-associated anemia and iron deficiency (ID).

Relevant articles were identified by screening the PubMed database for articles on IDA or ID in the context of GI or liver disease and associated illnesses. Data reported in abstract form only were identified by manual search through abstracts from major congresses in the field. In addition, the authors' own literature databases were screened for suitable publications. The results were filtered for articles with information on anemia prevalence and/or anemia management. The last search was conducted in June 2015.

ANEMIA AND ID IN DIFFERENT CONDITIONS

Esophagitis and hiatal hernia

Gastric bleeding from Cameron lesions in large diaphragmatic or hiatal hernia is an established cause of IDA[23,24]. However, axial and paraesophageal hernia without Cameron lesions can also be associated with IDA[25]. The reported incidence of IDA for all types of hernia ranges from 8% to 42%, with an average of 20%[26].

Hiatal hernia increases the risk of IDA independent of comorbid esophagitis[27]. Suggested causes of hernia-related IDA are mechanical trauma plus esophagitis, erosions or gastro-esophageal acid reflux[25,26].

Notably, the absence of endoscopic evidence of erosions in the majority of patients with hernia-related IDA does not exclude their causal role[26]. Therefore,

Table 1 Overview of diseases considered to be associated with iron deficiency/iron deficiency anemia

Conditions	Anemia or IDA prevalence	Predominant pathological contributors to anemia			Association with anemia and ID
		Bleeding	Malabsorption	Inflammation	
Nonvariceal upper GI bleeding[33]	80%	√			> 80% of patients admitted to hospital with nonvariceal AUGIB were anemic at the time of discharge
Celiac disease[87-89]	32%-69%		√	√	Well-established relationship between celiac disease and IDA Most widely cited cause of IDA is abnormal iron absorption, but bleeding and inflammation are also known contributory factors
Intestinal parasitic infections[151]	33%-61%	√	√		*T. trichiura* and hookworm infections are closely associated with IDA
GI cancers[107,108,111,117,121-120]	50%-60%	√		√	CRC: IDA associated with greater tumor diameter and with cancers of the right side of the colon Polyps: IDA much more common with malignant polyps than benign polyps GIST: Most frequent presentation is GI bleeding, which can result in anemia. In pediatric GIST, anemia is the most frequent clinical finding Gastric cancers: 6.8-fold relative risk of gastric cancer in patients with Pernicious anemia Small bowel malignancies: Anemia among most common presenting symptoms Esophageal cancers: Patients with Fanconi anemia at increased risk
Esophagitis and hiatal hernia[23-26]	8%-42%	√			Gastric bleeding from hernia is an established cause of IDA Even in absence of visible lesions, large hernia may be a possible cause of IDA with unexplained etiology
Bariatric surgery[77,196]	10%-40%		√		ID and anemia are well-known risks after bariatric procedures, but causes are multifactorial and vary depending on exact procedure and patient population
Intestinal failure[101-103]	30%-37%	√	√		Intestinal failure is associated with ID due to malabsorption, GI blood loss, and multiple surgery
Diverticular disease[144]	25%	√			One of the most common causes of lower GI bleeding leading to IDA Increasing prevalence due to rise in elderly population
Restorative proctocolectomy[153]	6%-21%	√	√		IDA due to mucosal bleeding and impaired iron absorption in patients developing symptomatic or asymptomatic pouchitis
NSAID-associated fecal blood loss[1]	10%-15%	√			Even low dose aspirin and non-aspirin-NSAIDs increase mean fecal blood loss 2-4-fold compared with normal
Angiodysplasia[1]	5%	√			Most common cause of lower GI bleeding in the elderly
Gastric antral vascular ectasia (GAVE)[1,48,55]	1%-2%	√			Chronic, slow bleeding is typically associated with IDA
Gastritis[57,66]	NA		√	√	*H. pylori* infection suggested to play important role in development of IDA
Peptic ulcer[197]	NA		√	√	H. pylori infection and IDA as above. Additionally, bleeding from ulcer
Chronic hepatitis and liver conditions with GI bleeding[155]	75%	√			Chronic liver disease can be complicated by anemia, particularly due to bleeding
Non-alcoholic fatty liver disease (NAFLD)[171]	NA			√	One-third of adult NAFLD subjects are reported to be iron deficient, defined by a TSAT < 20%

H. pylori: *Helicobacter pylori*; AUGIB: Acute upper gastrointestinal bleeding; CRC: Colorectal cancer; GI: Gastrointestinal; GIST: Gastrointestinal stromal tumors; ID: Iron deficiency; IDA: Iron deficiency anemia; NA: Not available.

even if no lesions are visible during endoscopy, larger hiatal hernia should still be considered as a possible cause of IDA with unexplained etiology.

Surgery in combination with proton pump inhibitor (PPI) therapy is evidently no better than PPI therapy alone in treating and preventing the recurrence of IDA, even in the case of larger hiatal hernia[26].

Nonvariceal upper GI bleeding

Acute upper gastrointestinal bleeding (AUGIB) is a common disorder associated with a high mortality rate of 3% to 15%[28-30]. While peri-endoscopic management of AUGIB, including blood transfusions, has been well characterized and standardized[31,32], guidelines for the monitoring and treatment of IDA in

patients after non-variceal AUGIB are still lacking.

Recently, a retrospective study showed that more than 80% of patients admitted to hospital with non-variceal AUGIB were anemic at the time of discharge[33]. Of these, only 16% received a recommendation to begin oral iron supplementation while intravenous iron was not even considered, demonstrating that post-discharge anemia is often disregarded.

Studies analyzing the clinical impact and risks associated with anemia after AUGIB are scarce. One study revealed that patients with hemoglobin (Hb) values < 10 g/dL after AUGIB had two-fold greater risks of re-bleeding and mortality than patients with Hb values ⩾ 10 g/dL[34].

A double-blind, placebo-controlled trial, recently demonstrated that patients with IDA after non-variceal AUGIB clearly benefit from iron supplementation[9]. In this study, oral and intravenous iron appeared to be equally effective in raising Hb levels, probably since most patients were not iron deficient at enrolment. However, iron stores (measured as serum ferritin) were replenished most effectively with intravenous iron supplementation.

Regarding the transfusion of red blood cell concentrates (RBC), a recent study in patients with AUGIB (TRIGGER)[35] suggests that the Hb threshold for RBC transfusion can be safely lowered without adversely affecting clinical outcomes. This is in line with results in other indications such as cardiac surgery, critical care and hip surgery. Accordingly, restrictive Hb thresholds (< 8.0 g/dL) should be considered except for patients with ischemic heart disease as pre-existing comorbidity[35,36].

NSAID-associated fecal blood loss

The administration of nonsteroidal anti-inflammatory drugs (NSAIDs) is known for its association with upper and lower GI injury[37-39]. This injury can include bleeding[40-42] which may be severe enough to result in hospitalization[43,44]. Even low dose aspirin as well as non-aspirin-NSAIDs increase mean fecal blood loss from roughly 0.5 mL/d to 1-2 mL/d (*i.e.,* 0.5-1.0 mg iron loss/d)[42]. Among patients treated with aspirin doses ⩾ 1800 mg/d, 31% had a blood loss of ⩾ 5 mL/d (*i.e.,* ⩾ 2.5 mg iron loss/d). Although cyclooxygenase-2 (COX-2) inhibitors are associated with fewer GI injuries than traditional NSAIDs, long-term use of a COX-2 inhibitor may also induce GI injuries and require concomitant medication for associated anemia and small intestinal injuries[45]. Notably, routine endoscopic examination may not reveal NSAID-induced GI injuries. Therefore, capsule endoscopy is recommended to screen for GI injuries in patients taking NSAIDs and presenting with unexplained anemia or ID[41,45,46].

Portal hypertensive gastropathy and gastric antral vascular ectasia

Portal hypertensive gastropathy (PHG) and gastric antral vascular ectasia (GAVE), although being distinct entities[47], can cause chronic gastrointestinal blood loss in patients with liver cirrhosis[48,49]. Most frequently found in association with liver cirrhosis, PHG can also occur in non-cirrhotic patients (*e.g.,* splanchnic venous thrombosis)[50]. The management of PHG is based on reducing hepatic venous pressure gradients and iron replacement therapy and/or blood transfusions. Severe cases may require shunt procedures[51-53].

GAVE, first described in 1953[54] in a patient with chronic IDA, accounts for up to 4% of all non-variceal upper gastrointestinal bleedings. Although cirrhosis is found in up to 30% of GAVE patients and occurs in about 2% of patients awaiting liver transplantation[49,55,56], portal hypertension does not seem to play an important role in the development of GAVE. Treatment comprises, in general, endoscopic interventions (*e.g.,* Argon plasma coagulation, Nd: YAG laser) and surgical procedures (*e.g.,* antrectomy and Billroth I anastomosis) to manage lesions, and symptomatic therapy with iron supplementation or blood transfusions, depending on the severity of anemia[49-52].

Autoimmune atrophic gastritis

Autoimmune gastritis (AIG) is implicated in 20%-30% of IDA cases that are refractory to oral iron[57,58].

AIG, first described by Faber in 1909 as *achlorhydric gastric atrophy*, is a chronic progressive inflammatory condition leading to the decrease or disappearance of parietal cells, which results in reduced or absent acid production (hypochlorhydria or achlorhydria)[59].The lack of gastric acidity has only recently been confirmed as key factor for impaired intestinal iron absorption[60]. IDA is more often associated with AIG than classical pernicious anemia and frequently precedes vitamin B_{12} deficiency (at least in fertile women)[61,62].

Helicobacter pylori gastritis

IDA is a recognized extragastric manifestation of *Helicobacter pylori* (*H. pylori*) infection[63]. Over 50% of patients with unexplained refractory IDA have active *H. pylori* infection[57,58]. Data, showing that *H. pylori* eradication reverses IDA, were confirmed by several observational and interventional trials, subsequently summarized in two meta-analyses of randomized controlled trials[57,64,65]. Accordingly, the Maastricht IV *H. pylori* consensus report[66] and other national and international guidelines[67-69] recommend *H. pylori* eradication for the treatment of IDA of unknown origin. Notably, Bismuth-based eradication therapy is more effective in terms of increasing hemoglobin and iron stores than first line PPI-based triple therapy in patients with IDA and *H. pylori* infection[63].

Discussed mechanisms underlying the pathogenesis of *H. pylori*-related IDA include occult chronic GI bleeding due to gastric mucosal microerosions, competition for dietary iron by the bacteria, reduced

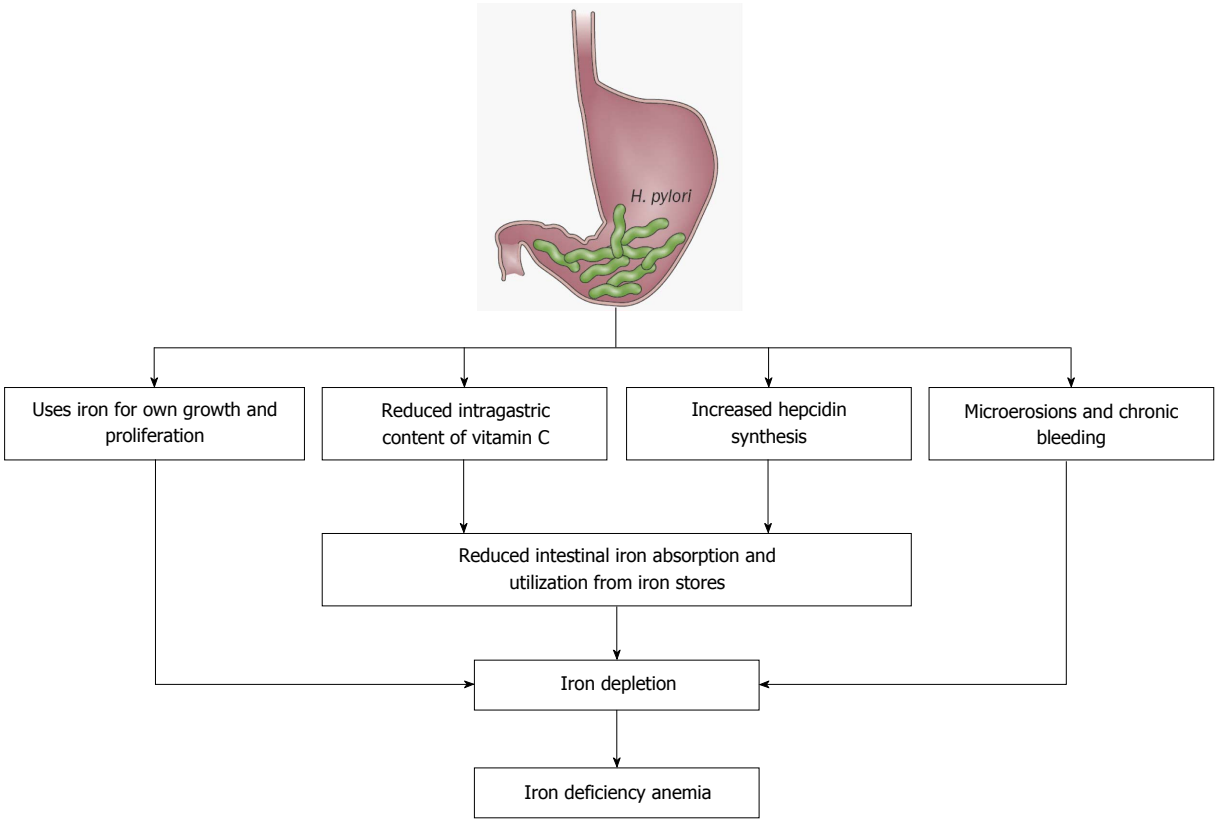

Figure 1 Pathogenic mechanisms proposed to be involved in the association of iron deficiency anemia and *Helicobacter pylori* infection[63].

ascorbic acid concentration in the gastric juice, affecting the absorption of dietary iron and upregulation of proinflammatory cytokines and hepcidin, the key regulator of iron homeostasis (Figure 1)[57,70-72]. In one study, *H. pylori* strains retrieved from patients with IDA exhibited faster, iron-dependent cell growth and an enhanced iron uptake than strains from patients without IDA[73]. Furthermore, *H. pylori* accelerates the development of inflammation, dysplasia and adenocarcinoma (mediated by the H. pylori virulence factor cytotoxin-associated gene A [CagA]) in an ID environment[74-76]. CagA also facilitates *H. pylori* colonization through iron acquisition, indicating that CagA provides a survival advantage for *H. pylori* in this setting.

Bariatric surgery

There is growing evidence of potentially severe, occasionally even life-threatening, nutritional and pharmacological consequences of bariatric surgery[77]. ID and IDA after bariatric procedures can result from intestinal bleeding (*e.g.*, from marginal ulcers) or reduced iron absorption due to postoperative intolerance for red meat, diminished gastric acid secretion or exclusion of the duodenum from the alimentary canal[78,79].

The reported incidence of ID in patients following gastric bypass surgery ranges from 12% to 47%[77]. However, the interpretation of reported incidence rates is complicated by different definitions of ID and IDA, postsurgical follow-up periods, types of interventions and patient populations[80]. In a large, patient record review of 959 patients who underwent laparoscopic Roux-en-Y gastric bypass (RYGB) between 2001 and 2011, 51.3% were iron deficient and 6.7% required intravenous iron therapy[81]. Amongst these patients, the prevalence of ID is significantly higher in premenopausal women than in postmenopausal women and males (72% *vs* 35% and 20%). Comparing different types of surgery, a cross-sectional pilot study of 95 patients showed no significant difference in ID rates after RYGB or sleeve gastrectomy (30% *vs* 36.4%)[82].

Since oral iron substitution has been shown to be relatively ineffective following bariatric surgery, and tolerance to oral iron preparations is often poor, intravenous iron treatment has been put forward as a preferable option[77]. Some authors suggest that repeated doses of intravenous iron may be required over the course of a year[83].

Ferric carboxymaltose (FCM) showed promise for the treatment of IDA in five phase 3 clinical trials involving 281 patients who had undergone bariatric surgery[84]. FCM exhibited similar or improved efficacy in terms of increasing hemoglobin, ferritin and transferrin saturation (TSAT) values and a favorable safety profile compared with standard medical care (iron sucrose, ferric gluconate, iron dextran or oral

iron). In addition, FCM offered the possibility for larger single-dose administrations at fewer visits compared to iron sucrose, ferric gluconate and oral iron[84].

Celiac disease

Celiac disease is one of the most common chronic inflammatory conditions of the GI system, affecting about 1% of the population[85]. There is a well-established relationship between celiac disease and IDA[86]. Anemia is the most common presenting symptom of celiac disease, found in 32%-69% of adult patients[87-89]. Approximately 80% of anemic patients with celiac disease are also iron-deficient[88,89]. In 49% of anemic patients with celiac disease, ID was found to be the only detectable abnormality[87]. Conversely, among patients presenting with unexplained IDA, 5% have histologically-confirmed celiac disease[57,90,91].

Impaired iron absorption (due to villous atrophy of the intestinal mucosa) and blood loss are important pathological contributors to anemia in celiac disease[92]. Occult GI bleeding was detected in about half of patients with celiac disease adhering to a gluten-free diet[93]. In some patients, nutritional deficiencies may also be a (contributing) causative factor[94]. Inflammation is a major contributor to IDA, with interleukin (IL)-1, IL-6, IL-10, interferon (IFN)-γ and tumor necrosis factor (TNF)-α as inducers of hepcidin, the main regulator of iron homeostasis[92,95]. Accordingly, celiac disease-related IDA is refractory to oral iron treatment[57,92,96], and even after switching to a gluten-free diet, it takes 6-12 mo until most patients recover from anemia[97]. Notably, half of patients remain iron-deficient even after 1-2 years on a gluten-free diet. The slow or lacking recovery from ID may be due to the low absorption rate of nutritional iron (1-2 mg/d), which hinders the repletion of severely depleted iron stores, and the potentially low content of iron and other micronutrients in a gluten-free diet[98].

Therefore, patients with celiac disease clearly benefit from immediate intravenous iron treatment instead of switching to intravenous iron only after (foreseeable) non-response and/or intolerance to oral iron[96].

Intestinal failure

Intestinal failure (IF) results from obstruction, dysmotility, surgical resection, congenital defect, or disease-associated loss of absorption, and is characterized by the inability to maintain protein-energy, fluid, electrolyte, or micronutrient balance[99]. In patients suffering from IF, total parenteral nutrition (TPN) is a life-saving intervention until full or partial recovery of enteral nutrition (EN)[100]. Nevertheless, patients with IF are prone to ID as a result of malabsorption, gastrointestinal blood loss and multiple surgical procedures. Accordingly, ID is the most common micronutrient deficiency during and after transition from TPN to EN, with reported incidences of 60%-80% for ID, and 30%-37% for IDA[101-103]. A study from the Mayo Clinic, including 185 patients, showed that IDA developed much more rapidly in patients with fistula and bowel obstruction than in those with short bowel syndrome (SBS) and dysmotility[104]. Despite the high prevalence of ID, iron is not routinely added to parenteral nutrition formulations because of the risk of anaphylaxis and concerns about incompatibilities. Although data describing the compatibility of iron supplementation with parenteral formulations are conflicting[105], iron dextran has been found to be compatible with lipid-free solutions at an amino acid concentration > 2%[106]. A safer approach would prescribe intermittent infusion of therapeutic iron doses.

GI cancers

Anemia and IDA are common in patients with colorectal cancer (CRC), with a prevalence of 50%-60%[107-111]. Risk factors for anemia in patients with CRC are greater tumor diameter and cancer in the right side of the colon[108,112]. CRC is a cause of lower GI bleeding in 11% to 14% of cases[113], and malignant polyps are associated with greater blood loss and more frequent occurrence of IDA than benign polyps[114].

Anemia has also been described in the context of gastrointestinal stromal tumors (GIST), which are frequently associated with acute or chronic bleeding[115,116]. In pediatric GIST, anemia is the most frequent clinical finding (86% with symptomatic anemia)[117]. Notably, anemia is also one of the most frequent side effects of imatinib, the standard treatment for advanced/metastatic GIST, including small bowel cancers[118,119]. In addition to IDA, other specific forms of anemia such as pernicious anemia and Fanconi anemia are also increased in patients with gastric cancers (6.8-fold relative risk of pernicious anemia)[120], small bowel malignancies[121] and esophageal cancers[122].

Notably, ID (with or without anemia) is associated with an increased risk of GI malignancy 2 years after diagnosis of ID[123]. Therefore, unexplained IDA is an important measure for detection of GI malignancy[108]. In patients with advanced CRC, Hb levels < 11 g/dL are a poor prognostic factor[124] and prompt referral as well as investigation of IDA are recommended in patients with CRC[124-126] and cancers in general[127]. However, treatment options for IDA are not discussed, as the guidelines primarily focus on surgical follow-up for CRC.

Since CRC surgery may result in significant blood loss, perioperative allogeneic blood transfusion (ABT) has often been used in CRC patients[128,129]. However, ABT is associated with certain risks, such as an increased infective complication rate and increased disease recurrence[130-132]. Furthermore, ABT involves significant cost[133], and RBCs are an increasingly limited resource. Therefore, alternative options such as perioperative intravenous iron administration have been examined[19,128,129,134] and a multicenter

randomized controlled trial comparing intravenous ferric carboxymaltose with oral iron as preoperative anemia treatment in colorectal cancer patients is ongoing[135]. One randomized prospective placebo-controlled pilot study showed preoperative intravenous iron sucrose (total iron dose 600 mg) to have no effect on serum Hb concentration or the rate of blood transfusion in 62 patients scheduled for resection of suspected colorectal cancer[128]. However, the trial included only 11 patients with confirmed anemia, while 22 had a normal Hb, and in 29 patients, there was no recent record of anemia status at all. Furthermore, a Hb increase of 0.5 g/dL, defined as the primary endpoint, is clinically insignificant and unlikely to have an impact on perioperative transfusion requirements[136]. In general, a Spanish expert panel on alternatives to allogeneic blood transfusion suggests perioperative intravenous iron administration to anemic patients scheduled for gastrointestinal surgery[137]; yet overall, there are only few high-quality prospective studies of sufficient power[138].

Diverticular disease

Diverticular disease, one of the most common causes of lower GI bleeding[113,139], accounts for 30%-50% of massive lower GI bleeding cases[140]. Diverticulitis is a major healthcare problem which often requires surgical management to optimize patient outcomes[141]. Despite reports of IDA associated with clinical cases of diverticulitis[142,143], information on prevalence is lacking. In a study of 1124 cases of colonic diverticular disease seen at a hospital clinic during a 15-year period, 44 (3.9%) had diverticular hemorrhage and 25% of these patients had anemia (Hb < 12 g/dL)[144]. Anemia was most frequent in elderly patients (60 years and upwards) and those with acute bleeding.

Angiodysplasia

Angiodysplasia is a poorly understood clinical condition involving fragile, thin-walled vascular malformations which are susceptible to rupture, and may thus cause severe GI bleeding[145]. Angiodysplasia accounts for up to 5% of cases of GI bleeding overall and up to 40% of obscure GI bleeding cases[146,147]. Angiodysplasia has been found to be present in 61% of patients over the age of 60, often with co-existing conditions[148]. Chronic angiodysplasia can be difficult to manage due to frequent rebleeding of multiple lesions clustered in different localizations of the GI tract and therefore frequently results in chronic IDA[149].

In the past, patients with angiodysplasia commonly had numerous and frequent blood transfusions and suffered end-organ damage due to refractory anemia[149]. Modern intravenous iron preparations can be considered a valuable treatment option if blood loss exceeds 10 mL/d (*i.e.*, around 5 mg iron)[149].

Intestinal parasitic infections

Several studies have shown parasitic infections, especially *T. trichiura* and hookworm infections, to be closely associated with IDA[150-152]. Hookworm infections are associated with mucosal damage and endogenous loss of iron[151], while *T. trichiura* and *E. histolytica* cause bleeding and dysentery by invading the mucosa of the large intestine. Accordingly, intestinal parasitic infections are recognized as predictors of IDA.

Restorative proctocolectomy

A frequent complication of restorative proctocolectomy is pouchitis, which in turn is associated with IDA (6%-21% of patients with functional pouches) due to mucosal bleeding and impaired iron absorption[153]. Notably, pouchitis can be asymptomatic but still be associated with IDA, as can pouches in the abscence of pouchitis. In patients that are intolerant or unresponsive to oral iron, intravenous iron and erythropoiesis-stimulating agent (ESA) treatment can correct the anemia. Another deficiency, vitamin B_{12} deficiency, occurs in 25%-53% of pouch recipients (compared to 3%-40% in the general population), being also a frequent cause of anemia. In general, vitamin B_{12} deficiency can be resolved with oral cyanocobalamin[153,154], suggesting a post-procedural change in dietary habit as the main reason for this deficiency.

Chronic hepatitis and liver conditions

Among patients with chronic liver disease, 75% are anemic[155], mainly due to acute or chronic GI hemorrhage which may lead to iron deficiency as a consequence. Acute gastrointestinal hemorrhage is a potentially serious complication of portal hypertension and the second most common cause of mortality in patients with cirrhosis. The increased risk of bleeding in severe hepatocellular disease can result from impaired blood coagulation due to reduced synthesis of blood coagulation factors by hepatocytes, and lower thrombocyte numbers. Initial treatment aims to correct hypovolemia and restore stable hemodynamic function (*e.g.,* gelatin-based colloids, solutions of human albumin or red blood cell transfusion)[155]. In addition, IDA caused by chronic blood loss may be treated with oral iron or intravenous iron in cases of advanced chronic liver disease.

Notably, anemia is frequently associated with both peginterferon (PEG-IFN) and ribavarin (RBV) in the treatment of chronic hepatitis C virus (HCV) infection, particularly when these drugs are administered in combination[156-158]. According to the WHO guidelines, grade 1 anemia (Hb 10-11 g/dL) has been reported in up to 30% and grade 2 (< 10 g/dL) in 9%-10% of cases. The addition of direct-acting anti-virals (DAAs) such as telaprevir (TVR) or boceprevir (BOC) as part of

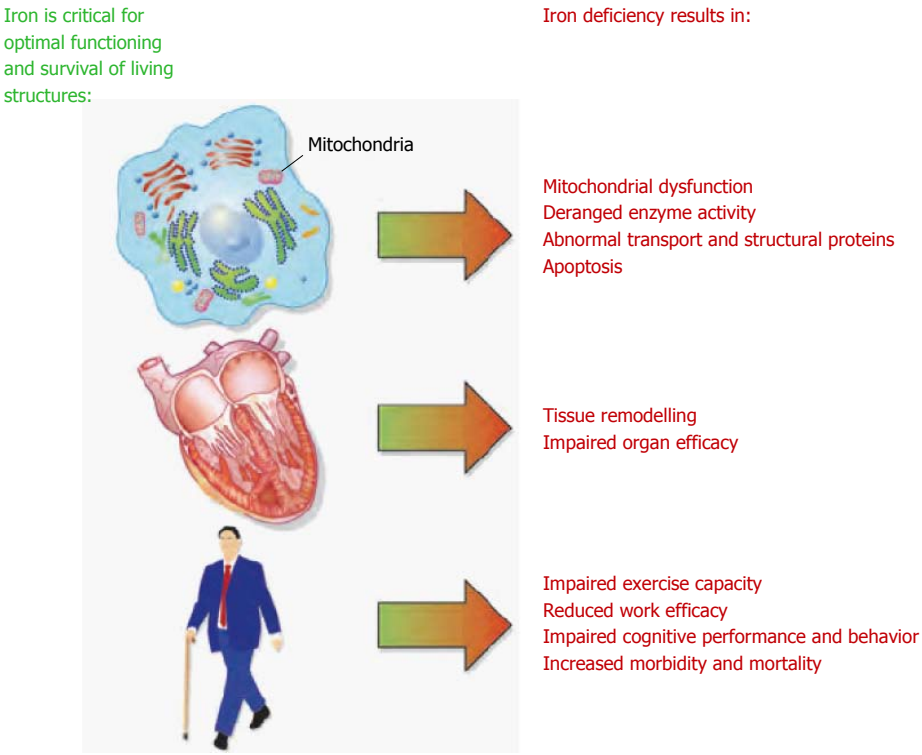

Figure 2 Role of iron in essential cellular functions[178].

the more effective antiviral triple combination therapy has been shown to increase anemia by up to 20% compared to PEG-IFN/RBV in both treatment-naïve and -non-naïve patients[159-163]. Since this treatment-induced anemia is mainly due to hemolysis, dose reduction of RBV by up to 50% is recommended, followed by administration of recombinant erythropoietin[156,164-166]. Evaluation of inosine triphosphatase polymorphisms may help to predict the risk of anemia and response to treatment[156,167-169]. Second generation DAAs, including simeprevir (SMV), sofosbuvir (SOF), daclatasvir (DCV), and ledipasvir (LDV), approved in combination (*e.g.*, SOF/SMV) as IFN-free regimens for the treatment of genotype 1 HCV infection, offer significantly greater cure rates and shorter treatment duration, and have been associated with lower incidence rates of anemia, ranging from 5% to 20%[170].

Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is becoming the most common liver disease worldwide (estimated prevalence of 25%-30%), with one-third of adult patients being iron deficient (TSAT < 20%)[171]. ID was significantly associated with female gender, obesity, increased BMI, lower alcohol consumption, non-white race and increased levels of IL-6 and IL-1ß. In contrast to patients with obesity-related, low-grade inflammation, serum hepcidin levels were low in NAFLD subjects with ID, reflecting an appropriate response of hepcidin signaling to ID. The authors concluded that initially, obesity-induced systemic inflammation may increase hepcidin levels and contribute to ID, but hepcidin is appropriately downregulated after ID is established[171]. Similar results of decreased intestinal iron absorption that are inversely associated with serum and urinary hepcidin levels have been reported in dysmetabolic iron overload syndrome (DIOS)[172] which is associated with half of NAFLD cases. Based on hepatic gene expression studies in pediatric patients with non-alcoholic steatohepatitis (NASH), it is hypothesized that, (1) a decreased level of transferrin receptor Ⅰ in NASH patients is an indicator of reduced erythropoietic activity in the bone marrow, a typical feature of anemia of chronic inflammation; and (2) that elevated expression of transferrin and transferrin receptor Ⅱ may result in the upregulation of hepcidin, leading to impaired duodenal iron absorption. In addition, the authors demonstrated that elevated serum ferritin levels do not reflect increased hepatic iron stores in patients with NASH, but are rather a consequence of hepatic and/or obesity-related inflammation[173].

DISCUSSION

While the origin of IDA is often multifactorial, a close relationship with various GI conditions has been established (Table 1)[1,2]. Nevertheless, management of patients with IDA often remains inadequate[1,4].

Even without anemia, ID can have a substantial impact on physical and cognitive function and quality of life (*e.g.*, fatigue)[174-177] (Figure 2). This supports

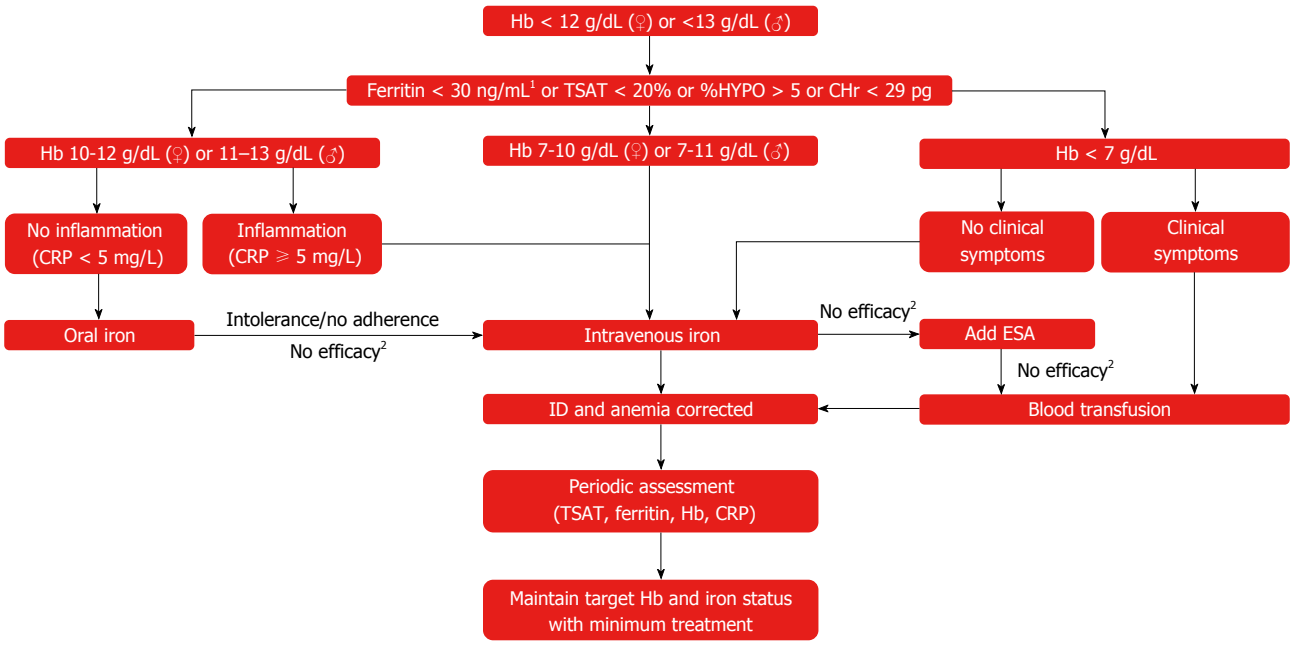

Figure 3 Suggested approach for the assessment and treatment of iron deficiency/iron deficiency anemia in clinical practice. [1]In patients with inflammation, ferritin levels < 100 ng/mL should be considered as iron-deficient; [2]Hb increase < 2 g/dL in 4 wk. Stein *et al*[6]. CHr: Hemoglobin content of reticulocytes; CRP: C-reactive protein; ESA: Erythropoiesis-stimulating agent; Hb: Hemoglobin; %HYPO: Percent hypochromic red blood cells; ID: Iron deficiency; IDA: Iron deficiency anemia; TSAT: Transferrin saturation.

the need for regular assessment of iron status and consideration of the clinical consequences of any disturbance of iron status. Patients with typical GI symptoms, such as epigastric pain, change in bowel habit, weight loss, early satiety, or poor appetite, should be assessed for ID and anemia, since these symptoms are often associated with acute or chronic blood loss, malabsorption and/or chronic inflammation[5]. In the planning of treatment for ID/IDA and the selection of the iron administration route, the frequency and magnitude of blood loss as well as the known side effects of oral iron should be considered[4].

Although chronic GI bleeding and malabsorption in GI conditions are well-recognized causes of ID/IDA[5,12,13], other factors such as age and chronic inflammation that inhibit iron availability via increased hepcidin levels should also be borne in mind[2,15,179]. Normal iron homeostasis is based on two pillars: absorption of nutritional iron by enterocytes in the duodenum and upper jejunum (1-2 mg/d), and recycling of iron *via* phagocytosis of senescent red blood cells (20-25 mg/d)[179]. Absorbed or recycled iron is transiently stored in the monocytes/macrophages of the reticuloendothelial system, from where it is released *via* ferroportin, loaded on transferrin and transported to the bone marrow for erythropoiesis. Hepcidin is a key regulator of iron homeostasis, blocking the ferroportin-mediated release of iron from enterocytes and macrophages, and impairing the utilization of nutritional or supplemental oral iron. In patients with inflammation, iron release from the reticuloendothelial system is reduced to 44% of that measured in normal subjects[180].

Diagnosis of anemia and ID involves standard laboratory tests (Hb, serum ferritin, TSAT) and blood counts, with low serum Hb or abnormal red blood cell indices usually being the initial finding in a routine complete blood count[5,181]. In some regions, anemic patients should be tested for hemoglobinopathies to exclude genetic reasons for anemia. Since little consensus exists among guidelines on different GI conditions as to the level of anemia that requires follow-up, it has been recommended that any degree of anemia should be further investigated for the presence of ID (Figure 3)[1].

In clinical practice, iron status is mainly assessed on the basis of serum ferritin levels[182]. However, serum ferritin is subject to gender differences and falsely elevated levels in populations with inflammatory reactions since it is also an acute-phase reactant[181]. Therefore, the diagnostic workup of anemic patients (*i.e.*, men with Hb < 13 g/dL or non-pregnant women with Hb < 12 g/dL) should include CRP, to detect underlying inflammatory reactions (suggested cut-off 5 mg/L), and TSAT (suggested cut-off 20%), a marker of low iron availability that is less affected by inflammatory reactions[181,182].

Additional markers of ID include the percentage of hypochromic red cells (%HYPO, suggested cut-off 5%) and the hemoglobin content of reticulocytes (CHr, suggested cut-off 29 pg) as well as serum levels of soluble transferrin receptors (sTfR) and zinc protoporphyrin (ZPP)[17,181]. Since sTfR levels reflect the erythropoietic activity rather than the iron status, sTfR

Table 2 Estimated total iron deficit (mg elemental iron) based on hemoglobin and body weight

Degree of iron deficiency	Hemoglobin level (g/dL)	Iron deficit (mg)	
		Body weight < 70 kg	Body weight ⩾ 70 kg
Moderate	10-12 (women) 10-13 (men)	1000	1500
Severe	7-10	1500	2000
Critical	< 7	2000	2500

Simplified scheme for estimation of total iron requirements[6].

cannot be used in patients treated with ESAs.

Analogous to patients with IBD, iron replacement should also be initiated in non-IBD patients, once IDA is clearly ascertained or deemed likely based on assessed iron markers. Treatment options for IDA include oral and parenteral iron, erythropoiesis-stimulating agents and blood transfusions. There is widespread support for iron supplementation both for the correction of anemia and for replenishment of body iron stores[1]. Currently, the first-line approach for treating IDA is oral iron; usually, 200 mg iron is administered twice daily, but lower doses may be as effective and better tolerated[1]. However, the efficacy of oral iron may be limited when GI uptake is impaired (*e.g.,* due to chronic inflammatory conditions, celiac disease or duodenal resection) or patient compliance is poor (*e.g.,* due to gastrointestinal side effects such as nausea, flatulence and diarrhea[8]). Also large iron deficits that result from chronic or acute GI bleeding or perisurgical blood loss cannot be adequately and quickly counteracted with oral iron. Notably, oral iron can exacerbate existing symptoms of GI disease[4,7], and particularly oral ferrous salts lead to oxidative stress, as evidenced by increased levels of non-transferrin bound iron (NTBI)[183].

Intravenous iron has proven its efficacy and tolerability in a wide range of therapeutic areas, and is recommended in respective treatment guidelines[1,8,127,184-186]. In particular, parenteral iron is considered advisable for patients with GI conditions who cannot be treated adequately with oral iron supplements due to severe GI side effects, inadequate absorption, or anemia requiring urgent correction. Intravenous iron replacement facilitates faster correction of ID and avoids GI side effects by bypassing the GI tract. Although intravenous iron is more costly than oral treatment, administration by a medical professional ensures compliance and more reliable repletion of iron stores which in turn may prevent anemia recurrence and related treatment costs in the long term.

The underutilization of intravenous iron is largely based on past experience with high molecular weight iron dextran (HMWID) that is associated with anaphylactic reactions and therefore has been removed from the market in the United States and Europe. In recent years, safety of intravenous iron has been vastly improved by new, well-tolerated preparations[57,149,187]. A review issued by the United States Food and Drug Administration (FDA) studying serious adverse reactions across different intravenous compounds (iron sucrose, ferric gluconate and low molecular weight iron dextran) showed a cumulative rate of only < 1:200000[188,189]. In 2013, the European Medicines Agency (EMA) published an assessment report[190] concluding that the benefits of intravenous iron-containing medicinal products continue to outweigh the risks in the treatment of iron deficiency when the oral route is insufficient or poorly tolerated. Notably, the EMA removed the necessity of a test dose, yet trained staff and resuscitation facilities to manage anaphylactic or anaphylactoid reactions should be available when any intravenous iron product is administered.

Traditional calculation of iron deficits (iron doses) with the Ganzoni formula is error-prone, inconvenient and underestimates iron requirements[191]. Accordingly, a more simple fixed-dose regimen (of ferric carboxymaltose) based on Hb and body weight (Table 2) was tested in IBD patients, and found to be superior to the Ganzoni-calculated dosing (of iron sucrose) in terms of efficacy and compliance[192]. This novel dosing scheme can equally be utilized as a simple dosing guide for other patient groups and iron formulations that can be given at doses of 1000 mg per administration for efficient and rapid iron replenishment. Most clinical trial and observational data on high dose iron administration have been generated with ferric carboxymaltose and low molecular weight iron dextran. In cases of severe anemia, the iron dose should be increased by 500 mg.

In response to parenteral iron administration, serum ferritin is greatly elevated for the first 8 wk after infusion. Therefore, ferritin should be monitored only after 8-12 wk, and in case of iron overload (TSAT > 50%), treatment should be adjusted accordingly.

Treatment response to intravenous iron replacement can be defined as an increase in hemoglobin levels of ⩾ 2 g/dL within approximately 4-8 wk of infusion and restoration of appropriate iron availability (TSAT ⩾ 30%). Patients who show limited or no response to intravenous iron therapy, especially those with anemia of chronic inflammation, should be considered for adjunctive treatment with ESAs (target Hb level ⩽ 12 g/dL). Overall, intravenous iron replacement is increasingly recommended by gastroenterologists[1,5,8,149].

Regardless of the route chosen, iron therapy must continue after resolution of anemia until iron stores are completely replenished[4]. Once Hb levels and red cell indices have been normalized, they should be monitored at regular intervals[1]. The authors of the British Society of Gastroenterology guidelines propose assessments at 3-monthly intervals for one year, then after a further year, and immediately if symptoms of anemia reoccur[1].

Blood transfusions should be used only as a last

option and as rescue treatment when faced with a life-threatening situation (*e.g.*, in severe cases of acute bleeding)[4,5]. There is a wealth of evidence concerning post-operative mortality and morbidity following blood transfusion, even after transfusion of a single RBC unit[193-195]. Consequently, a restrictive approach to blood transfusion is warranted except for patients who present with ischemic heart disease as pre-existing comorbidity[35,36,195]. In patients with GI disease, transfusions should aim to restore Hb to a safe level, but not necessarily up to normal values, and iron supplementation should be given subsequently to replenish stores[1].

CONCLUSION

IDA is a common comorbidity in patients with GI or liver disorders. In general, the origin of IDA can be multifactorial, with bleeding, malabsorption and inflammation playing important roles in the context of different GI conditions. IDA can contribute substantially to the morbidity and mortality of the underlying disorder and even ID without anemia can reduce quality of life, exercise capacity and cognitive function. Therefore, effective treatment of ID and IDA as well as prevention of recurrence are necessary and may provide an important alleviation of the overall disease burden. The lack of guidelines on diagnosis and treatment of IDA in the field of GI disease results in suboptimal assessment and management of IDA.

The standard laboratory approach used to investigate IDA would benefit from inclusion of TSAT assessment, which is less affected by inflammatory reactions than the commonly-used acute-phase protein serum ferritin.

Oral iron, often selected as the initial treatment option, has considerable limitations in GI patients due to severe GI side effects, inadequate absorption and a slow course of action. Furthermore, patient compliance with oral iron therapy is often poor. If oral therapy fails or is inadvisable, intravenous iron replacement is a valuable option. Intravenous iron therapy is more efficient than oral iron, and faster at increasing Hb levels and replenishing iron stores. Iron therapy should be continued until iron stores are completely replenished. During subsequent follow-up visits for their GI disorder, patients should be routinely monitored for any signs of ID or IDA.

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REVIEW

HER2 aberrations and heterogeneity in cancers of the digestive system: Implications for pathologists and gastroenterologists

Nicola Fusco, Silvano Bosari

Nicola Fusco, Silvano Bosari, Division of Pathology, Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico, 20122 Milan, Italy

Nicola Fusco, Silvano Bosari, Department of Pathophysiology and Organ Transplantation, University of Milan, 20122 Milan, Italy

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Correspondence to: Silvano Bosari, MD, Professor, Division of Pathology, Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico, Via Francesco Sforza, 35, 20122 Milan, Italy. silvano.bosari@unimi.it
Telephone: +39-2-55032415

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Abstract

Management of cancers of the digestive system has progressed rapidly into the molecular era. Despite the significant recent achievements in the diagnosis and treatment of these patients, the number of deaths for these tumors has currently plateaued. Many investigations have assessed the role of *HER2* in tumors of the digestive system in both prognostic and therapeutic settings, with heterogeneous results. Novel testing and treatment guidelines are emerging, in particular in gastric and colorectal cancers. However, further advances are needed. In this review we provide a comprehensive overview of the current state-of-knowledge of *HER2* alterations in the most common tumors of the digestive system and discuss the operational implications of *HER2* testing.

Key words: HER2; Digestive system; Gastrointestinal tract; Gastric cancer; Colon cancer; Esophageal cancer; Gastroesophageal junction cancer; Biliary tract cancer; Gallbladder cancer; Liver cancer; Pancreas cancer

Core tip: Numerous studies have broadened our understanding of *HER2* as a critical oncogene in many human cancers, including tumors of the digestive system. Due to the increasing importance of *HER2* testing in this heterogeneous group of tumors, in this review we seek to outline the current state of knowledge of *HER2* alterations in the most common malignancies occurring in the digestive system, to examine the operational implications of *HER2* testing as a biomarker and potentially targetable gene, and discuss immediate future perspectives for pathologists and gastroenterologists.

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INTRODUCTION

The epidermal growth factor receptor 2 is a proto-oncogene that was first identified in the early 1980s in rodent neural tumor cell lines and therefore named *neu*[1]. Given the homology between the human gene and that of the rodent, adherence to appropriate nomenclature is pivotal to avoid any confusion. In this review, *HER2/neu* will refer to the gene across both species, while *HER2* and erbB2 will be used specifically to indicate the human gene and its protein product, respectively[2]. *HER2/neu* belongs to one of the most studied growth factor receptor systems in cancer, the erbB tyrosine kinase family[1-4]. This family consists of four members encoding the homologous epidermal growth factor receptor proteins erbB1, 2, 3, 4 that are ubiquitously expressed in epithelial, mesenchymal, and neuronal normal cells and their cellular progenitors[5,6]. Each of these receptors is composed of an extracellular ligand-binding domain, a transmembrane segment and an intracellular protein kinase domain with a carboxyl terminal segment holding site of phosphorylation or tyrosine residues (Figure 1)[5,7,8]. Among the four erbB proteins, erbB2 is functionally characterized by an extraordinarily strong catalytic kinase activity, representing a key oncoprotein that triggers cornerstone intracellular signaling events for cell growth and survival, ultimately leading to increased signal transduction and activation of the MAPK and PI3K/Akt pathways[4,6,9]. Importantly, erbB2 is not involved in ligand binding of the growth factors unless is overexpressed[10], while the other members of its family represent active receptors in basal conditions also[5,9], as outlined in Figure 1.

Numerous preclinical and clinical studies, beginning with the intuition of Slamon and collaborators on the role of *HER2* in breast cancer, have broadened our understanding of this oncogene in many human cancers, including digestive system cancers (DSC)[8,11]. While *HER2* represents a prognostic marker of aggressive behavior in many DSC[8,12,13], the importance of this oncogene remains closely related to its role as a potentially targetable cancer gene[4,14]. To date, anti-*HER2* antibodies such as trastuzumab, pertuzumab, the new conjugate ado-trastuzumab emtansine, and *HER2*-inhibitors (*e.g.,* lapatinib) have received the United States Food and Drug Administration (FDA) approval not only in *HER2*-positive breast cancers but also in *HER2*-positive metastatic gastric cancer (GC)[15]. Massively parallel sequencing studies have recently revealed that a substantial proportion of DSC are genetically characterized by *HER2* alterations (Figure 2)[16]. However, highly different percentages in the incidence of these molecular aberrations, ranging from 0 to 50%, have been reported even within the same anatomic site, such as the pancreas (Table 1)[16-19]. These partially discordant observations could have been, at least in part, responsible for the nihilistic view of *HER2* in the targeted therapeutic regimens for extra-gastric DSC. Many groups are currently establishing the role of *HER2* in DSC in both prognostic and therapeutic settings. However, targeting of tumors that overexpress erbB2, albeit representing the reality for advanced GC and gastroesophageal junction (GEJ) cancer, is considered a reasonable future option[8,20]. At present, the role of trastuzumab in DSC is being explored by a variety of translational research molecular pathology studies, as well as clinical trials.

Management of DSC has progressed rapidly into the molecular era[21-30]. However, the "trastuzumab-revolution" that we have experienced in the breast has yet to be realized in the digestive system tract and its accessory organs[8,31]. Due to the increasing importance of *HER2* testing in cancer and the new exciting challenges that precision medicine is providing, in this review we seek to describe the current state of knowledge of *HER2* alterations in the most common DSC, to discuss the operational issues of *HER2* testing, and to outline forthcoming clinical perspectives, in particular focusing on the cutting-edge tools available for *HER2* characterization and targeting in the digestive system.

HER2 TESTING IN THE DIGESTIVE SYSTEM

Esophageal cancer

Esophageal cancer (EC), excluding GEJ tumors, is among the ten most prevalent tumors worldwide and ranks fifth in cancer mortality in men and eighth in women[32]. Squamous cell carcinoma (SCC) represents the most frequent histological type[20]. The poor prognosis of EC results from the delayed diagnosis and poor efficacy of current treatments, being in most cases limited to a palliative role[23]. In the largest meta-analysis of the prognostic significance of erbB2 overexpression and gene amplification in EC patients, 22% of tumors were *HER2*-positive, regardless of histotype[33]. However, these data are likely to be overestimated. Indeed, the overexpression of erbB2 has been observed in 12%-17% of adenocarcinomas (ADC) in more recent studies[34], whereas less than 4% of esophageal SCCs are *HER2*-amplified[35]. Taken together, no significant differences in survival rates have been reported in patients diagnosed with *HER2*-positive esophageal ADC compared with the *HER2*-negative cases. However, the great heterogeneity among indexed studies on *HER2* prognostic role in these malignancies demands further investigations. Interestingly, the prognostic influence of *HER2* amplification as a biomarker is slightly greater in SCC compared to ADC[33,35]. On the other hand, the small number of *HER2*-positive SCCs, the lack of large-

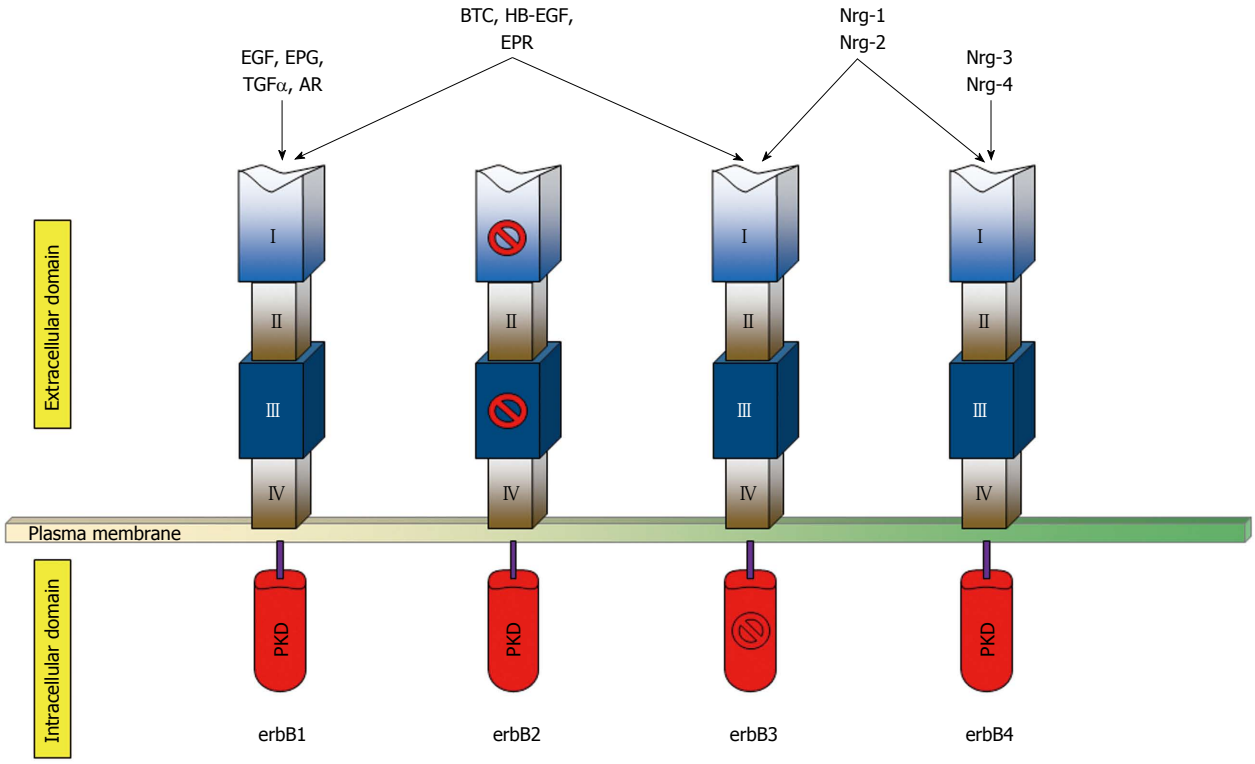

Figure 1 Schematic representation of the human erbB receptors in basal condition. The extracellular portion of each receptor consists of four domains (Ⅰ-Ⅳ). Both domains Ⅰ and Ⅲ, which are related leucine-rich segments, actively participate in ligand binding, except for those of erbB2. Domains Ⅱ and Ⅳ contain numerous cysteine residues and participates in dimer formation. The kinase domain of erbB3 is kinase-impaired. The growth factor groups that bind each receptor are indicated on the top. PKD: Protein kinase domain; EGF: Epidermal growth factor; EPG: Epigen; TGFα: Transforming growth factor-α; AR: Amphiregulin; BTC: Betacellulin; HB-EGF: Heparin-binding epidermal growth-factor like growth factor; EPR: Epiregulin; Nrg-1/2/3/4: Neuregulin-1/2/3/4.

cohort studies, and the absence of standardized methods for *HER2* testing limit our knowledge of *HER2* significance in SCC of the esophagus[36]. At present, the optimal treatment for EC remains controversial. In this regard, neoadjuvant chemotherapy with subsequent surgery represent the standard approach in the United Kingdom[37,38], whereas in Europe and United States neoadjuvant chemo-radiotherapy followed by surgery is preferred[39]. However, the individualization and optimization of therapy for EC might come across *HER2* and its epistatic interactions with other potentially actionable cancer genes. Indeed, it has recently been reported that possible alterations in epidermal growth factor receptor (*EGFR*), telomerase reverse transcriptase (*TERT*), and *HER2* are *bona fide* predictor of response to *HER2*-target therapy in EC, particularly in SCCs[35,40]. At present, RTOG 1010 (Radiation Therapy, Paclitaxel, and Carboplatin With or Without Trastuzumab in Treating Patients With EC) is the only ongoing phase Ⅲ trial (https://clinicaltrials.gov/ct2/show/study/NCT01196390) randomizing patients with *HER2*-positive esophageal ADC to chemoradiation with or without trastuzumab.

Gastric and gastroesophageal junction cancer

GC, including GEJ cancer, is closely related to environmental factors, reflecting its characteristic geographical distribution[32]. Although GC rates have gradually decreased during the past decades, this tumor still represents the third leading cause of cancer-related death globally[32]. The vast majority of GCs can be divided into three distinct subtypes based on Lauren's histopathologic classification: intestinal-type, showing glandular architecture, diffuse-type, with poorly cohesive cells arranged in an infiltrative pattern, and mixed-type, bearing hybrid characteristics[20]. This morphologic heterogeneity replicates an intrinsic molecular complexity. Recently, The Cancer Genome Atlas (TCGA) network proposed a novel molecular classification of GC, dividing these tumors into four major molecular subtypes, namely tumors positive for Epstein-Barr, microsatellite unstable tumors, genomically stable tumors, and tumors with chromosomal instability[41]. Among these molecular subgroups, microsatellite unstable tumors preferentially occur in the body and antrum, and are characterized by an extraordinarily high number of mutations with the lack of targetable amplifications, including *HER2* amplification. Chromosomal instability subtype encompasses the majority of GC, has a predilection for the GEJ, is associated with intestinal-type histology, and exhibit the highest rates of *HER2* amplification among all molecular subtypes[41]. Overall, GCs overexpressing erbB2 and/or showing *HER2* amplification, range from 13% to 22% of cases[12,42]. Meta-analysis data suggest that GC harboring *HER2* amplification fares worse[43];

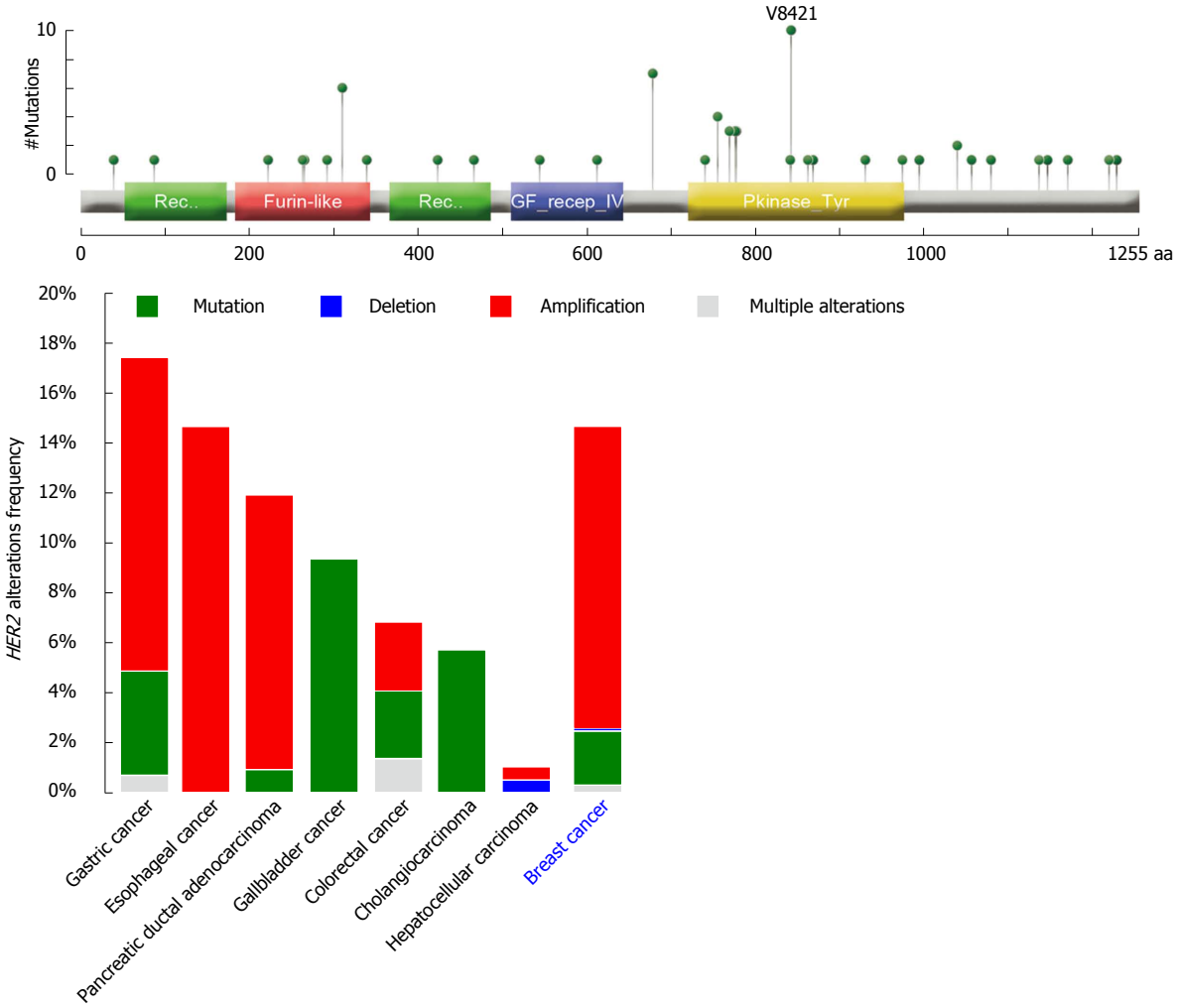

Figure 2 *HER2* alterations frequencies from public datasets accessible from cBioPortal[16] in the most common tumors occurring in the digestive system compared to breast cancer. Overall, *HER2* amplifications occur more frequently in gastric, esophageal, and pancreatic cancers, whereas gallbladder cancer and cholangiocarcinoma characteristically show *HER2* mutations (10% and 6% of cases, respectively). The domain structure of the erbB2 protein and gene alterations identified in primary carcinomas of the digestive system available from cBioPortal[16] (reported the top) show the presence of hotspot somatic mutations in the furin-like (binding site) and protein kinase domains. Mutation types are color-coded on the basis of the legend on the bottom right.

Table 1 erbB2 overexpression and gene amplification reported frequencies in tumors of the digestive system

Primary tumor	erbB2 overexpression frequencies	*HER2* amplification frequencies	Ref.
Esophageal cancer	4%-22%	4%-14%	[33-36,38,39]
Gastric cancer	13%-22%	10%-18%	[12,41-47]
Colorectal cancer	2%-11%	3%	[48-57]
Biliary tract cancer	5%-76%	1%-8%	[27,75,86,88-90]
Pancreatic cancer	0%-50%	2%-29%	[17-19,64-66,68]
Liver cancer	2%-5%	0-1%	[72-79]

however, the prognostic value of *HER2* remains controversial in the stomach[44-46]. This is probably due to the heterogeneous *HER2* status patterns in tumors arising in the stomach that basically mirror the intratumor morphologic and molecular heterogeneity of GC (Figure 3)[12]. Indeed, in contrast to breast carcinoma, up to 90% of erbB2-positive GCs are reported to harbor erbB2 overexpression in less than 5% of tumor cells[12,46]. From a therapeutic perspective, it is currently recommended to administer trastuzumab in combination with cytotoxic therapies in *HER2*-positive GC patients[42]. In this setting, the addition of trastuzumab to chemotherapy increased the objective response rate from 35% to 47%, improving progression-free survival from 5.5 mo to 6.7 mo and overall survival from 11.1 mo to 13.8 mo[5,42,47]. Clinical trials aiming to examine the efficacy of lapatinib in combination with paclitaxel compared with paclitaxel alone in the treatment of *HER2*-positive GC are on-going (https://clinicaltrials.gov/ct2/show/study/NCT01705340). Other clinical trials are currently exploring the effect of adding pertuzumab to chemotherapy (https://clinicaltrials.gov/ct2/show/study/NCT01461057) and comparing trastuzumab to paclitaxel or docetaxel as second-line treatment (https://clinicaltrials.gov/ct2/show/study/NCT01641939).

Colorectal cancer

Colorectal cancer (CRC) is a major contributor to

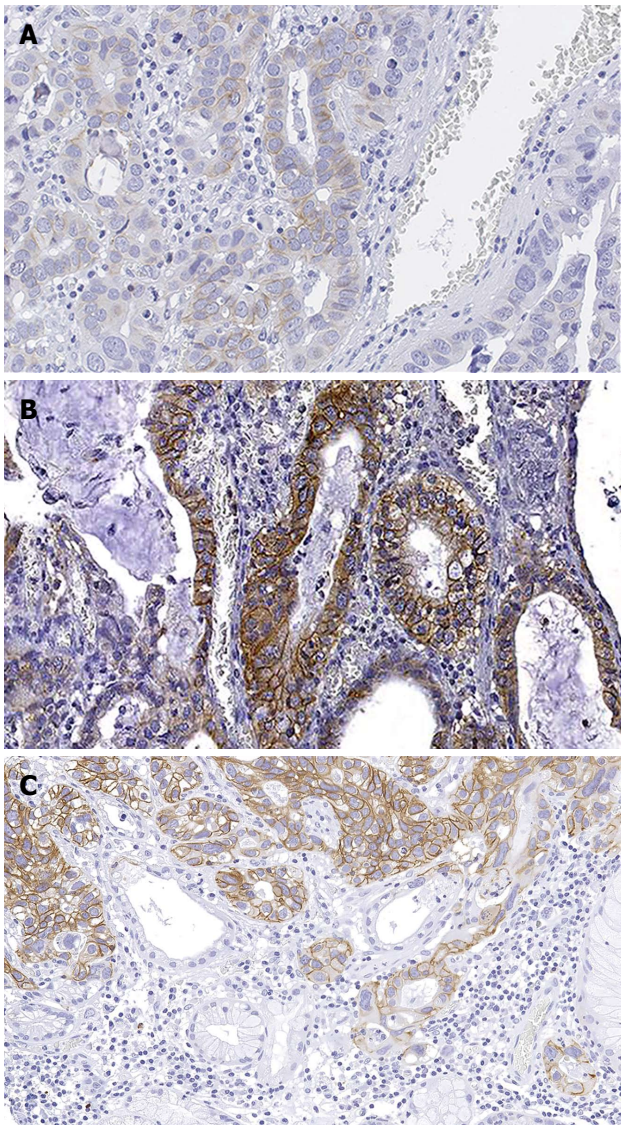

Figure 3 Representative micrographs of a gastric adenocarcinoma showing heterogeneous erbB2 immunohistochemical expression. In this paradigmatic example of *HER2* heterogeneity in gastric cancer, the tumor showed the coexistence of 2+ (A), 3+ (B, C), and negative areas (C), with the former immunohistochemical pattern involving the majority of the tumor cells. Original magnification × 200.

cancer morbidity and mortality, with 1.36 million new cases and more than half million deaths per year worldwide[32]. Several studies assessed *HER2* status in CRC, with some authors reporting membranous erbB2 overexpression in 2%-11% and others reporting cytoplasmic overexpression in 47%-68% of cases[48-52]. However, it is currently acknowledged that the amplification of *HER2* occurs in only 3% of CRC[53]. In accordance to this notion, a recent comprehensive genomic characterization of CRC revealed a recurrent amplicon at 17q21.1 in 4% of these tumors[54]. This locus contains seven genes, including *HER2*[55]. The operational implications of *HER2* amplification in CRC, however, remain elusive, with a number of studies reporting contradictory links to prognosis[53,56,57]. How to assess *HER2* status in CRC cancer remains a matter of debate among pathologists; however, a panel of *HER2* experts recently provided a reproducible and rigorous testing algorithm[57]. Intriguingly, no intra-tumor heterogeneity is described in CRC except for anecdotic reports[57]. Albeit *HER2* seems not to represent a reliable prognostic marker in CRC, this molecular alteration is strongly associated with wild-type status of Kirsten rat sarcoma viral oncogene homolog (*KRAS*) and amplification DNA topoisomerase 2-alpha (*TOP2A*)[16,58,59]. This observation is not trivial, raising the hypothesis that *HER2* amplification might be a *bona fide* alternative driver of Ras-Raf-MEK-ERK pathway activation in CRC. In contrast to *HER2*, intratumor heterogeneity of *KRAS* mutation status is reported and crucial in selecting patients for anti-*EGFR* therapy in CRC[12]. For these reasons, and given the homogeneity in erbB2 expression, it has recently been proposed that *HER2* status should be assessed as a putative biomarker of resistance to anti-EGFR therapy in *KRAS* wild-type patients and, if further studies confirm that *TOP2A* amplifications are associated with anthracycline sensitivity, as a predictor to response[57,59,60]. At present, results from phase Ⅱ and Ⅲ trials suggest that *HER2*-positive CRC should be treated with trastuzumab[61].

Pancreatic ductal adenocarcinoma

Pancreatic cancer (PC) accounts for approximately 2% of new cancers, and is responsible for 7% of cancer-related death yearly worldwide[32]. The vast majority of PCs is represented by invasive ductal ADC arising in the head of the gland; 20% of cases involve the body or the tail[20]. The poor prognosis for these patients is attributed to delayed diagnosis, early metastasis, and the limited efficacy of available systemic treatments[62]. Systemic therapies are only modestly effective; however, there is emerging evidence that small groups of patients may respond well to specific treatments. Amplification of *HER2* gene and/or overexpression of its product have been implicated in the development of PC[63]. However, the reported rates of erbB2 overexpression in these neoplasms are extremely variable, ranging from 0 to 50% of cases[17-19,64]. Furthermore, the prognostic role of *HER2* amplification in PC has been investigated in numerous studies, again, with heterogeneous results[63]. As a consequence, the diagnostic criteria and prevalence of *HER2* amplification in pancreatic ductal ADC remain unclear. Preclinical studies support the potential efficacy of trastuzumab in PC[65,66], although clinical trials have been disadvantaged by small cohorts[67,68]. In one phase Ⅱ trial, 17 patients showing *HER2* amplification were treated with capecitabine combined with trastuzumab[68]. Although the therapy was well tolerated, progression-free and overall survivals were not favorable compared to standard chemotherapy. At present, there is no consensus on the treatment modalities of *HER2*-positive pancreatic ductal ADC.

Hepatocellular carcinoma

Hepatocellular carcinoma (HCC), is the fifth most common cancer in men and the seventh most common cancer in women, resulting in approximately 700000 deaths yearly[32]. In recent years, these tumors showed increasing incidence, albeit variable throughout the world[32,69]. The asymptomatic nature of early disease and the limited use of screening protocols in high-risk individuals often lead to diagnosis in advanced stages, with subsequent requirement of systemic therapy[70,71]. To date, sorafenib (a kinase inhibitor) is the only approved drug in patients with advanced HCC, with modest effectiveness at prolonging patients' overall survival[72,73]. Given the scarce therapeutic armamentarium currently available, capturing the complexity underpinning HCC biology represents a high-priority goal[70]. In this setting, the role of *HER2* in HCC has been explored in several studies yielding, however, to extremely discrepant results due to the diverse methods used for *HER2* testing, even including cytoplasmic expression[74-76]. It is currently recognized that there is a low frequency of erbB2 strong membranous IHC overexpression in HCC[77-79]. Among these few cases, only a minority of tumors is reported to harbor *HER2* amplification[80,81]. Therefore, it is unlikely that patients with HCC would benefit from treatment with trastuzumab. In addition, recent studies suggest that there is also little indication for using *HER2* as a prognostic biomarker in these tumors[79].

Biliary tract cancer

Biliary tract cancer (BTC) encompasses a heterogeneous group of rare tumors originating in either the intra- or extrahepatic ducts[20]. This collection of neoplasms includes cholangiocarcinoma, gallbladder cancer (GBC), and ampulla of Vater cancer[20]. Among them, GBC is the most frequent type with an annual incidence of 2.5 cases per 100000 individuals[32]. Taken together, BTC mortality varies between geographic areas, accounting for higher mortality rates in South America and South-East Asia[69]. The current state-of-knowledge on BTC, regrettably, can be summarized briefly: complete tumor resection is the best chance of survival[82]. However, most cases are detected at an advanced stage, when surgical approaches are no longer feasible, and recurrences and distant metastases are common, with subsequent poor survival rates[82]. In addition, adjuvant chemotherapy has not shown sufficient benefit for BTC, while the efficacy of molecular targeted agents is still extremely disappointing[83,84]. To improve the prognosis of BTC, identification of prognostic markers and effective therapeutic targets is essential[85]. BTCs showing erbB2 overexpression and/or *HER2* amplification range between 5% and 76%[86], including an estimated 13% of erbB2-positive GBC[87]. This wide range is largely dependent upon the lack of standardized methods used among different studies[27,88]. Intratumor erbB2-expression heterogeneity has not been investigated thoroughly in BTC[75,86,88-90]. However, 51% of cases in a small cohort study showed erbB2 positivity using 50% of positive tumor cells as a threshold, suggesting the presence of heterogeneous protein expression[27]. Furthermore, in this subset of tumors, 83% of cases with heterogeneous erbB2 immunohistochemical (IHC) overexpression displayed *HER2* amplification by fluorescent *in situ* hybridization (FISH)[27]. Nevertheless, no data regarding *HER2* amplification heterogeneity have been reported in literature. The highest concordance between erbB2 expression and gene amplification was demonstrated for advanced BTC with high scores of erbB2 expression in the vast majority of tumors cells, present only in a minority of cases[68,88]. Taking into account overall and disease-free survival, *HER2* amplification seems not to have a prognostic role in BTC[27]. On the other hand, form a therapeutic standpoint, a subset of *HER2*-positive GBC responded well to trastuzumab treatment, both in monotherapy and in combination with taxane[85,90-92]. Despite these encouraging observations, an early phase clinical trial of trastuzumab for *HER2*-positive locally advanced or metastatic GBC was terminated in the United States because of the lack of participants (https://clinicaltrials.gov/ct2/show/study/NCT00478140). In this respect, it would be extremely beneficial to involve countries with a higher incidence of BTC in large-cohort clinical trials.

FROM TRADITIONAL PATHOLOGY TO MOLECULAR CHARACTERIZATION

Determination of HER2 status and its clinical significance

These data highlight that erbB2 overexpression and/or *HER2* amplification occur more frequently in ADC of the upper gastrointestinal tract compared to the rest of the DSC. In routine diagnostic practice and in research settings, erbB2 IHC scoring, along with the assessment of other prognostic and predictive factors, remains a cornerstone in the DSC pathology[44,57,93,94]. At present, it is taking place a consensus in extending the scoring system currently adopted in gastric ADC for all other DSC[57]. Compared to the breast, erbB2 IHC in DSC has a few substantial differences not only from intra- and inter-tumor heterogeneity standpoints but also in terms of cellular staining patterns. Indeed, erbB2 expression is mainly restricted to intestinal-type, gland-forming GC, and incomplete, often basolateral or even only lateral membranous IHC staining is the rule rather than an exception for *HER2*-positive GC[93,95]. Hence, circularity of IHC staining is no longer a criterion for erbB2 IHC scoring in the digestive tract[95]. A cornerstone work on esophageal ADC aimed to compare the erbB2 scoring system routinely employed in the breast to that used in GC[96,97]. However, no similar studies have been performed on other DSCs, therefore further analyses are warranted. In this era

Table 2 Mechanisms of resistance occurring in erbB2-tageting agents[107]

Type	Agent	Target	Mechanisms of resistance	Factors involved
Monoclonal antibodies	Trastuzumab	erbB2	Alterations in tyrosine kinase domain; overexpression of alternative erbB isoforms and dimerization receptors; loss of downstream checkpoints; dimerization and interaction with other receptors	p95HER2, *MUC4 EGFR*, erbB ligands (TGFα, EGF, HB), *PTEN IGF1R*, MET
	Pertuzumab	erbB2		
	T-DM1	erbB2		
Tyrosine kinase inhibitors	Lapatinib	erbB1, erbB2	Alterations in tyrosine kinase domain; acquisition of *HER2* mutations; activation of further downstream signaling pathways	KIT and PDGFRA receptor signaling pathway; PI3K-AKT, mTOR
	Neratinib	erbB2, erbB4		
	Afatinib	erbB1, erbB2, erbB4		
	Canertinib	erbB1, erbB2, erbB4		
Inhibitors of the downstream targets	Everolimus	mTOR	Activation of further downstream signaling pathways	PI3K-AKT, mTOR, *MEK*, *MAPK*
	BKM120	PI3K/AKT		
	BEZ-235	PI3K/AKT/mTOR		
	GS-1101	PI3K		
	NVP-BKM120	PI3K		
	GDC-0941	PI3K		
	GSK458	PI3K/mTOR		
	GDC-0980	PI3K/mTOR		
	PI-103	PI3K/mTOR		
hsp90 inhibitors	Tanespimycin	hsp90	Up-regulation of alternative pathways	NF-κB, *MAPK*
	Retaspimycin	hsp90		
	AUY922	hsp90		

of precision medicine, there are increasing evidences that digital image analysis tools are able to capture the whole spectrum of erbB2 IHC expression patterns and therefore represent useful tools for determining *HER2* status and its heterogeneity in DSC[12,98,99]. Both the membranous and nuclear features of the cells should be identified and scored, while the settings for cell count and differentiation between stroma and neoplasia should take into account the morphologic features of the cells (*e.g.,* curvature, color intensity, size, roundness, compactness, elongation) as well as each histologic pattern (*e.g.,* glandular, solid). Digital image analysis technologies grant a rapid and quantitative record of the percentage of stained tumor cells and their membrane staining distribution, allowing a precise and reproducible patients' stratification also capturing intra-tumor heterogeneity[12]. To verify equivocal IHC results, FISH, silver *in situ* hybridization (SISH), chromogenic *in situ* hybridization (CISH) assays are widely performed[8,12,57,93,96]. In particular, FISH identifies the number of *HER2* gene copies in conjunction with the number of chromosome 17 centromere (CEP17) copies[94]. This scoring is considered more objective and quantitative than IHC, however FISH reproducibility is strictly dependent on technical issues (*e.g.,* thickness of tissue sections)[8,100-103]. On the other hand, CISH is re-emerging as a more cost-effective assay, using conventional enzymatic reactions and being applicable to standard formalin-fixed paraffin embedded (FFPE) tissues[104]. This method shows high levels of quality and reproducibility, particularly in GC[105]. In a way akin to CISH, SISH is a rapid automated assay that can be interpreted using conventional microscopies, allowing pathologists to evaluate *HER2* status within the context of tissue morphology[103,106].

Overcoming resistance to erbB2-targeted therapies

Resistance to trastuzumab and other anti-erbB2 therapies is an event that may occur during the course of therapy or *de novo* (Table 2)[107]. Drug resistance has been widely studied in breast cancer but not in the DSC, with subsequent lack of a detailed molecular characterization of this phenomenon. Intra-tumor and tumor-to-metastasis heterogeneity are among the most important characteristics that determine resistance to anti-erbB2 therapy in DSC and should always be taken into account when selecting patients eligible for these treatments and clinical trials[12,19,52,96,108]. Indeed, there are several molecular evidences that genetic heterogeneity is not restricted to passenger genes but that also *bona fide* driver genetic alterations such as *HER2* gene amplification can be heterogeneously distributed within a given tumor[109]. The therapeutic implications of this concept are yet to be ascertained, although it is intuitive that only the *HER2*-positive neoplastic population would be sensitive to anti-erbB2 drugs[110]. Furthermore, it is not clear whether *HER2* amplification is an early event and subsequently lost in the *HER2*-negative components, or whether *HER2* amplification might be subclonally acquired at a relatively late stage of tumorigenesis[12,27]. In addition, somatic mutations in *HER2* have been described in a small subset of DSC and there are functional evidences that at least a subset of mutations targeting *HER2* might be responsible for the development of resistance to trastuzumab therapy, in a way akin to breast cancer[8,109]. For example, alterations leading to increased heterodimerization of *HER2* with *EGFR* or *HER3* are thought to induce resistance to trastuzumab therapy[107]. Furthermore, cleavage of the full-length erbB2 protein produces a truncated membrane-

associated fragment called p95HER2 with increased kinase activity in GC cell lines[111,112]. Up to 30% of breast cancers may harbor this alteration, showing poor prognosis and lower rates of response to trastuzumab therapy compared to patients with full-length *HER2*[111]. Furthermore, 11% of HCC but none of BTC have been found to harbor *HER2* (H878Y) somatic mutation occurring in the tyrosine kinase domain, resulting in c.2632C>T[81,113,114]. This specific mutation has been proposed as a predictor of response to *HER2*- and/or *EGFR*-targeted therapy in HCC. Interestingly, a phase Ⅱ trial observed that the dual *EGFR*/*HER2* tyrosine kinase inhibitor lapatinib was active in HCC but not in BTC, suggesting that mutations in the tyrosine kinase domain of *HER2* in HCC may underlie responsiveness to agents that target *HER2* and/or *EGFR*[115]. Generally, erbB2-directed therapy appears to be beneficial in erbB2-positive gallbladder cancers; however, tumors harboring *HER2* mutation (V777L), in the kinase domain, followed into the non-responders category[91]. In addition to the alterations in erbB2 receptors, mutations in genes involved in the signaling pathways activated by these receptors are also correlated with failure of therapeutic response to erbB2 inhibitors[116]. A preclinical trial is testing the hypothesis that *HER2* amplification might be used, under certain conditions, as a "molecular bait" for trastuzumab-emtansine precision chemotherapy to overcome anti-erbB2 resistance in *HER2*-positive metastatic CRC[117]. Moreover, *PIK3CA* mutations and *PTEN* inactivation could affect the effectiveness of erbB2-targeting therapy. Thus, it might be advantageous to clarify not only *HER2* alterations but also the PI3K-Akt pathway status to optimize HER2-targeting therapy[118]. In this regard, massively parallel sequencing and bioinformatic analyses are likely to represent the next frontier in the identification of complex mechanisms of trastuzumab resistance in this broad group of tumors[8,118-120]. A better knowledge of the biology underpinning *HER2* status in the digestive system should be regarded as a priority for the development of effective strategies to overcome resistance.

CONCLUSIONS: BUILDING UP INDIVIDUAL THERAPEUTIC SCHEMES

Substantial progress has been made in the management of patients with advanced-stage DSC in recent years, with the realization of tangible improvements in terms of outcome and life quality. In particular, trastuzumab has greatly improved the therapeutic approach to patients with advanced GC but not yet in other DSC. However, no validated *HER2* testing strategies are available for non-gastric DSC and subsequently tailored treatments are yet to be implemented in this broad group of malignancies. Several drugs targeting erbB2 or its downstream signals are under development in CRC, GBC, and EC, including ongoing phase 3 clinical trials in CRC. Nowadays, the focus on *HER2* expression/amplification status alone is not able to capture the underlying mechanisms of disease progression and resistance. In this setting, *PIK3CA* mutation or *PTEN* loss has been evaluated as a possible predictive biomarker and has also been used as one of the inclusion criteria. Understanding the interplay between *HER2* and the PI3K-Akt pathway alterations would be pivotal in the development of new therapeutic strategies. Further studies focused on the epistasis between molecular alterations and associations between molecular alterations and tumor microenvironment are warranted to accurately and robustly predict anti-erbB2 treatment outcome in DSC. Thus, a comprehensive clinical and pathogenomic approach is fundamental in appropriately characterizing *HER2* status in DSC at an individualized level for both precision therapy and accurate prognostication.

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REVIEW

Therapeutic aspects of c-MYC signaling in inflammatory and cancerous colonic diseases

Ferenc Sipos, Gábor Firneisz, Györgyi Műzes

Ferenc Sipos, Gábor Firneisz, Györgyi Műzes, 2nd Department of Internal Medicine, Semmelweis University, H-1088 Budapest, Hungary

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Correspondence to: Ferenc Sipos, MD, PhD, 2nd Department of Internal Medicine, Semmelweis University, Szentkirályi Street 46., H-1088 Budapest, Hungary. dr.siposf@gmail.com
Telephone: +36-1-2660926
Fax: +36-1-2660816

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Abstract

Colonic inflammation is required to heal infections, wounds, and maintain tissue homeostasis. As the seventh hallmark of cancer, however, it may affect all phases of tumor development, including tumor initiation, promotion, invasion and metastatic dissemination, and also evasion immune surveillance. Inflammation acts as a cellular stressor and may trigger DNA damage or genetic instability, and, further, chronic inflammation can provoke genetic mutations and epigenetic mechanisms that promote malignant cell transformation. Both sporadical and colitis-associated colorectal carcinogenesis are multi-step, complex processes arising from the uncontrolled proliferation and spreading of malignantly transformed cell clones with the obvious ability to evade the host's protective immunity. In cells upon DNA damage several proto-oncogenes, including *c-MYC* are activated in parelell with the inactivation of tumor suppressor genes. The target genes of the c-MYC protein participate in different cellular functions, including cell cycle, survival, protein synthesis, cell adhesion, and micro-RNA expression. The transcriptional program regulated by c-MYC is context dependent, therefore the final cellular response to elevated c-MYC levels may range from increased proliferation to augmented apoptosis. Considering physiological intestinal homeostasis, c-MYC displays a fundamental role in the regulation of cell proliferation and crypt cell number. However, *c-MYC* gene is frequently deregulated in inflammation, and overexpressed in both sporadic and colitis-associated colon adenocarcinomas. Recent results demonstrated that endogenous c-*MYC* is essential for efficient induction of p53-dependent apoptosis following DNA damage, but *c-MYC* function is also involved in and regulated by autophagy-related mechanisms, while its expression is affected by DNA-methylation, or histone acetylation. Molecules directly targeting c-*MYC*, or agents acting on other genes involved in the c-*MYC* pathway could be selected for combined regiments. However, due to its context-dependent cellular function, it is clinically essential to consider which cytotoxic drugs are used in combination with c-*MYC* targeted agents in various tissues. Increasing our knowledge about MYC-dependent pathways might provide direction to novel anti-inflammatory and colorectal cancer therapies.

Key words: c-MYC; Therapy; Apoptosis; Autophagy; Colon; Inflammation; Colorectal cancer

Core tip: The c-*MYC* gene is frequently deregulated in colonic inflammation, and overexpressed in both sporadic and colitis-associated colon adenocarcinomas. Endogenous c-*MYC* is essential for efficient induction of p53-dependent apoptosis following DNA damage, moreover its function is also involved in and regulated by autophagy-related mechanisms, and its expression is affected by DNA-methylation, or histone acetylation. Increasing our knowledge about MYC-dependent pathways might provide direction to novel colonic anti-inflammatory and anti-cancer strategies.

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INTRODUCTION

Chronic, non-infectious inflammatory and cancerous colonic diseases currently represent a major threat to human health worldwide. Inflammation is required to fight microbial infections, heal wounds, and maintain tissue homeostasis, however, it could lead to cancer. As the seventh hallmark of cancer it may affect all phases of tumor development, including tumor initiation, promotion, invasion and metastatic dissemination, and also evasion immune surveillance[1]. Inflammation acts as a cellular stressor and may trigger DNA damage or genetic instability, and, further, chronic inflammation can provoke genetic mutations and epigenetic mechanisms that promote malignant cell transformation[1,2]. Both sporadical and colitis-associated colorectal carcinogenesis are multi-step, complex processes arising from the uncontrolled proliferation and spreading of malignantly transformed cell clones with the obvious ability to evade the host' s protective immunity[3,4]. Therefore to develop more effective therapeutic strategies for colorectal cancer (CRC) it is quite challenging due to its heterogeneity and phenotypic diversity.

The *MYC*-family of cellular proto-oncogenes encodes three highly related nuclear phosphoproteins, namely c-MYC, N-MYC, and L-MYC[5]. c-MYC is a basic-helix-loop-helix-leucine zipper protein with a proto-oncogene function, being involved in cell proliferation, transformation, and death[6]. Data from chromatin immunoprecipitation studies demonstrate that c-MYC protein occupies regulatory regions of up to 15% of all genes, and can both activate or repress the expression of several target genes[7,8] (Figure 1A). The target genes of c-MYC participate in different cellular functions, including cell cycle, survival, protein synthesis, cell adhesion, and microRNA (miRNA) expression[7] (Figure 1B). The transcriptional program regulated by c-MYC is context dependent, therefore the final cellular response to elevated c-MYC levels may range from raised proliferation to augmented apoptosis[7]. In the absence of c-MYC cell cycle kinetics is strongly reduced[9].

As a result of synergistic or sequential damage of DNA in normal colonic epithelial cells, several proto-oncogenes, including *c-MYC* are activated in parallel with the inactivation of tumor suppressor genes, leading finally to the alteration of DNA repair systems and apoptosis regulation. Accumulation of the damaged DNA may ultimately cause cellular transformation. In this article we try to summarize the complex interactions of *c-MYC*-signaling within physiological intestinal epithelial homeostasis, inflammatory and cancerous colonic diseases, and the related therapeutic aspects.

CONTROL AND EFFECTS OF *MYC* GENE EXPRESSION

During recent years, several basic cellular functions of MYC have been established[10]. MYC plays a master regulator role of cell growth and proliferation, and it also controls stemness by maintaining pluripotency and self-renewal. On the other hand, MYC can sensitize cells to apoptosis, regulate cellular senescence, and is involved in DNA damage responses[10].

As a central, dual-faced regulator gene, *MYC* is controlled by several different mechanisms. Growth factor-dependent signals have been identified to control *MYC* expression. Growth factors like Ets-1 or E2F1 enhance transcription from the *MYC* promoter[11]. The β-catenin/TCF site also mediates the induction of the *MYC* promoter in regards to the Wingless type (Wnt)-signaling pathway[12]. Additionally, growth factor-dependent pulse of phosphoinositol (PI)3-kinase protects c-MYC protein from proteosomal degradation[13]. In contrast, the Smad and E2F4 containing repressor complex which forms on the *MYC* promoter after transforming growth factor (TGF)-β stimulus suppresses *MYC* expression and enhances the anti-proliferative effects of TGF-β[14,15].

Elevated levels of c-MYC protein strongly sensitize epithelial cells toward proapoptotic stimuli like DNA damage[16]. As a result, downregulation of c-*MYC* is necessary for cell cycle arrest, and survival of cells in response to DNA damage[17]. Since in the presence of strong mitogenic signals the downregulation of *MYC*-expression is required for proto-oncogene-induced cellular senescence[18], c-MYC may be involved in tumor-suppressive mechanisms as well. In case of epithelial and mesenchymal stem cells, however, *MYC*-

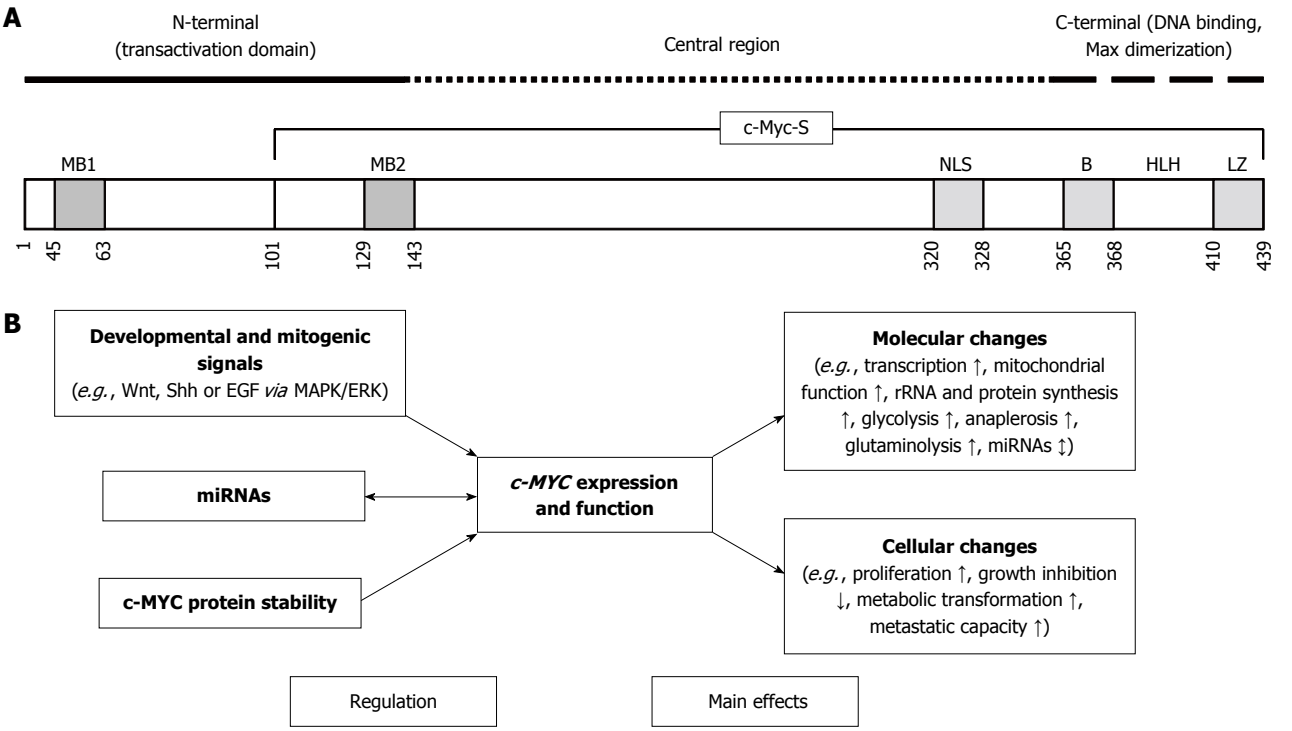

Figure 1 Schematic representation of the structure, regulation and main effects of c-MYC. A: The c-MYC protein consists of three domains: N-terminal, Central region, and C-terminal. The Central region and the C-terminal domain of c-MYC are responsible for protein-protein interactions that result in transcriptional repression by c-MYC. The C-terminal domain contains a basic (B) helix-loop-helix (HLH)-leucine zipper (LZ) motif that is necessary for interaction with different proteins (such as Max), and physiological recognition of DNA target sequences[7,58]; B: Developmental and mitogenic signals tightly regulate *c-MYC* gene expression both in normal (nontransformed) and in transformed cells *via* the MAPK/ERK pathway. MicroRNAs display a dual-faced role in the c-MYC regulatory network; both as regulators and as targets of c-MYC. The stability of the c-MYC protein also represents a particularly effective mechanism of gene regulation. c-Myc-S: Truncated c-Myc protein; MB1 and MB2: Evolutionarily conserved Myc Box sequences; NLS: Nuclear localization signal; Shh: Sonic hedgehog; EGF: Epidermal growth factor; MAPK: Mitogen-activated protein kinase; ERK: Extracellular signal-regulated kinase.

expression can be restricted even in the presence of several growth factors and cytokines[19]. These observations indicate that *MYC*-expression plays a dual-faced role regarding cellular survival and tissue homeostasis.

In physiological circumstances, negative feedback regulatory loops also play an important role in decreasing cellular c-MYC levels[20]. Negative feedback regulation is frequently disturbed in the course of tumorigenic transformation, permitting transformed cells to overexpress *MYC*[20]. Epigenetic factors, such as miRNAs, are also involved in downregulation of *MYC* in response to DNA damaging agents[17].

Regarding the colon, the protein kinase MK5 have been also identified as a negative regulator of *MYC* expression[15]. Expression of MK5 itself is regulated by MYC, since MYC binds to the promoter of the *MK5* gene, therefore activates its expression. As a result, MYC and MK5 form a negative feedback loop, in which FoxO proteins have been identified as key mediators[15].

c-MYC IN PHYSIOLOGICAL INTESTINAL HOMEOSTASIS

Considering intestinal homeostasis, c-MYC expressed in the entire intestinal tract displays a fundamental role[21]. In the small intestine c-MYC regulates the appropriate number of epithelial cells within the crypts[9]. Muncan *et al*[9] reported that upon conditional deletion of *c-MYC* gene crypt epithelial cells become smaller as compared to normal ones. Moreover, in the absence of c-MYC protein epithelial cell proliferation became reduced[9]. On the other hand, it was unexpectedly found in mice that conditional deletion of c-*Myc* in adult intestinal epithelium by utilizing a *Cre*-estrogen receptor fusion transgene driven by the intestine-specific villin promoter did not induce an overt phenotype[22]. According to this result the proliferation and expansion of intestinal epithelial progenitors can occur in a *Myc*-independent manner, as well. The difference between the studies of Muncan *et al*[9] and Bettess *et al*[22] most likely relates to deletion efficiency accomplished with the different Cre transgenes in the earliest crypt progenitors. Regarding apoptotic cell death, c-MYC does not influence epithelial apoptotic rate in the small intestine, it induces apoptosis only in the colon[9,23].

In the intestine cell proliferation and differentiation are under the tight control of the Wnt/β-catenin signaling[24]. In mice c-MYC is a critical downstream effector of cellular proliferation induced by the Wnt/β-catenin pathway[25,26]. Following epithelial injury,

the *c-Myc* 3'Wnt responsive DNA elements (WRE)-dependent regulation of the expression of the *c-Myc* gene seems to be essential for maintaining intestinal homeostasis and regeneration[27].

In colonic epithelial cells, c-MYC-induced apoptosis can be either p53-dependent or independent[28-30]. Basically, in cells the level of p53 expression is low, but its expression is elevated upon stress responses[31,32]. By promoting proteosomic degradation mouse double minute (Mdm)-2 is a negative regulator of the p53 protein[33]. As a regulatory loop, p53 transcriptionally upregulates Mdm2[33]. Alternative reading frame (Arf) also has a role in this regulatory mechanism, since it inhibits the function of Mdm2 and c-MYC[33,34]. By increasing Arf expression, c-MYC protein displays a prominent role in p53 regulation leading finally to p53-dependent apoptosis[35]. The crosstalk between c-MYC and p53 is essential in inducing pro-survival or pro-death responses to apoptotic stimuli.

Upon modulating apoptotic signals c-MYC is able to regulate intrinsic apoptosis independently from p53, as well[36,37]. c-MYC can also alter the balance between the pro- and antiapoptotic members of the Bcl2 (B-cell/lymphoma 2)-family[16,38]. Bcl2 can inhibit c-MYC mediated apoptosis, however, on the other hand, *c-MYC* overexpression suppresses the antiapoptotic Bcl2 protein and mRNA levels[39]. To suppress the antiapoptotic Bcl2 expression the DNA-binding activity of c-MYC is required[40]. In mice, c-MYC may induce apoptosis *via* the activation of the proapoptotic protein, Bax[41]. c-MYC also participates in the extrinsic apoptotic pathways[38,42,43]. Therefore, it is difficult to predict which c-MYC target genes are responsible for the final biological effects. It is likely that the current status of cell physiology ultimately influences the outcome of *c-MYC* overexpression, and affects c-MYC regulating the apoptotic process in colonic epithelial cells.

c-MYC IN COLONIC INFLAMMATION

As a hallmark of cancer, inflammation may lead to tumor formation. Acute and chronic colonic inflammation disrupts the integrity of the epithelial layer, moreover can lead to regenerative cell proliferation, and even fibrosis. In animal colitis models the use of glycogen synthase kinase (GSK)3β inhibitors mitigated disease symptoms by reducing pro-inflammatory immune response[44]. It has been shown, that during the recovery phase of dextran sulfate sodium (DSS)-induced colitis GSK3β inhibition by lithum chloride promotes colonic regeneration. The explanation of this effect is that lithium treatment increased the expression of *Myc* transcripts, MYC proteins, and the expression of several Wnt/MYC target genes in the colonic epithelium[45].

Additionally, in humans the steady-state levels of several nuclear proto-oncogenes including c-*MYC* and N-*MYC* were demonstrated to be lower in epithelial cells from involved or uninvolved inflammatory disease bowel (IBD) samples than in normal epithelial cells from either sporadic colon cancer or diverticulitis patients[46]. In active inflammation the downexpression of c-MYC in IBD epithelium may result in attenuated cell proliferation, therefore may contribute to mucosal ulceration. On the other hand, c-MYC may also be involved in epithelial regeneration after inflammatory damage by altering apoptotic cell death.

It is a known fact, that patients with chronic, longstanding IBD have an increased risk for developing colitis-associated cancer (CAC). By using whole-exome sequencing analysis it has been recently demonstrated that -among others- the *MYC* genomic locus is more frequently amplified in CAC than sporadic colorectal cancers[47]. Moreover, genomic alterations observed in CAC are distinct form those found in sporadic CRCs, and vary by type of IBD[48]. Proteomic network analyses have identified proteins related to mitochondria, oxidative activity, calcium-binding proteins, and c-MYC that play roles in early and late stage colitis-associated neoplastic progression, respectively[49]. *c-MYC* is often overexpressed in dysplastic cells in chronic longstanding ulcerative colitis, the precursor to CAC[50,51]. Taking together these data, it seems that the complex role and final effects of c-MYC in inflammatory colon mucosa are context- and microenvironment dependent.

c-MYC IN COLORECTAL CANCER

The *c-MYC* oncogene is frequently deregulated in human cancers and is overexpressed in up to 70%-80% of colon adenocarcinomas[52]. Since *c-MYC* is a downstream target of the *APC* (adenomatous polyposis coli) gene, and *APC* itself is inactivated in most colorectal cancers[53], it is not surprising that in early and advanced stages of colorectal carcinogenesis *c-MYC* is overexpressed at both the mRNA and protein levels[54,55].

The imbalance of cell proliferation and apoptosis is a key component in initiation of colorectal tumorigenesis. Basically, overexpression of *c-MYC* could lead to apoptosis[38], indicating its crucial role for determining cell survival and/or apoptotic pathways[36]. Under pathological conditions deregulated Wnt/β-catenin signaling promotes CRC by activating the expression of *c-MYC*[56]. Moreover, c-MYC-triggered apoptosis provides an inherent "fail-safe" program to check unlimited cell growth. The extent of apoptotic cell death is in correlation with the level of *c-MYC* expression[57].

In case of early to late colorectal adenomas significant correlation of nuclear β-catenin and c-MYC nuclear expression was found with the size of colon adenomas, but not with their cellular proliferative activity[58]. This phenomenon implies a dose-dependent function of β-catenin. Without nuclear β-catenin, T-cell factor family (TCF) proteins are bound by a co-

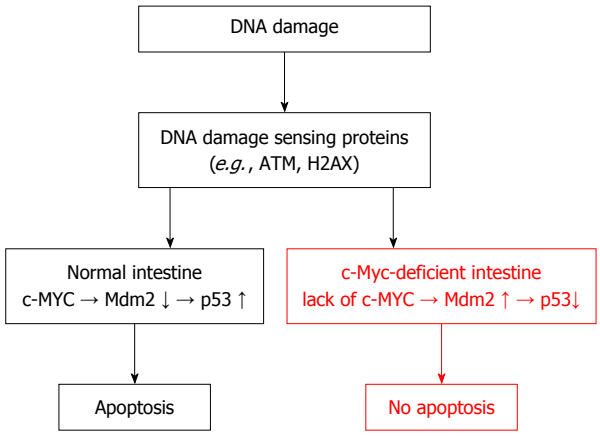

Figure 2 Schematic illustration of the relation of c-MYC to p53 following DNA damage in the intestine.

repressor, and this complex acts as transcriptional repressor of the target genes[59,60]. Nuclear β-catenin competes with the co-repressor for TCF binding in a dose-dependent manner. In colorectal cancer cells the disruption of β-catenin/TCF-4 activity induces a rapid G1 arrest and blocks the physiologically active genetic program in the proliferative compartment of colonic crypts. Simultaneously, an intestinal differentiation program is induced, in which c-MYC plays a switch role by direct repression of the p21CIP1/WAF1 promoter. Following disruption of β-catenin/TCF-4 activity, the decreased expression of c-MYC results in p21CIP1/WAF1 transcription, which in turn mediates G1 arrest and differentiation[12].

Though several *in vitro* studies proved that c-MYC has the ability to sensitize or induce apoptosis[61,62], its role in apoptotic cell-death is not well established and unclear *in vivo.* In a recent article, Phesse *et al*[63] demonstrated for the first time in an *in vivo* model that endogenous c-MYC is essential for efficient induction of p53-dependent apoptosis following DNA damage. It has been long known that p53 serves a key element in the development of sporadic colorectal cancer[64], and further, it is also involved in colitis-associated carcinogenesis[65]. Until now, in the gut *c-MYC* was considered as a fundamentally expressed gene responsible for epithelial regeneration and the regulation of the number of crypt cells. Phesse *et al*[63] concluded that c-MYC serves as a universal regulator of apoptosis in *in vivo* systems suggesting an important and new aspect of colorectal carcinogenesis (Figure 2). On the other hand, the exact mechanisms linking c-MYC levels to Mdm2 expression still remain unclear. In accordance with recent results[63,66,67], one can speculate that c-MYC may directly inhibit Mdm2 transcription. The induction of the c-MYC-dependent apoptosis program requires *c-MYC* expression to exceed a threshold, which is defined by Bcl2 family proteins in a cell-, tissue type and milieu-specific fashion[23]. In the colon, however, the different behaviour of the apoptosis regulator Bax, controlled by c-MYC may suggest the existence of a different apoptotic program of epithelial cells.

A recent report has demonstrated a role of AMBRA1 (activating molecule in Beclin-1 regulated autophagy) in both the autophagic pro-survival response and Beclin-1-dependent autophagy in embryonic stem cells[68]. AMBRA1 has been shown to be a crucial regulator of autophagy and apoptosis in colorectal cancer cells that maintains the balance between these cellular mechanisms[69]. AMBRA1 promoted dephosphorylation and degradation of c-MYC, and favors the interaction between c-MYC and PP2A (a c-MYC phosphatase), leading finally reduced cell divison rate[70]. AMBRA1 has been recently characterized as a target of mTOR (mammalian target of rapamycin) in the autophagy process[71]. Furthermore, the AMBRA1/PP2A-mediated regulation of c-MYC is also under mTOR control[70], indicating the key role of mTOR in regulating cellular fate by interfering with its metabolic status (Figure 3).

The MK5 kinase regulates the translation of c-MYC, since it is required for the expression of miR-34b/c that bind to the 3'UTR of *MYC*. The MK5-MYC negative regulatory feedback loop has been found to be disrupted during colorectal tumorigenesis[15]. Two changes may explain the disruption of this regulatory circuit. First, silencing of the miR-34B/c gene promoters by DNA methylation[72]. Second, the expression of MK5 is downregulated in colorectal tumors by a currently unknown mechanism[15]. Depletion of MK5 regulates *Ephrin B1*, a MYC-repressed gene that is involved in the progression of p53-deficient colorectal tumors[73].

ANTI-INFLAMMATORY THERAPEUTIC ASPECTS OF c-MYC IN THE COLON

Mesenchymal stem cell transplantation (MSCT) has been reported effective in the treatment of IBD as it can restore epithelial barrier integrity, induce immune suppression, and stimulate regeneration of endogenous host progenitor cells[74-78]. Mesenchymal stem cells can be engrafted into the damaged mucosa and even differentiated into colonic interstitial cells[79]. The pathobiologic background of this reparative process, however, is not well known. In an IBD-MSCT rat model, when intestinal epithelium was inflamed, the canonical Wnt signaling was found to be activated by Wnt3a and inhibited by GSK-3β and APC[78]. Shortly after MSCT, the elevated *c-Myc* and downregulated *Apc* gene expressions facilitate mesenchymal stem cell proliferation, and then differentiation into intestinal epithelial cells in the anaphase, by reducing the expression of *c-Myc*. These changes promoted intestinal stem cell proliferation and repaired the intestinal mucosa. Though, MSCT is a useful therapeutic possibility in IBD models, the parallel use of GSK3β inhibitors after MSCT may be therapeutically useful to enhance MYC-signaling, hence promoting reparative cell proliferation[45].

Traditionally, the pathomechanism of Crohn's

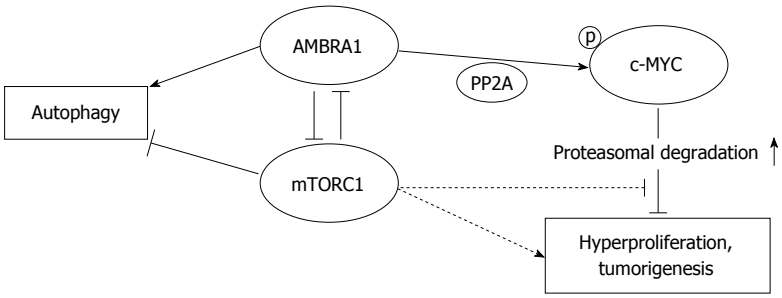

Figure 3 Interplay of AMBRA1, c-MYC and mTOR in colorectal cancer cells. AMBRA1 links autophagy to cell proliferation by facilitating c-MYC demolition. AMBRA1 promotes c-MYC phosphorilation and proteasomal degradation, therefore prevents hyperprolifearion and tumorigenesis. mTORC1 negatively controls the function of AMBRA1, thus finally supporting c-MYC-driven cell proliferation. Arrows represent stimulation or increase; blocked arrows represent inhibition; broken lines represent indirect effects. AMBRA1: Activating molecule in Beclin-1 regulated autophagy; mTORC1: Mammalian target of rapamycin complex 1; PP2A: Protein phosphatase 2A.

Table 1 Therapeutic options based on c-MYC targeting are represented by various strategies in inflammatory and cancerous colonic disorders

Main mode of action	Potential pathways/agents
Inflammatory colonic disorders	
Upregulation of c-MYC expression	GSK inhibitors ± MSCT
Inhibition of c-MYC signaling (suppression of Th1 function)	BET inhibitors ± c-MYC inhibitors
Cancerous colonic diseases	
Downregulation of c-MYC expression	dose-dependent gene and protein expression suppression; PPAR-γ (5-ASA, mesalazine)
	suppressing protein expression by UDCA
	crosstalk with integrins
	E2F1 inhibition (downregulatin GCN5 expression)
	FGFR kinase inhibition
	epigenetic regulation by miR-320b
	siRNA blocking of ABC-transporters
	lncRNAs (blocking of PARROT or CCAT1-L)
	siRNAs using PEI-PGMA platform
	modified ODC promoter
Promoting c-MYC degradation	26S proteosomal pathway (aspirin)
	SIRT2 inhibition
Inhibition of c-MYC signaling	Omomyc
	BET (+ Wnt/MAPK) inhibitors

GSK: Glycogen synthase kinnase; MSCT: Mesenchymal stem cell transplantation; PPAR-γ: Peroxisome proliferator-activated receptor-γ; 5-ASA: 5-aminosalicylate; UDCA: Ursodeoxycholic acid; E2F1: E2F Transcription factor 1; GCN5: Histone acetyltransferase; FGFR: Fibroblast growth factor receptor; miR-320b: Micro-ribonucleic acid-320b; siRNA: Small interfering ribonucleic acid; ABC: Adenosine triphosphate-binding casette; lncRNA: Long noncoding ribonucleic acid; PARROT: Proliferation associated RNA and regulator of translation; CCAT1-L: Longer isoform of colon cancer associated transcript 1; PEI-PGMA: Polyethileneimine-polyglycidal methacrylate; ODC: Ornithine decarboxylase; SIRT2: Sirtuine2; BET: Bromo- and extra-terminal domain; MAPK: Mitogen-activated protein kinase.

disease has been associated with Th1 cytokine profile. In an experimental autoimmune inflammation model it was demonstrated that inhibiting the functions of BET (bromo and extra-terminal domain)-family proteins during early T-cell differentiation resulted in long-lasting suppression of the pro-inflammatory functions of Th1 cells[80]. These effects were mimicked by an inhibitor of c-MYC function, as well, implicating reduced expression of c-*Myc* as one avenue by which BET-inhibitors suppressed the inflammatory functions of T-cells. hypothetically, BET and c-MYC inhibition may have therapeutic potential in Crohn's disease (Table 1).

ANTI-CANCER THERAPEUTIC ASPECTS OF c-MYC IN THE COLON

Cancer cells have been reported to display cell cycle arrest, differentiation, senescence or cell death after MYC inhibition *via* different molecular mechanisms (Table 1).

5-aminosalicylic acid has been identified as an agonist of peroxisome proliferator-activated receptor (PPAR)-γ[81]. Activation of PPAR-γ induces apoptosis by downregulating *c-MYC*[82,83]. Regrading aspirin the involvement of the 26S proteasomal pathway has been found in decreasing *c-MYC* expression in a concentration-dependent fashion[84]. Due to its oncogenic activities including cell growth, proliferation, angiogenesis, genomic instability and blocking differentiation, the downregulation of *c-MYC* would be expected to have important clinical implications[85].

Ursodeoxycholic acid (UDCA) displays chemo-preventive action against chemical and colitis-associated colonic carcinogenesis[86]. One possible explanation of this effect is the inhibition of cell proliferation by suppressing c-MYC protein expression and, as a consequence, cell cycle regulatory molecules including cyclin-dependent kinase-4 and -6[86]. According to this result, c-MYC is a target molecule of UDCA in colon carcinoma cells. However, mapping the benefits of UDCA administration for CRC chemoprevention at population level needs further studies.

Integrins, containing noncovalently associated α/β heterodimers provide dynamic cell to cell linkage and cell attachment to matrix molecules. While in normal human intestinal epithelium α1β1 integrins are usually expressed in the lower third of crypts[87],

in colorectal cancers and colon cancer cell lines integrin-α1 is expressed up to 65% of cases[88]. c-MYC regulates several integrin subunits, thus influences various functions of integrins regarding colon cancer cell proliferation, migration, and survival[89-92]. A combination of anti-MYC and -integrin targeted therapies hence may represent novel aspects of anti-tumor strategies in colon cancers.

Aberrant kinase activation originated from mutation, amplification, or translocation can drive growth and survival in several human cancers[93,94]. In gastric cancer, the crosstalk between fibroblast growth factor receptor (FGFR)2 and CD44 has been found to maintain cancer stemness by reciprocically regulating their expression *via* differentially regulating *c-MYC* transcription[95]. Since FGFR2 has been found to be amplified in the NCI-H716 colorectal cancer cell line[96], this result suggests that emerging FGFR inhibitor therapeutics may have efficacy in a subset of colon cancer driven by FGFR2 amplification.

It has been shown that the inhibition of BET protein family impairs the proliferation of several cancer cell lines[97-99]. These effects are partly mediated by *c-MYC* repression[98]. In a recent study of Tögel *et al*[100] the authors investigated the effect of BET inhibitors on proliferation and *c-MYC* expression within 20 CRC cell lines. They have found that JQ1, a BET inhibitor, administered together with Wnt or MAPK inhibitors sufficiently downregulates the expression of *c-MYC*, thus inhibits CRC cell proliferation. Based on these results, this kind of combined therapy seems to be effective in CRC treatment.

Upon recent results, targeting c-MYC can also be considered as a promising anti-cancer therapeutic strategy[23,101-104]. c-MYC inhibition with a protein fragment called Omomyc has been shown to be very effective to regress epithelial cell-derived tumors in mice models[23,105]. Omomyc has been found to be a pan-MYC family (c-, N- and L-MYC) inhibitor, potentially useful for cancers carrying any MYC family member amplification[106].

In case of cancers in which cell growth is not dependent on amplified *MYC* family genes, *MYC* suppression alone is not enough for a sufficient therapeutic effect. In animal models of *Myc*-driven cancers, reversion of the tumor by *Myc* suppression has been impeded by the parallel repression of TP53 or retinoblastoma-1 proteins underlining the relevance of these pathways to be intact for the treatment of cancers by MYC targeting[107-109].

Using a focused RNA interference library for genes involved in epigenetic regulation, sirtuin2 (SIRT2), an NAD(+)-dependent deacetylase, has been identified as a modulator of the therapy response to EGFR inhibitors in colon and lung cancers[110]. Thiomyristoyl lysine compound (TM), a SIRT2 inhibitor with high potency and specificity, has broad anti-cancer activity. SIRT2 inhibition was found to promote c-MYC ubiquitination and degradation, hence it may be a potential target for c-MYC-driven cancers including colorectal carcinoma[111].

Recent studies have suggested that the elevated expression of general control nonrepressed protein 5 (GCN5), a histone acetyltransferase can often be detected in human cancers[112]. GCN5 expression is elevated in colon cancer, and its overexpression is regulated by c-MYC[113]. By suppressing GCN5 human colon cancer cell growth can be inhibited. Furthermore, the suppression of the proapoptotic transcription factor E2F1-induced GCN5 transcription facilitates E2F1-induced cell death, implying a negative feedback in apoptosis regulation[113]. According to these results, GCN5 seems to be a potential therapeutic target for human colon cancers.

Regarding transcription factor-based therapies of tumorous diseases inhibition of c-MYC may also represent a promising option[101,114]. Numerous cytotoxic agents, and ionizing radiation have been shown to induce apoptosis following DNA damage. Since most of the anti-cancer drugs are used in combination with the potential of genotoxicity, it is of importance to further assess the role of c-MYC in response to DNA damage.

Therapeutic approaches that would allow the reprogramming and returning of altered c-MYC activity within tumor cells are also promising therapeutical strategies. RNA interference technology is one of these modalities. MiRNAs are key post-transcriptional regulators of genetic networks. Single-stranded mature miRNAs associated with Argonaute proteins form the core of a gene regulatory complex [*i.e.* RNA-induced silencing complex (RISC)]. MiRNA-RISC-mediated gene inhibition can be materialized by three processes: (1) site-specific cleavage; (2) enhanced mRNA degradation; and (3) translational inhibition[115]. Evidences indicate that post-transcriptional miRNA-mediated gene expression regulation can act as tumor suppressor or onogene in CRC[116]. Currently, miR-320b has been found to be significantly down-regulated in CRC tumor tissues. In addition, miR-320b overexpression has been found to correlate with decreased cell growth both *in vitro* and *in vivo*. Moreover, it has been also demonstrated that miR320b directly targets *c-MYC*, and its overexpression in SW-480, SW-620, HCT-116, LoVo, and HK293 CRC cell lines decreases *c-MYC* expression at gene and protein level as well[117]. According to these results, increasing miR-320b gene expression may represent a potential therapeutic approach in CRC.

Colorectal cancer stem cells (CSCs) has an important role in tumor initiation, progression, and recurrence. c-MYC was found to be highly expressed in CD133+ colon CSCs[118]. The overexpression of ATP-binding casette (ABC) transporters in cancer cells can result in therapy resistance by exporting anti-tumor drugs[119]. Recently, *c-MYC* expression has been effectively blocked on mRNA and protein level by *c-MYC* small interfering RNA (siRNA), moreover *c-MYC*

silencing sensitized CD133+ CSCs to chemotherapy-induced cytotoxicity by downregulating the expression of ABC transporter proteins[120].

In eukaryotic cells a vast number of noncoding RNA species are transcribed. Among them, long noncoding RNAs (lncRNAs) have been widely implicated in post-transcriptional gene expression regulation. The expression level of lncRNAs is usually very low and tissue-specific[121]. c-MYC can regulate the expression of lncRNAs, some of these may also contribute to the transcription of c-MYC target genes[122]. It has been reported that proliferation associated RNA and regulator of translation (PARROT), an lncRNA dynamically expressed in both transformed and normal cells contributes to proliferation in senescence and cancer. PARROT has been also identified as an upstream regulator of c-MYC. Its depletion results in the depletion of *c-MYC* mRNA and protein expression, subsequently altering cell growth and proliferation[121]. In gastric cancer c-MYC activates the expression of colon cancer associated transcript 1 (CCAT1) lncRNA, leading to an increased proliferation and migration of cancer cells[123]. CCAT1-L, a longer isoform of CCAT1, has been reported to regulate *MYC* expression in colon cancer. It is supposed that CCAT1-L allows the interaction between the enhancer and the *c-MYC* promoter thus promotes tumorigenesis[124].

Achieving effective intracellular delivery of therapeutic RNA interfering molecules such as siRNAs or short hairpin RNAs (shRNAs) is quite challenging. In a recent study, spherical nucleic acid-gold nanoparticle conjugates have been shown to selectively induce apoptosis in glioma cells *in vivo*[125]. However, the used 21 base siRNA duplexes were quite unstable. ShRNAs with a transient period of expression are better suited for long-term effectiveness, due to their ability to produce siRNAs continuously within cancer cells, thus resulting in prolonged suppression of target genes[126]. Until today, shRNAs have been delivered effectively *in vivo* using viral vectors. Among nonviral vectors, polyethileneimine (PEI) is the most widely used, gold-standard agent[127]. However, the major disadvantage of PEI is its cytotoxicity[128]. On the other hand, it has been demonstrated that anchoring multiple PEI chains to macromolecule polyglycidal methacrylate (PGMA) nanoparticles dramatically reduces their cytotoxicity, while achieving efficient nanoparticle endocytosis[129]. Using the PGMA platform effective delivery of small oligos (anti-miRs and mimics) and larger encoding shRNAs were performed in a wide variety of cancer cell lines including colorectal ones. Furthermore, the effectiveness of this therapy was validated for *in vivo* tumor suppression using transgenic mouse models. It was found that oral delivery of the *c-Myc*-conjugated nanoparticles to an *Apc*-deficient crypt progenitor colon cancer model resulted in an increased host survival and re-entered intestinal tissue to a non-*Wnt*-deregulated state[126]. According to these results, it seems that careful design of nonviral nanoparticles may help to made RNA interference technology an affordable and amenable therapy for CRC.

Regarding tumor-specific cytotoxicity, viral-directed enzyme prodrug therapy may also represent an ideal alternative[130]. However, the viruses used to deliver cDNAs encoding prodrug-activating enzymes can transduce normal cells, not just tumor cells. To achieve tumor-specific expression of the delivered cDNAs is to regulate transcription of the prodrug-activating enzyme with a promoter that is preferentially activated by tumor cells. *MYC*-responsive, modified ornithine decarboxylase (ODC) promoter/enhancer sequences have been identified that upregulate target protein expression in SW480 an HT29 colon cancer cells overexpressing the c-MYC protein. The modified ODC promoter may be useful in achieving tissue-specific expression of target proteins in cancers overexpress *c-MYC*[131].

CONCLUSION

The incidence of inflammatory colonic disorders is increasing worldwide. Though inflammation is required to heal infections, wounds, and maintain tissue homeostasis, as the seventh hallmark of cancer, however, it may affect all stages of tumor development. c-MYC, with its dual-faced role in cell proliferation and death, is implicated in several aspects of inflammatory tissue damage and repair. Since the therapeutic potential of c-MYC influencing therapies has not studied yet in the clinic, additional studies are needed to determine whether long-term treatment with c-MYC targeting agents can therapeutically suppress ongoing inflammation.

Colorectal carcinogenesis is a complex, multistep process that is driven by the accumulation of multiple genetic alterations. *c-MYC* is overexpressed in several types of malignant tumors including colorectal cancer, and is necessary for the uncontrolled proliferation of cancer cells. Single or combined therapies based on c-MYC targeting are represented by various strategies. Molecules directly targeting c-MYC, or agents acting on other genes involved in the c-MYC pathway could be selected for combined regiments. However, due to its context-dependent cellular function, it is clinically essential to consider which cytotoxic drugs are used in combination with c-MYC targeted agents in various tissues. Noncoding small RNAs have been recently implicated in anti-cancer therapies[132]. Regardless of the therapy applied, it is important to first determine the molecular pathways underlying the agents to inform the therapy design. Combining c-MYC-targeting agents with specific noncoding RNAs may lead to the development of novel colorectal cancer therapies.

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REVIEW

Role of miRNAs and their potential to be useful as diagnostic and prognostic biomarkers in gastric cancer

Kelly Cristina da Silva Oliveira, Taíssa Maíra Thomaz Araújo, Camila Inagaki Albuquerque, Gabriela Alcantara Barata, Carolina Oliveira Gigek, Mariana Ferreira Leal, Fernanda Wisnieski, Fernando Augusto Rodrigues Mello Junior, André Salim Khayat, Paulo Pimentel de Assumpção, Rommel Mário Rodriguez Burbano, Marília Cardoso Smith, Danielle Queiroz Calcagno

Kelly Cristina da Silva Oliveira, Taíssa Maíra Thomaz Araújo, Camila Inagaki Albuquerque, Gabriela Alcantara Barata, Fernando Augusto Rodrigues Mello Junior, André Salim Khayat, Paulo Pimentel de Assumpção, Danielle Queiroz Calcagno, Núcleo de Pesquisas em Oncologia, Universidade Federal do Pará, Hospital Universitário João de Barros Barreto, Belém, PA 66073-000, Brazil

Carolina Oliveira Gigek, Mariana Ferreira Leal, Fernanda Wisnieski, Marília Cardoso Smith, Disciplina de Genética, Departamento de Morfologia e Genética, Universidade Federal de São Paulo, São Paulo 04021-001, Brazil

Rommel Mário Rodriguez Burbano, Laboratório de Citogenética Humana, Instituto de Ciências Biológicas, Universidade Federal do Pará, Belém, PA 66073-000, Brazil

Author contributions: da Silva Oliveira KC and Calcagno DQ performed the review design; da Silva Oliveira KC, Thomaz Araújo TM, Albuquerque CI and Calcagno DQ collected the data; Thomaz Araújo TM, Albuquerque CI, Barata GA, Rodrigues Mello Junior FA and Calcagno DQ wrote the paper; Gigek CO performed corrections and suggestions; de Assumpção PP, Rodriguez Burbano RM and Smith MC revised the paper critically; all the authors contributed to this manuscript.

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Correspondence to: Danielle Queiroz Calcagno, PhD, Núcleo de Pesquisas em Oncologia, Universidade Federal do Pará, Hospital Universitário João de Barros Barreto, 2º Piso da UNACON, Av. Mundurucus, Belém, PA 66073-000, Brazil. danicalcagno@gmail.com
Telephone: +55-91-32016776

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Abstract

Alterations in epigenetic control of gene expression play an important role in many diseases, including gastric cancer. Many studies have identified a large number of upregulated oncogenic miRNAs and downregulated tumour-suppressor miRNAs in this type of cancer. In this review, we provide an overview of the role of miRNAs, pointing to their potential to be useful as diagnostic and/or prognostic biomarkers in gastric cancer. Moreover, we discuss the influence of polymorphisms and epigenetic modifications on miRNA activity.

Key words: Gastric cancer; Epigenetic; Diagnostic biomarkers; miRNAs; Prognostic biomarkers

Core tip: Accumulating evidence indicates that dysregulated miRNAs play important roles in gastric cancer pathogenesis. In this context, we provide an overview of the role of miRNAs, pointing to their potential to be used as diagnostic and prognostic biomarkers in gastric cancer. Moreover, we discuss the influence of polymorphisms and epigenetic modifications on miRNA activity.

da Silva Oliveira KC, Thomaz Araújo TM, Albuquerque CI, Barata GA, Gigek CO, Leal MF, Wisnieski F, Rodrigues Mello Junior FA, Khayat AS, de Assumpção PP, Rodriguez Burbano RM, Smith MC, Calcagno DQ. Role of miRNAs and their potential to be useful as diagnostic and prognostic biomarkers in gastric cancer. *World J Gastroenterol* 2016; 22(35): 7951-7962 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i35/7951.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i35.7951

INTRODUCTION

Gastric cancer (GC) is the fifth most frequent cancer, besides being the third leading cause of cancer-related death worldwide[1]. According to Laurén, GC is classified into intestinal and diffuse types[2], which are a consequence of an accumulation of genetic and epigenetic modifications[3].

Epigenetic events refer to alterations that promote gene expression variation without changing the DNA sequence yet leading to transcriptional activation or silencing of the gene[4].

Epigenetic alterations, mainly aberrant DNA methylation, histone modifications and microRNA (miRNA) expression play a central role in many diseases, including GC[5-7].

miRNAs are a class of small non-coding RNAs (19–25 nucleotides) that act as important epigenetic players in many cellular processes, such as differentiation, proliferation and apoptosis, exerting a great influence in cancer pathogenesis[8,9].

In general, miRNA genes are located in intergenic regions, suggesting that most miRNA genes are transcribed as autonomous transcription units[10]. Moreover, these molecules are usually transcribed by RNA polymerase II, generating long primary transcripts (pri-miRNAs). The pri-miRNAs are processed to pre-miRNAs (70 nucleotides) by Drosha. Then, these pre-miRNAs are processed by Dicer and generate a double-stranded RNA, which includes the mature miRNA[8].

The mature miRNAs repress protein translation through binding to the target protein-coding mRNAs by base-pairing to partially complementary regions frequently located at the 3'-untranslated regions (3'-UTR) of the target transcript[8,11-13].

A large number of miRNAs with different biological functions have been found altered in correlation with clinico-pathological features and/or prognosis in GC[5,7]. Ribeiro-dos-Santos *et al*[14] and Moreira *et al*[15] suggested the existence of gastric tissue and organ miRNA expression signatures. Accordingly, Gomes *et al*[16] observed a specific expression signature of let-7b, miR-21, miR-29c, miR-31, miR-192, miR-141, miR-148c and miR-451 in GC.

In this review, we describe the role and clinical significance of miRNAs, highlighting their use as potential prognostic and/or diagnostic biomarkers in GC. Moreover, we discuss the influence of polymorphisms and epigenetic modifications on miRNA activity.

ROLES AND CLINICAL SIGNIFICANCE OF miRNAs IN GASTRIC CANCER

In cancer, miRNAs can function as oncogenes and/or tumour suppressor genes depending on the outcome of the target mRNA (oncomiRNA or tsmiRNA, respectively). Increased activity of an oncomiRNA leads to inhibition of apoptosis and cell proliferation. In contrast, decreased activity of a tsmiRNA leads to increased tumour formation[17].

Because *in vitro* and *in vivo* introduction of tsmiRNAs promotes antitumoural activity by restoring lost tumour suppressor activity[18,19] and the use of antagomirs inhibits the pro-tumourigenic activity of oncomiRNAs[20], improved understanding of miRNAs' role in cancer could be helpful for providing novel insights into the role of miRNAs as molecular targets, whose modulation might hold therapeutic promise.

Both the overexpression of oncomiRNAs and the decreased expression of tsmiRNAs play pivotal roles in GC, and many studies in the literature have identified a large number of upregulated and downregulated miRNAs and their potential targets in this type of cancer. Therefore, aberrant expression of miRNAs has been significantly related to clinico-pathological features such as tumour stage, size, differentiation, metastasis and *H. pylori* status (Table 1)[21-118].

In GC, studies have consistently reported that miR-106a has oncogenic activity through suppressing the expression of *TIMP2, PTEN, FAS* and *RUNX3* genes[45-50]. Zhu *et al*[50] demonstrated that miR-106a is frequently upregulated in human GC and is closely associated with local tumour invasion and distant spreading by directly regulating its functional target *TIMP2*, a metastasis associated gene. Similarly, Xiao *et al*[45] stated that the level of miR-106a in GC tissues was significantly higher than that in non-tumour tissues, with an average increase of 1.625-fold and was significantly associated with tumour stage, size and differentiation, lymphatic and distant metastasis and invasion.

On the other hand, let-7a is one of the most important tsmiRNAs involved in gastric carcinogenesis,

Table 1 Deregulated miRNA in gastric cancer tumor

miRNA/role	Targets	Clinicopathological features	Ref.
OncomiRNAs			
miR-17	*UBE2C* *FBXO31*	Tumor size Tumor infiltration Clinical grade Prognosis Tumor stage	[21-24]
mir-19a	*MXD1* *SOCS1* *PTEN*	Migration Invasion Metastasis Proliferation Multidrug resistance	[25-28]
miR-20a	*EGR2* *E2F1*	Overall survival Relapse-free survival Self-renewal and proliferation of GC stem cells Chemoresistance of GC cells to cisplatin and docetaxel	[29-31]
miR-21	*PTEN* *PDCD4* *RECK* *SERPINI1*	Differentiation Lymph node metastasis *H. pylori* infection Tumor stage Tumor size	[29,32-37]
miR-25	*FBXW7* *TOB1* *RECK*	Proliferation Invasion Migration Metastasis Aggressive phenotype Poor long-term survival	[38-41]
miR-27a	*PHB* *ZBTB10* *HOXA10* *CCND1*	*H. pylori* infection Proliferation Drug resistance	[42-44]
miR-106a	*TIMP2* *PTEN* *FAS* *RUNX3*	Invasion Differentiation Distant metastasis Lymph node metastasis Tumor stage Tumor size	[45-50]
miR-106b	*P21* *E2F5* *E2F1*	Lymph node metastasis Depth of infiltration	[29,46,51-54]
miR-200b	*ZEB1* *ZEB2* *SUZ12* *DNMT3A* *DNMT3B* *SP1* *WNT-1*	Diffuse-type Poor overall survival *H. pylori* infection Metastasis Tumor size	[55-58]
miR-215	*RB1* *RUNX1*	Tumor stage	[59-61]
miR-222	*PTEN* *RECK*	Shorter metastasis-free survival Proliferation	[38,62-65]
tsmiRNAs			
let-7a	*RAB40C* *CDKN1* *SPHK2* *FN1*	Differentiation Lymph node metastasis Cell cicle arrest Growth suppression Overall survival Relapse-free survival	[66-71]
miR-143	*COX-2*	Invasion Haematogenous metastasis Lymph node metastasis Tumor stage	[69,72-75]
miR-148a	*ROCK1* *MMP7* *p27* *DNMT1* *SMAD4*	Clinical stage Lymph node metastasis Poor clinical outcome Epithelial-mesenchymal transition	[76-80]
miR-200c	*RND3* *DNMT3A* *DNMT3B* *SP1*	Lymph node metastasis Poor overall survival Sensitivity of chemotherapy to cisplatin Clinical stage Invasion	[56,57,81,82]
miR-204	*SIRT1* *BCL-2* *EZR*	Epithelial-mesenchymal transition Anoikis resistance Migration Invasion Colony forming ability	[83-85]
miR-218	*VOPP1* *ROBO1*	Proliferation Migration Metastasis	[86-89]
miR-433	*RAB34* *KRAS*	Tumor stage Overall survival Proliferation Migration Invasion	[90-92]
Controversial			
miR-9	*CCND1* *ETS1* *CDX2* *GRB2* *NF-kappaB1* *RAB34*	Proliferation Invasion Metastasis	[90,93-96]
miR-107	*FOXO1* *DICER1* *CDK6*	Invasion Differentiation Lymph node metastasis Tumor size Tumor stage Overall survival	[97-100]
miR-146a	*EGFR* *IRAK1* *L1CAM* *CARD10* *COPS8* *NASF2* *SMAD4* *WASF2*	Tumor size Poor differentiation Lymph node metastasis Venous invasion Overall survival time Apoptosis	[101-106]
miR-155	*SMAD2* *CDC73* *CYCLIN D1*	Invasion Lymph node metastasis *H. pylori* infection Cell viability Apoptosis	[38,107-111]
miR-181b	*CREB1* *BCL2*	Proliferation Migration Invasion Colony formation Apoptosis Multidrug resistance	[112-115]
miR-223	*EPB41L3* *STMN1* *FBXW7* *HMGA2*	Poor metastasis-free survival Apoptosis Proliferation Invasion Poor clinical prognosis	[116-118]

and studies in the literature have reported RAB40C, *CDKN1, SPHK2* and *FN1* as its targets[66-71]. Yang *et al*[68] demonstrated that GC tumour and cell lines with lower expression of let-7a tended to have poor differentiation. Furthermore, they demonstrated that induced overexpression of let-7a resulted in a decrease in cell proliferation, G_1 arrest and significant suppression of anchorage-dependent growth *in vitro* and tumourigenicity of GC cells in a nude mouse xenograft model.

Several studies have reported on miRNAs with a controversial role in gastric carcinogenesis such as miR-107 and mir-181b. For example, Guo *et al*[114] stated that the proliferation, migration and invasion of GC cells significantly increased after miR-181b transfection, probably due to downregulation of protein levels of TIMP3. Conversely, Chen *et al*[115] showed that miR-181b is downregulated in human GC cell lines in comparison with gastric epithelial cells. They observed that overexpression of miR-181b suppressed the proliferation and colony formation rate of GC cells, suggesting that miR-181b may function as a tumour suppressor in gastric adenocarcinoma cells through negatively regulating the *CREB1* gene.

The dual role of this and other miRNAs could be explained by the fact that a single miRNA is capable of targeting multiple genes, repressing the production of hundreds of proteins, directly or indirectly. Additionally, each gene can be regulated by multiple miRNAs, so the final effect will depend on these complex interactions[119,120].

Because miRNAs have thousands of predict targets in a complex regulatory cell signalling network, it is important to study multiple target genes simultaneously. Thus, a research group at Federal University of Pará (UFPA) developed the web tool TargetCompare (http://lghm.ufpa.br/targetcompare) to analyse multiple gene targets of pre-selected miRNAs. The described tool is useful for reducing arbitrariness and increasing the chances of selecting target genes having an important role in the analysis[121].

CIRCULATING miRNAs AS POTENTIAL GASTRIC CANCER BIOMARKERS

In cancer, it has been shown that primary tumour cells can release specific cancer miRNAs into the tumour microenvironment as well as into the circulation[122,123]. In recent years, studies have reported that miRNAs detectable in plasma or serum are more stable among individuals of the same species in comparison with other circulating nucleic acids[124].

This finding could be explained by the fact that circulating miRNAs exhibit resistance to endogenous ribonuclease activity by binding certain proteins such as Argonaute2 and high-density lipoproteins, besides being packaged in secretory particles including apoptotic bodies and exosomes, which allow them to be protected from existing ribonucleases[125-127]. Thus, it is plausible to use circulating miRNAs as biomarkers for early detection of various diseases, including GC.

Several studies have described circulating miRNAs as reproducible and reliable potential biomarkers as well as therapeutic targets in GC (Table 2)[128-137]. Tsujiura *et al*[130] suggested that miR-18a, which is a component of the miR-17-92 cluster, could be considered a novel plasma biomarker in GC patients. In addition to observing that the plasma miR-18a concentrations were significantly higher in GC patients than in healthy controls, they also stated that the plasma miR-18a levels were significantly reduced in postoperative samples compared to preoperative samples.

Recently, Wang *et al*[138] assessed the diagnostic performance of circulating miRNAs for the detection of gastrointestinal cancer in a meta-analysis including 21 GC studies. The majority of the GC studies were of Asian ethnicity, and the most frequent miRNAs found in plasma or serum were miR-106b and miR-21. In Caucasian patients with GC, they described miR-203, miR-146b-5p, miR-192 and miR-200c as potential biomarkers in plasma. However, many of these biomarkers have been tested in very restricted parameters and are highly influenced by ethnic and environmental factors, thus making it even more difficult to find specific biomarkers for GC.

EPIGENETIC FACTORS INFLUENCING miRNA EXPRESSION IN GASTRIC CANCER

Many molecular mechanisms lead to miRNA deregulation such as genetic mutation and epigenetic aberration. Approximately half of miRNA genes are located next to CpG islands, and the expression of these miRNAs is regulated by alterations in DNA methylation and histone modification[139-143].

DNA methylation is involved in silencing expression of tumour suppressor genes by establishing and maintaining a repressive status at gene promoters[5-7,144]. The basic transcription mechanism of miRNAs is fundamentally similar to that of classical protein-coding genes, and aberrant DNA hypermethylation has been shown to silence tsmiRNAs in cancer.

Many miRNAs have been reported to be downregulated due to hypermethylation of the CpG islands in GC, such as miR-9, miR-34b/c, miR-129, miR-137, miR-181c, miR-199a, miR-212, miR-338, miR-512, miR-516, miR-941 and miR-1247[142,143,145-150].

Several studies have shown that the miRNA methylation level was positively associated with the clinicopathological features of GC[147]. Low expression levels of miR-34b and miR-129-3p are associated with a poor clinical outcome in GC patients, and hypermethylation of miR-129-2 and miR-34b CpG islands tends to correlate with poor clinico-pathological features[148].

miRNAs can also be decontrolled as a consequence

Table 2 Circulating miRNA as diagnostic and prognostic biomarkers

miRNA	Samples	Potential biomarker type	Method	Clinical implication	Ref.
miR-1	164 GC/127 C Serum	Diagnostic	Solexa sequencing qRT-PCR	GC detection	[128]
miR-16	40 GNCA/40 C Plasma	Diagnostic	Taqman low-density array qRT-PCR	Early detection of GNCA	[122]
miR-17-5p	79 GC/30 C Plasma	Diagnostic	qRT-PCR	GC detection	[46]
	79 pre-operative GC/30 post-operative GC/6 relapse Plasma	Prognostic	qRT-PCR	Prediction of prognosis and monitoring of chemotherapeutic effects	[129]
miR-18a	104 GC/65 C Plasma	Diagnostic Prognostic	qRT-PCR	Screening GC and monitoring tumor dynamics	[130]
miR-20a	164 GC/127 C Serum	Diagnostic	Solexa sequencing qRT-PCR	GC detection	[128]
	90 GC/90 C Plasma	Diagnostic	qRT-PCR	Early detection of GC	[131]
	79 pre-operative GC/30 post-operative GC/6 relapse Plasma	Prognostic	qRT-PCR	Prediction of prognosis and monitoring of chemotherapeutic effects	[129]
miR-21	69 GC Plasma	Prognostic	qRT-PCR	Prognostic marker	[132]
	16 LN-metastasis positive/15 LN-metastasis negative/10 C Serum	Prognostic	qRT-PCR	Predicting LN metastasis	[133]
	79 GC/30 C Plasma	Diagnostic	qRT-PCR	GC detection	[46]
	70 GC/70 C Plasma	Diagnostic	qRT-PCR	GC detection	[134]
miR-25	40 GNCA/40 C Plasma	Diagnostic	Taqman low-density array qRT-PCR	Early detection of GNCA	[122]
miR-34	164 GC/127 C Serum	Diagnostic	Solexa sequencing qRT-PCR	GC detection	[128]
miR-92a	40 GNCA/40 C Plasma	Diagnostic	Taqman low-density array qRT-PCR	Early detection of GNCA	[122]
miR-106a	79 GC/30 C Plasma	Diagnostic	qRT-PCR	GC detection	[46]
miR-106b	79 GC/30 C Plasma	Diagnostic	qRT-PCR	GC detection	[46]
	90 GC/90 C Plasma	Diagnostic	qRT-PCR	Early detection of GC	[131]
miR-191	57 GC/58 C Serum	Diagnostic	qRT-PCR	GC detection	[135]
miR-218	70 GC/70 C Plasma	Diagnostic	qRT-PCR	GC detection	[134]
miR-221	90 GC/90 C Plasma	Diagnostic	qRT-PCR	Early detection of GC	[131]
miR-223	70GC/70C Plasma	Diagnostic	qRT-PCR	GC detection	[134]
miR-378	61GC/61C Serum	Diagnostic	miRNA microarray qRT-PCR	Early detection of GC	[136]
miR-423-5p	164GC/127C Serum	Diagnostic	Solexa sequencing qRT-PCR	GC detection	[128]
miR-451	56GC/30C Plasma	Diagnostic	miRNA microarray qRT-PCR	Screening GC	[137]
	40GNCA/40C Plasma	Diagnostic	Taqman low-density array qRT-PCR	Early detection of GNCA	[122]
miR-486	56GC/30C Plasma	Diagnostic	miRNA microarray qRT-PCR	GC Screening	[137]
miR-486-5p	40GNCA/40C Plasma	Diagnostic	Taqman low-density array qRT-PCR	Early detection of GNCA	[122]
let-7a	79GC/30C Plasma	Diagnostic	qRT-PCR	GC detection	[46]

C: Control; GC: Gastric cancer; LN: Lymph node; GNCA: Gastric non-cardia adenocarcinoma; qRT-PCR: Quantitative real time polymerase chain reaction.

of aberrant expression of specific epigenetic regulators such as polycomb repressor complexes and histone deacetylases (HDACs). Wisnieski *et al*[151] demonstrated HDAC1 downregulation in gastric tumours compared with adjacent non-tumour samples. According to Scott *et al*[152], inhibition of HDACs results in transcriptional changes in approximately 40% of miRNAs expressed in a breast cancer cell line (SKBr3).

In 2009, Saito *et al*[153] analysed the miRNA expression profile in human GC cells treated with 5-aza-2′-deoxycytidine (5-Aza-CdR) and 4-phenylbutyric acid (PBA), and they suggested that chromatin remodelling at Alu repeats by DNA demethylation and HDAC inhibition can induce expression of silenced *miR-512-5p*. Moreover, activation of *miR-512-5p* can lead to suppression of *Mcl-1*, resulting in apoptosis of gastric cancer cells. Thus, epigenetic treatment, by using synthetic miRNAs, can serve as an "endogenous silencer" of target oncogenes in GC cells, blocking their activity as tumour enhancers.

SINGLE-NUCLEOTIDE miRNA POLYMORPHISMS IN GASTRIC CANCER

Single-nucleotide polymorphisms (SNPs) in miRNA have also been associated with alteration of GC susceptibility

Table 3 miRNA related to the risk of gastric cancer

miRNA	SNP	Country	Population	Number of cases/ controls	Ref.
miR-27a	rs895819	China	Asian	304/304	[43]
		China	Asian	295/413	[154]
		China	Asian	278/278	[155]
	rs11671784	China	Asian	892 /978	[156]
		China	Asian	278/278	[155]
miR-146a	rs2910164	China	Asian	304/304	[157]
		Japan	Asian	583/1637	[158]
		Japan	Asian	90/90	[101]
		China	Asian	1686/1895	[159]
		South Korea	Asian	461/447	[160]
		Japan	Asian	552/697	[161]
miR-196a	rs11614913	Japan	Asian	552/697	[161]
		China	Asian	213/213	[162]
		South Korea	Asian	461/447	[160]
		Greece	Greak	163/480	[163]
miR-499	rs3746444	Japan	Asian	697/552	[161]
		South Korea	Asian	461/447	[160]
		China	Asian	363/969	[164]
miR-149	rs2292832	China	Asian	274/269	[165]
		South Korea	Asian	461/447	[160]
		Greece	Greak	163/480	[163]
miR-24	rs4819388	China	Asian	183/348	[166]
miR-570	rs4143815	China	Asian	205/393	[167]
miR-200c	rs12904	China	Asian	522/501	[168]
miR-505	rs111638916	China	Asian	857/748	[169]
Pre-miR-30c	rs928508	China	Asian	240/240	[170]
Pri-let-7a-2	rs629367	China	Asian	107/124	[171]

and modification of target gene expression. However, the role of these genetic variants in GC susceptibility remains essentially unidentified[7]. Table 3[154-171] summarizes described SNPs in miRNA in GC.

One of the most described miRNA SNPs associated with elevated risk in GC is SNP rs2910164 of miR-146a. Ahn *et al*[160] demonstrated that the C/G polymorphism in miR-146a decreases miR-146a expression and subsequently leads to reduced regulation of the target genes *TRAF6*, *IRAK1* and *PTC1* by the C allele. Moreover, some studies reported that miR-146a rs2910164 also affects susceptibility to gastric lesions. Song *et al*[172] found that the G/C polymorphism in miR-146a rs2910164 may play a role in the evolution of *H. pylori*-associated gastric lesions. Thus, SNP rs2910164 may be used as a genetic biomarker to predict GC risk.

SNPs in pri-miRNAs and pre-miRNAs could affect the maturation process and function of the miRNA, which may affect the expression of many proteins in the interaction pathway. Recently, Xu *et al*[171] found that upregulation of pri-let-7a-2 expression by the rs629367 C/C genotype was associated with increased risk and low survival in GC, probably by affecting the expression of mature let-7a.

The binding capacity of a miRNA with its target can be modified by SNPs affecting the miRNA TAG sequence. Additionally, a SNP in an mRNA sequence could influence the complementarity between the miRNA and the target mRNA. This could result in alteration of susceptibility to tumorigenesis. Wang *et al*[167] described that a SNP in the *PDL1* (rs4143815) could affect its protein expression by interfering with miR-570 negative regulation. Furthermore, this SNP was significantly related to the risk of GC and depth of tumour infiltration, differentiation grade, lymph node metastasis, tumour size and staging.

Hence, SNP data could be useful to improve our understanding of the contribution of individual susceptibility to GC pathogenesis.

FUTURE PERSPECTIVES

Accumulating evidence indicates that the dysregulation of miRNAs plays important roles in GC pathogenesis. In this context, miRNA expression profiles have been shown to correlate with GC development, progression and response to therapy[173,174], suggesting their possible use as diagnostic, prognostic and predictive biomarkers.

Moreover, miRNA-based anticancer therapies have recently been explored, either alone or in combination with current targeted therapies[175,176]. However, a big challenge in using miRNAs in cancer therapeutics is the considerable number of genes that a single miRNA can target, leading to a pleiotropic effect that may limit their manipulation at the systemic level. Nevertheless, the increasing capability of producing synthetic interfering miRNAs with higher affinity to the desired target is minimizing this barrier.

Thus, the strategy of using miRNAs for targeted therapy in the near future is probably over-optimistic, considering that the studies of miRNA-based the-

rapeutics are still premature; however, the number of discoveries, increasing so fast in the past few years, is surely extremely promising.

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REVIEW

Periodontal and inflammatory bowel diseases: Is there evidence of complex pathogenic interactions?

Ronaldo Lira-Junior, Carlos Marcelo Figueredo

Ronaldo Lira-Junior, Carlos Marcelo Figueredo, Department of Periodontology, Faculty of Odontology, Rio de Janeiro State University, Rio de Janeiro, RJ 20551-030, Brazil

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Correspondence to: Dr. Carlos Marcelo Figueredo, Department of Periodontology, Faculty of Odontology, Rio de Janeiro State University, Boulevard 28 de Setembro 157, 20 Andar, Vila Isabel, Rio de Janeiro, RJ 20551-030, Brazil. cmfigueredo@hotmail.com
Telephone: +55-21-28688282

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Abstract

Periodontal disease and inflammatory bowel disease (IBD) are both chronic inflammatory diseases. Their pathogenesis is mediated by a complex interplay between a dysbiotic microbiota and the host immune-inflammatory response, and both are influenced by genetic and environmental factors. This review aimed to provide an overview of the evidence dealing with a possible pathogenic interaction between periodontal disease and IBD. There seems to be an increased prevalence of periodontal disease in patients with IBD when compared to healthy controls, probably due to changes in the oral microbiota and a higher inflammatory response. Moreover, the induction of periodontitis seems to result in gut dysbiosis and altered gut epithelial cell barrier function, which might contribute to the pathogenesis of IBD. Considering the complexity of both periodontal disease and IBD, it is very challenging to understand the possible pathways involved in their coexistence. In conclusion, this review points to a complex pathogenic interaction between periodontal disease and IBD, in which one disease might alter the composition of the microbiota and increase the inflammatory response related to the other. However, we still need more data derived from human studies to confirm results from murine models. Thus, mechanistic studies are definitely warranted to clarify this possible bidirectional association.

Key words: Periodontal disease; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Inflammation

Core tip: The prevalence of periodontal disease seems to be increased in patients with inflammatory bowel disease (IBD). Moreover, the induction of periodontitis seems to result in gut dysbiosis and altered gut epithelial cell barrier function. This review points to a complex pathogenic interaction between periodontal disease and IBD, in which one disease might alter the composition of the microbiota and increase the inflammatory response related to the other disease.

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INTRODUCTION

Periodontal disease is a biofilm-induced chronic inflammatory condition that affects the tooth-supporting tissues, which in its severe form may lead to tooth loss and negatively affect systemic health. Although host immune and inflammatory responses are crucial in the control of this biofilm, their persistence and dysregulation may lead to destruction of periodontal tissues[1], where neutrophils and macrophages might play an important role[2,3]. Moreover, it has been shown that periodontitis is associated with several chronic inflammatory diseases, among which inflammatory bowel disease has raised special attention[4,5].

Inflammatory bowel disease (IBD) is a chronic inflammatory condition of the gastrointestinal tract, which comprises two main forms, Crohn's disease and ulcerative colitis[6]. The pathogenesis of IBD involves genetic and environmental factors, such as diet, smoking, stress, and microorganisms[7], and it is characterized by intestinal inflammation and epithelial injury[8,9]. Crohn's disease (CD) is characterized by macrophage aggregation, frequently forming non-caseating granulomas and transmural inflammation. The terminal ileum is the most common site affected, but the disease can involve any site of the gastrointestinal tract. Ulcerative colitis (UC) is characterized by a significant infiltration of neutrophils within the lamina propria and the crypts, forming micro-abscesses and superficial mucosal ulceration. The distal colon is the most affected region[6,7]. As previously mentioned, both cell types, macrophages and neutrophils, are also relevant to the pathogenesis of periodontal disease, suggesting that under a similar cytokine signalling, these diseases might share similar pathways.

Indeed, the presence of periodontal disease is more frequent in patients with IBD when compared to controls[4]. In addition, greater severity and extent of periodontitis have been found in IBD patients when compared to healthy controls[5]. This might be related to a higher expression of IL-18 in the serum of IBD patients with periodontitis[10]. However, different cytokine clustering patterns were observed in gingival tissues in comparison to those found in intestinal tissues[11]. This might suggest that although a common pathway may exist in serum, local cytokine behaviour may be slightly different.

Considering the complexity of both periodontal disease and IBD, it is very challenging to comprehend the possible pathways involved in their coexistence. Therefore, this review aimed to provide an overview of the evidence dealing with a possible pathogenic interaction between periodontal disease and IBD.

PERIODONTAL DISEASE

Periodontal disease is one of the most prevalent chronic diseases of mankind. Gingivitis, the initial lesion, is a reversible inflammatory condition of the soft tissue surrounding the teeth, induced by a direct immune response to the biofilm formed on the tooth surfaces on a daily basis. Periodontitis is a multifactorial inflammatory disease that destroys the tooth-supporting structures and may lead to tooth loss[12]. Its severe form affects over 740 million people worldwide[13]. Figure 1 depicts a simplistic view of healthy periodontal tissue on one side and diseased tissue on the other.

The pathogenesis of periodontal disease, similarly to that of IBD, involves a complex interplay between periodontopathogens and the host immune-inflammatory response, greatly influenced by genetic and environmental factors. Although the presence of microorganisms is required, it is not sufficient for disease initiation[14]. Rather, it is the unbalanced, persistent host inflammatory reaction against the pathogens that results in the destruction of periodontal tissues[14].

While it was once believed that a few specific microorganisms, mainly those forming the so-called "red complex" (*Porphyromonas gingivalis, Treponema denticola* and *Tannerella forsythia*), were involved in the aetiology of periodontitis, advances in technology and our deeper understanding of microbiome dynamics have pointed to a dysbiotic microbial community as responsible for eliciting a non-resolving chronic inflammation and tissue destruction[15,16]. This dysbiotic community provides a constant challenge to the innate immune system[17]. Bacterial components, such as lipopolysaccharides, peptidoglycans and proteases, induce an inflammatory response through stimulation of pattern recognition receptors on inflammatory cells as well as on resident cells.

This host inflammatory response is mediated mainly by neutrophils, monocytes/macrophages, and T and B lymphocytes. As a result, inflammatory mediators, including cytokines, chemokines and proteolytic enzymes, are produced and contribute to tissue degradation and bone resorption. Neutrophils are the first cells to arrive at the inflammatory inflitrate and predominate within the junctional epithelium and gingival crevice[18]. Previous studies from our group have shown that neutrophils from periodontitis patients are hyper-reactive and contribute to tissue destruction[3,19,20]. These neutrophils have also been shown to present a cytokine hyper-reactivity[21] and a dysfunctional chemotaxis[22].

When the resolution of inflammation is not achieved, antigen-presenting cells are activated by bacterial pro-

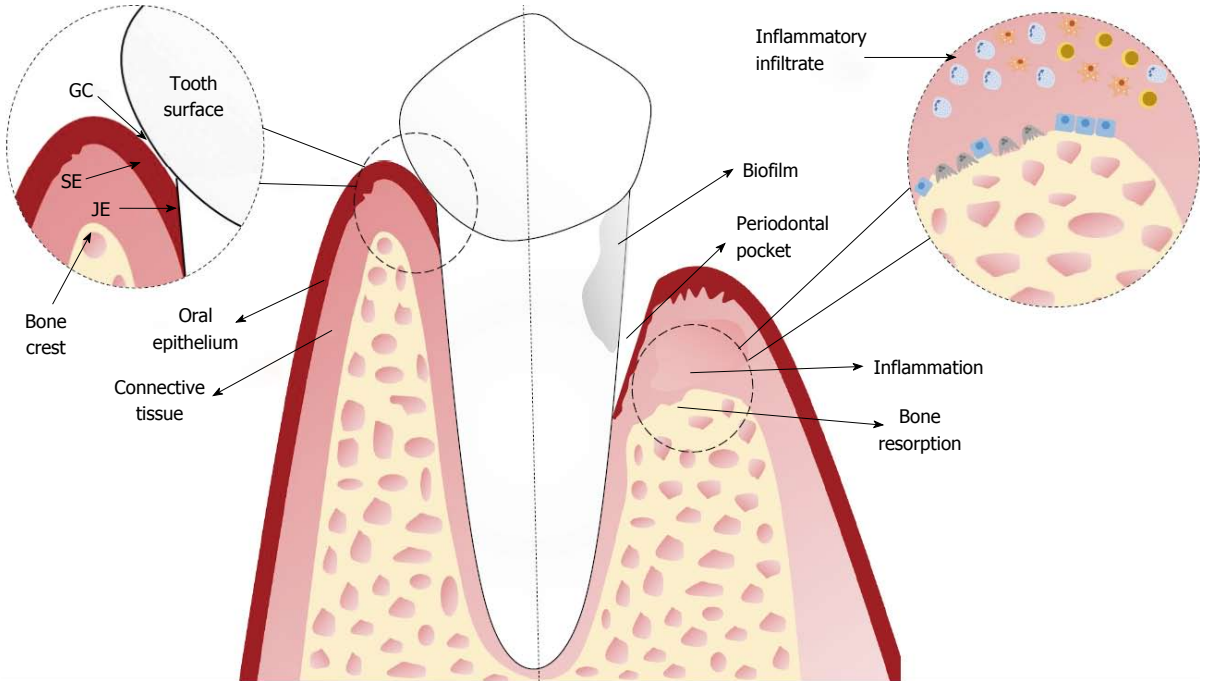

Figure 1 Depiction of the tooth inserted into the alveolar bone in a scenario of periodontal health (left) and periodontitis (right). In periodontal health, the alveolar bone and connective tissue are covered by the oral epithelium. The junctional epithelium (JE) connects the gingiva to the tooth, and the gingival crevice (GC) is the area between the sulcular epithelium (SE) and the tooth surface. In periodontitis, there is a periodontal pocket, a severe inflammatory infiltrate in response to the biofilm, and bone resorbtion.

ducts and interact with naïve T helper cells (Th0), driving their differentiation into several subsets, such as Th1, Th2, Th17, and Treg. These subsets are characterized according to the cytokines they produce[18]. For a long time, periodontitis lesions were conceptually defined based on a Th1/Th2 paradigm, with inconclusive studies pointing to both Th1 and Th2 responses as characterizing disease progression[23]. More recently, the Th17 subset has been implicated in periodontitis pathogenesis, mainly due to its involvement as the specialized lymphocyte linking T-cell activation to bone resorption[24]. In a non-human primate model of periodontitis, Ebersole *et al*[25] have shown an overexpression of the Th17/Treg responses (IL-1β, IL-6, TGF-β, and IL-21) in disease initiation, followed by a persistence of the Th17 response in periodontitis progression.

In conjunction with infiltrating inflammatory cells, gingival fibroblasts take part in the inflammatory process in the periodontium and contribute to the disease persistence[18]. These cells are able to produce cytokines, chemokines and matrix metalloproteinases[26-28]. Baek *et al*[26] have found that gingival fibroblasts from periodontitis patients expressed higher mRNA of IL-1β, IL-6 and TIMP-3, and lower mRNA of IL-4, than fibroblasts from healthy patients. Periodontal ligament fibroblasts also participate in the inflammatory response and play an important role in alveolar bone remodelling[29]. When in cell-cell contact with osteoclast precursors, periodontal ligament fibroblasts up-regulated osteoclastogenesis-related genes and significantly increased the number of osteoclast-like cells[30].

As a consequence of the unresolved inflammation and an increased concentration of inflammatory mediators, tissue destruction occurs. Matrix metalloproteinases (MMPs) are proteolytic enzymes involved in the homeostasis of connective tissue and the balance between MMPs and their endogenous inhibitors (tissue inhibitor of matrix metalloproteinases - TIMPs) controls the MMP activity[18,31]. MMPs play an important role in tissue degradation observed in periodontitis and there is strong evidence of their increased activity in periodontitis[32,33], as well as of an imbalance between MMPs and TIMPs[34].

Regarding bone loss, the main system regulating normal bone resorption and deposition activities that occur during bone remodelling is RANK/RANKL/OPG. RANKL (receptor-activator of nuclear factor-κB ligand) is expressed by several cell types and binds to RANK on osteoclast precursors, causing them to differentiate into active cells that secrete enzymes that degrade bone. OPG (osteoprotegerin) is a soluble decoy receptor of RANKL that prevents the RANK-RANKL interaction[17]. In periodontitis, higher levels of RANKL and lower levels of OPG have been detected in gingival crevicular fluid[35,36]. Several cytokines, such as IL-1β, TNF-α, IL-6, and IL-17, have the ability to stimulate bone resorption, whereas others, such as IL-4, IL-10 and TGF-β, act as inhibitors[37]. Therefore, the inflammatory periodontal milieu, which is rich in pro-resorptive cytokines, can directly affect bone loss

Table 1 Summary of the main epidemiological studies assessing the relationship between inflammatory bowel disease and periodontitis

Ref.	Methods	Principal findings
Flemmig *et al*[74] 1991	107 IBD patients (46 with CD and 61 with UC). Periodontal examination was carried out at two sites of all teeth in two quadrants. There was no control group and results were compared with the assessment of Oral Health of United States Adults	IBD patients presented an 11.9% higher prevalence, but lower severity
Grossner-Schreiber *et al*[42] 2006	62 patients with IBD (46 with CD and 16 with UC) and 59 healthy controls. Periodontal examination was performed in two quadrants	IBD patients had more sites with attachment loss of at least 4 and 5 mm, although periodontal disease was not clearly different from the control group
Brito *et al*[4] 2008	179 patients with IBD (99 with CD and 80 with UC) and 74 controls. Full-mouth periodontal examination was performed	CD and UC patients had higher prevalence of periodontitis than controls, but smoking was an effect modifier
Habashneh *et al*[5] 2012	160 patients with IBD (59 with CD and 101 with UC) and 100 control patients. Full-mouth periodontal examination was performed	Patients with IBD have higher prevalence, severity and extent of periodontitis compared with those having no IBD
Vavricka *et al*[75] 2013	113 patients with IBD (69 with CD and 44 with UC) and 113 controls	Gingivitis and periodontitis markers were higher in patients with IBD than in healthy control. No clear association was found between IBD clinical activity and periodontitis
Koutsochristou *et al*[76] 2015	55 children and adolescents with IBD and 55 controls. Community periodontal treatment needs indices were evaluated	More clinical signs of gingival inflammation and increased periodontal treatment needs were observed in children and adolescents with IBD

CD: Crohn's disease; UC: Ulcerative colitis; IBD: Inflammatory bowel disease.

by increasing RANKL/OPG ratio[17].

IMPACT OF IBD ON PERIODONTAL DISEASE

The clinical presentation of IBD is characterized by the co-existence of extra-intestinal manifestations, which may affect eyes, joints, skin, liver, pancreas, blood, and mouth[7]. These extra-intestinal manifestations may precede or follow the intestinal symptoms by years[38]. Oral manifestations of CD were first described in 1969[39], and can include pyostomatis vegetans, gingival hyperplasia, papillomatosis of the oral mucosa, vesicular eruptions such as in pemphigus vegetans, periodontitis, and caries[4,40]. Also, oral and gingival mucosa may be affected by hypertrophy and swelling of lips, cobblestone appearance of the oral mucosa and palate, presence of vesicles, erosions, ulcers, aphthous-like ulcerations, polypoid lesions, and areas of necrosis[38,41].

Some epidemiological studies have been performed to investigate a possible increase in the prevalence of periodontal disease in patients with IBD. These studies have presented conflicting results. Grössner-Schreiber *et al*[42] have shown that periodontal status was not significantly distinct from the control group, suggesting the IBD did not appear to enhance the susceptibility to periodontal disease. However, only partial periodontal examinations were performed in that study. Other studies using full-mouth periodontal examination have found an increased prevalence of periodontal disease[4,5], as well as higher severity and extent of periodontitis, in IBD patients[5]. These changes seemed to be more pronounced in UC than in CD patients[4,5]. A summary of the main epidemiological studies assessing the association between periodontitis and IBD is presented in Table 1.

The reasons why IBD patients presented poorer periodontal health have not been comprehensively investigated. Since both diseases share pathogenic similarities and their development is related to an aberrant immune response to a dysbiotic microbiota, disturbances of these factors are proposed as the mechanisms responsible for the interaction between the diseases.

Microbiological impact

An important topic that has been evaluated as a possible factor responsible for the altered susceptibility to periodontal disease in IBD patients is the oral microbiota. Van Dyke *et al*[43] have assessed the periodontal microflora of patients with IBD and found a microflora composed predominantly of small, motile, Gram-negative rods that were most consistent with the genus *Wolinella*. Another study has reported decreased overall diversity of the oral microbiota in pediatric patients with CD, but not in patients with UC[44]. Said *et al*[45] have found a significant difference in salivary microbiota composition in patients with IBD. The authors also observed a significant increase of the genus *Prevotella* in the salivary microbiota of IBD patients. In mouse models of colitis, changes were seen in the microbiota of the tongue, buccal mucosa and saliva. Also, the microbial community in saliva was more sensitive to change than that in tongue and buccal mucosa[46].

Our group has analysed the subgingival microbiota in patients with untreated periodontal disease and IBD. We found that several species, such as *Campylobacter gracilis* and *Treponema denticola* differed between

patients with CD, UC and controls in inflamed sites irrespective of the degree of periodontal destruction, and these differences were more pronounced in CD patients. These species might be harmful for the microbe-host interaction[47]. Kelsen *et al*[48], in a cohort of pediatric patients with CD, have shown that Capnocytophaga, Rothia, and TM7 were more abundant in CD relative to healthy controls. The importance of these alterations to the pathogenesis of periodontal disease needs further evaluation.

Immunological impact

The immune-inflammatory response is the main factor driving the tissue damage observed in IBD and periodontitis. Therefore, it is reasonable to assume that the inflammatory response could be the leading factor for posing an increased risk for periodontitis in the IBD population.

Our group investigated the expression of IL-1β, IL-4, IL-6, IL-10, IL-12p40, IL-12p70, IL-18, and INF-γ in gingival crevicular fluid and serum from patients with untreated periodontitis and IBD. We found a significantly decreased level of IL-4 in inflamed sites without tissue destruction from UC patients in comparison with controls. However, for the other cytokines analysed, the expression in gingival fluid was similar in all groups. In serum, IL-18 showed significantly higher levels in CD and UC patients when compared with controls[10]. Similarly to the gingival fluid results, we found that there were no significant differences in the expression of an array of cytokines (IL-1β, IL-4, IL-6, IL-10, IL-21, IL-22, IL-23, IL-25, IL-31, IL-33, IL-17A, IL-17F, IFN-γ, sCD40L, and TNF-α) between CD and UC when assessing the gingival tissue of these patients[11]. Unpublished data from our group suggests that IBD activity probably increases the inflammatory response in the gingival tissue of IBD patients with periodontitis, as evidenced by significantly higher levels of IL-4, IL-10 and IL-21 and a tendency towards higher levels of IL-1β.

Some investigations have focused on the salivary alterations in patients with IBD. Increased levels of pro-inflammatory cytokines have been found in IBD patients, especially those with active disease[49,50]. Aleksandra Nielsen *et al*[51] have reported increased salivary levels of IL-6 in patients with CD and not in patients with UC, but only seven patients were analysed in the UC group. Szczeklik *et al*[49] have found higher salivary levels of IL-1β, IL-6, and TNF-α in patients with active CD than in patients with inactive disease and in controls. Interestingly, CD patients also presented significant reductions in total antioxidant capacity, and increased TGF-β(1), nitric oxide, and lipid peroxidation[50]; UC patients presented higher TGF-β(1) and nitric oxide levels in comparison to the control group[52].

Decreased lysozyme and increased IgA and LL37 in saliva have also been reported in CD and UC patients[46]. It seems that the salivary inflammatory state tended to be slightly higher in UC than in CD group[46]. It is noteworthy that these studies have not always assessed the presence of periodontal disease, which could have entailed a strong confounding effect on the results, since periodontal disease could alter the level of biomarkers in saliva[53]. Though it is tempting to speculate that these salivary changes might account for the increased prevalence of periodontitis in IBD patients, how these alterations might affect the development and/or progression of periodontal disease still needs further investigation.

Interestingly, it has also been reported that buccal epithelial cells from pediatric patients with CD without oral lesions released increased amounts of chemokines (CXCL-8, CXCL-9, and CXCL-10) when compared to epithelial cells from healthy controls, children with UC and adults with CD. Adults with CD did not exhibit increased chemokine production. In addition, stimulation with lipopolysaccharide or zymosan resulted in increased chemokine production by epithelial cells from pediatric patients with CD[54].

Neutrophil behaviour, which plays an important role in the pathogenesis of periodontitis, has also been investigated in patients with IBD. Lamster *et al*[55] have shown that peripheral neutrophils from patients with active IBD displayed greater metabolic activity than neutrophils from patients with inactive IBD, which presented greater metabolic activity than neutrophils from patients without systemic disease. On the other hand, salivary neutrophils from IBD patients displayed an average of 45% less activity than salivary neutrophils from patients without systemic disease. The authors speculated that this might relate to a prior activation of peripheral neutrophils in the circulation of IBD patients. This activation in peripheral blood may compromise the ability of the neutrophils to respond to what becomes a second challenge. Van Dyke *et al*[43] have revealed a serum-mediated defect in neutrophil chemotaxis in IBD patients with periodontal disease, although neutrophil phagocytosis was normal. Interestingly, in this study, the levels of PGE2 in gingival fluid from IBD patients were four times higher than the levels of the control group[43].

The impact of single nucleotide polymorphisms on periodontal status in patients with CD has also been a matter of investigation. Stein *et al*[56] have found a decreased frequency of *Prevotella intermedia* in carriers of CARD15 mutations compared to the wild type, although the clinical periodontal parameters did not differ significantly between them. On the other hand, Schulz *et al*[57] have shown that CD patients carrying the A allele (cDNA-238G>A) or GA haplotype (cDNA-308G>A/cDNA-238G>A) of the TNF-α polymorphisms presented worse clinical periodontal symptoms: increased bleeding on probing, probing depth, and clinical attachment level.

It is interesting to note that in a mouse model

of progressive CD-like ileitis (SAMP1/YitFc), the occurrence of spontaneous periodontal disease was observed in the absence of any exogenous stimuli[58]. The authors have observed similar alveolar bone resorption on both sides of the mouth, suggesting a systemic phenomenon. Thus, they concluded that periodontal disease and IBD likely share similar aetiopathogenic features and multiple pathogenic mechanisms[58]. Previously, Oz *et al*[59] had shown that the oral administration of the low dose DSS (dextran sulphate sodium) induced alveolar bone loss and chronic colitis, as evidenced by severe shrinkage of the colonic tissue and infiltration of inflammatory cells into the colonic tissue. The authors pointed out that this model elicits chronic inflammatory responses in the gut and oral cavity that mimic aspects of IBD and periodontal disease progression in patients.

Furthermore, Park *et al*[60] have used a T-cell transfer model of IBD, using CD4+CD45RB^{High} T cells, to assess the alveolar bone metabolism. It was found that this T cell subset was sufficient for the induction of alveolar bone resorption. It was also reported that alveolar bone marrow stromal cells showed decreased osteogenic and increased adipogenic potential. The authors suggested that diseases such as IBD, through the induction of generalized inflammation, could potentially contribute to alveolar bone resorption. More studies are certainly warranted to further investigate these aspects.

When assessing the possible association between periodontitis and IBD, we have to consider that altered bone metabolism is frequent in IBD patients, where excessive bone loss is a common finding. The exact mechanisms for this are only partially understood, but it has been speculated that corticosteroid therapy, calcium and vitamin D deficiency, hypogonadism, malnutrition, smoking, alcohol consumption, and reduced physical activity are all contributory factors[61]. Also, systemic inflammatory activity is an important factor for the development of osteoporosis in IBD patients[62]. These factors could somehow take part in the association between IBD and periodontitis, contributing to the increased alveolar bone loss seen in this group of patients. Our previous study found that IBD patients taking immunosuppressive drugs had significantly lower concentrations of IL-4 and IFN-γ in the gingival fluid when compared with controls[10].

IMPACT OF PERIODONTAL DISEASE ON IBD

Periodontitis has been associated with other chronic inflammatory diseases for over 20 years. The inflammation evoked by periodontitis could result in low-grade systemic inflammation and thus it is plausible to speculate that periodontitis might influence IBD. Locally produced pro-inflammatory cytokines might enter the systemic circulation, induce an acute-phase response in the liver, and contribute to several processes, such as an atherosclerotic process[63].

Also, as large quantities of oral bacteria are constantly swallowed *via* the saliva into the gut, it has been proposed that swallowed *P. gingivalis* may cause alterations to the gut microbiota, thereby leading to increased gut epithelial permeability and endotoxemia, which causes systemic inflammation[63]. Arimatsu *et al*[64] have used a mouse model to evaluate whether endotoxemia is responsible for inflammation in several organs and tissues. Oral administration of *P. gingivalis*, a proposed periodontopathogen, induced changes of bacterial composition of the gut microbiota along with alterations of gut epithelial cell barrier function. Insulin resistance and change of gene expression in adipose tissue and liver were also observed. Interestingly, *P. gingivalis* was not detected in the gut. Thus, the mechanisms responsible for the changes in gut microbiota remain to be established.

The effects of a single administration of *P. gingivalis* on the gut microbiota, gut barrier function, and influx of gut bacteria into the liver were investigated in a mouse model. This single administration had a great impact on the gut microbiota, as evidenced by an increased proportion of the phylum Bacteroidetes and a decreased proportion of the phylum Firmicutes. In addition, the administration of *P. gingivalis* downregulated the expression of the tight junction protein 1 (tjp-1) and occluding (ocln) in the small intestine, in parallel with an influx of bacteria into the liver. IL-6 expression was significantly elevated and Rorγt expression was significantly decreased in the small intestine, whereas TNF-α expression was significantly increased in the large intestine[65]. Another study by the same group, using a ligature-induced periodontitis model, has shown that the ligature placement induced qualitative but not quantitative changes in the gut microbiota, together with trends toward lower expression of *tjp1* and *ocln* in the small intestine and of *ocln* in the large intestine[66].

Recently, Blasco-Baque *et al*[67] set up a mouse model of periodontitis by infecting the periodontal tissue with *P. gingivalis*, *F. nucleatum* and *P. intermedia*. The mice were fed with either a normal chow diet or a diabetogenic, high-fat, carbohydrate-free diet and were then assessed for periodontal and gut microbiota changes. The authors have found that in mice fed a normal chow diet, periodontitis was associated with modest changes of the gut microbiota which included increased members of the *Actinobacteria* and *Deltaproteobacteria* groups. Similarly, in mice fed a high-fat, carbohydrate-free diet, subtle changes of gut microbiota were also observed.

Another study has postulated that salivary microbiota can affect the development of gut microbiota to some extent, since saliva always flows into the gastrointestinal tract, and thus, salivary bacteria have many opportunities to reach the intestine[46]. In a

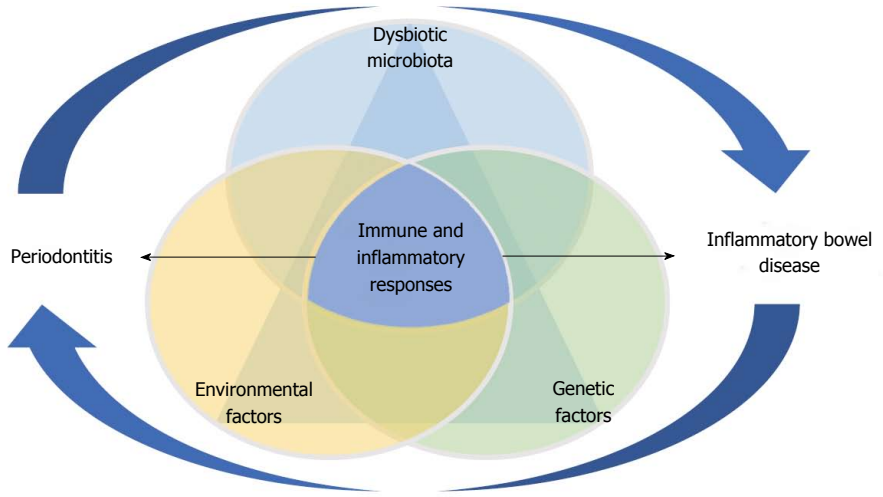

Figure 2 Model of pathogenesis of both periodontal disease and inflammatory bowel disease. This involves a complex interplay between the immune-inflammatory response and a dysbiotic microbiota under the influence of genetic and environmental factors, where the diseases might have a cyclic impact on each other.

study performed to assess the metatranscriptome and metagenome of the human gut microbiota, it was found that oral species, derived from saliva samples, were detectable in the gut at the DNA level, although they showed minimal transcriptional activity there[68]. Interestingly, it was reported that in patients with liver cirrhosis, most of the patient-enriched species in the gut microbiome are of oral origin, suggesting that oral bacterial species could invade the gut[69].

Our study that evaluated the subgingival microbiota found that IBD patients harbour higher levels of bacteria that are related to opportunistic infections, such as *S. aureus* and *S. anginosus*[47]. As shown by Van Dyke *et al*[43], the *Wolinella* isolates, the predominant genus in periodontal microflora of IBD patients, had a profound effect on neutrophil chemotaxis *in vitro*, suggesting that this oral pathogen could play a role in IBD as an infectious agent or as a host response modifier. The impact of these disturbances on IBD remains unknown.

In a study performed to assess the effects of probiotic supplementation on ligature-induced periodontitis and intestinal morphology in rats, Messora *et al*[70] have found that the animals with ligature-induced periodontitis showed alteration in the intestinal structure, such as defects of the villi, epithelial stratification, basal lamina degeneration, and neutrophil infiltration in the small intestine. Also, ligature-induced periodontitis seemed to have shortened and damaged the villi of the jejunum. Probiotic supplementation attenuated these alterations. Another study by the same group has also shown that ligature-induced periodontitis altered villous height and crypt depth in the small intestine[71]. Pietropaoli *et al*[58], in a mouse model of progressive CD-like ileitis (SAMP1/YitFc), have found evidence of a correlation between the severity of periodontal disease and the severity of ileal scores, and this correlation was independent of age. Furthermore, it was shown, in ApoE^{null} mice, that oral infection with *P. gingivalis*, *T. denticola* and *T. forsythia* impaired the BH_4/nNOS/NRF2 pathway in proximal, mid- and distal colon. These results raised the possibility that oral bacteria associated with periodontitis might contribute to colonic motility dysfunctions[72].

A recent study was performed in order to assess the global transcriptome of periodontitis, as well as its association with cardiovascular disease, rheumatoid arthritis and UC, using gingival biopsies. Processes related to immune responses, cell motion, cell death, and homeostasis were up-regulated in all the diseases, but only one gene, pleckstrin (*PLEK*), was commonly up-regulated in all four diseases, suggesting that it could be a key link between periodontitis and these inflammatory diseases. *MMP7* and B-cell lymphoma 2-related protein A1 (*BCL2A1*) were also up-regulated across periodontitis and UC[73].

Taken all together, the studies cited herein show that a model of the interaction of the pathogenesis of periodontitis and inflammatory bowel disease involves a complex interplay between the immune-inflammatory response and the dysbiotic microbiota, under the influence of environmental and genetic factors. We also suggest that there is an interplay between both diseases, where the diseases might have a cyclic impact on each other, which can be seen in Figure 2.

CONCLUSION

This review points to a complex pathogenic interaction between periodontal disease and IBD, in which one disease might alter the composition of the microbiota and increase the inflammatory response related to the other. However, we still need more data derived from human studies to confirm these preliminary results from murine models. Thus, mechanistic studies are definitely warranted to clarify the possible bidirectional association between periodontitis and IBD.

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REVIEW

Portal biliopathy

Mohammad S Khuroo, Ajaz A Rather, Naira S Khuroo, Mehnaaz S Khuroo

Mohammad S Khuroo, Sher-I-Kashmir Institute of Medical Sciences, Srinagar Kashmir 190010, India

Mohammad S Khuroo, Digestive Diseases Centre, Dr. Khuroo's medical Clinic, Srinagar, Kashmir 190010, India

Ajaz A Rather, Department of Surgery, SKIMS Medical College and Hospital, Bemina, Srinagar, Kashmir 190010, India

Naira S Khuroo, Consultant Radiology, Digestive Diseases Centre, Dr. Khuroo's Medical Clinic, Srinagar, Kashmir 190010, India

Mehnaaz S Khuroo, Department of Pathology, Govt. Medical College, Srinagar, Kashmir 190001, India

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Correspondence to: Mohammad S Khuroo, Director, Digestive Diseases Centre, Dr. Khuroo's Medical Clinic, Sector 1, SK Colony, Qamarwari, Srinagar, Kashmir 190010, India. khuroo@yahoo.com
Telephone: +91-194-2492398
Fax: +91-194-2491190

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Abstract

Portal biliopathy refers to cholangiographic abnormalities which occur in patients with portal cavernoma. These changes occur as a result of pressure on bile ducts from bridging tortuous paracholedochal, epicholedochal and cholecystic veins. Bile duct ischemia may occur due prolonged venous pressure effect or result from insufficient blood supply. In addition, encasement of ducts may occur due fibrotic cavernoma. Majority of patients are asymptomatic. Portal biliopathy is a progressive disease and patients who have long standing disease and more severe bile duct abnormalities present with recurrent episodes of biliary pain, cholangitis and cholestasis. Serum chemistry, ultrasound with color Doppler imaging, magnetic resonance imaging with magnetic resonance cholangiopancreatography and magnetic resonance portovenography are modalities of choice for evaluation of portal biliopathy. Endoscopic retrograde cholangiography being an invasive procedure is indicated for endotherapy only. Management of portal biliopathy is done in a stepwise manner. First, endotherapy is done for dilation of biliary strictures, placement of biliary stents to facilitate drainage and removal of bile duct calculi. Next portal venous pressure is reduced by formation of surgical porto-systemic shunt or transjugular intrahepatic portosystemic shunt. This causes significant resolution of biliary changes. Patients who persist with biliary symptoms and bile duct changes may benefit from surgical biliary drainage procedures (hepaticojejunostomy or choledechoduodenostomy).

Key words: Biliary disease; Extrahepatic portal venous obstruction; Portal cavernoma; Bile duct strictures; Bile duct calculi

Core tip: Extrahepatic portal vein obstruction is often encountered in children in India. It is caused by long standing thrombosis of portal vein and leads to cavernous transformation of the bridging venous collaterals. Cholangiographic abnormalities occur in majority of such patients, however, the entity stays asymptomatic in early stages. Biliopathy is a progressive disease and patients surviving to adulthood develop more severe biliary abnormalities and present with clinical disease. Now, portal biliopathy is an important clinical entity faced by hepatologists in India. Since the disease was described in early 90's, there have been important developments in definition, pathogenesis, diagnostic modalities and therapeutic interventions of portal biliopathy.

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INTRODUCTION

Portal biliopathy refers to cholangiographic abnormalities which occur in patients with portal cavernoma[1-3]. Biliary abnormalities do occur in patients with cirrhosis and idiopathic portal hypertension. These are primarily in the intrahepatic bile ducts and are caused by hepatic nodularity and fibrosis rather than portal cavernoma[4]. Some investigators have suggested that such cholangiographic abnormalities should also be defined as portal biliopathy. However, there is a general consensus that portal biliopathy should be restricted to those cholangiographic abnormalities which are caused by portal cavernoma. Other biliary diseases which may superficially resemble portal biliopathy and need to be excluded include choledocholithiasis, primary sclerosing cholangitis, biliary parasitosis, AIDS cholangiopathy, oriental-cholangio-hepatitis and cholangiocarcinoma *etc.*[1].

Historical background

Portal biliopathy as a clinical entity was established by 3 reports published in early 90's. In 1989, we were confronted with biliary disease in two patients of portal cavernoma[5]. Both patients exsanguinated and died at elective gall bladder surgery. We searched the literature around that time and could not find well-documented reports of biliary disease with extrahepatic portal venous obstruction (EHPVO). From December 1989 to November 1991, we[3] prospectively studied 21 consecutive such patients for evidence of biliary tract disease. Cholangiographic abnormalities were detected in 17 (80.9%) patients. Three adult patients had clinically manifest biliary disease. The pathogenesis of these changes and their relationship with portal cavernoma was critically evaluated. Another study reported on bile duct changes in 20 patients with portal cavernoma[6]. Sarin *et al*[7] observed similar changes in 80% patients with EHPVO. Since then, several investigators have reported on case series of patients with portal biliopathy (Table 1)[8-22]. Portal biliopathy has been described by a multitude of names in literature. Dilawari and Chawla[6] described these cholangiographic abnormalities as "pseudosclersoing cholangitis". Bayraktar[8] described these abnormalities resembling "pseudocholangiocarcinoma sign". Dhiman *et al*[16] named it portal hypertensive biliopathy, while others have used terms extrahepatic portal biliopathy[23], vascular biliopathy[24], portal ductopathy, portal cholangiopathy[25] and portal cavernoma cholangiopathy[1] to describe this entity. However, authors believe that portal biliopathy suggested by Sarin *et al*[7] is appropriate term to reflect the cholangiographic abnormalities in this entity.

BILIARY ANATOMY

Biliary tract has intrahepatic and extrahepatic components. The anatomy of the intrahepatic bile duct follows that of the portal system and segmentation of the liver. The Couinaud classification divides liver in to eight (Ⅰ to Ⅷ) segments. Right liver lobe, consisting of segments Ⅴ, Ⅵ, Ⅶ and Ⅷ is drained by the right hepatic duct. The left hepatic lobe consisting of Segments Ⅱ, Ⅲ and Ⅳ is drained by left hepatic duct. Caudate lobe (segment 1) receives small ducts from right and left lobes. The right and left hepatic ducts join in the hilum to form common hepatic duct, which continues as common bile duct from the point cystic duct joins it laterally. Common bile duct is 6.0-8.0 cm long and has four segments namely supraduodenal, retroduodenal, retropancreatic and intraduodenal[26,27].

Biliary tree including ampulla of Vater receive blood supply from branches of the coeliac trunk[27]. The common bile duct is palisaded by 3 o'clock (left) and 9 o'clock (right) marginal arteries, which derive blood supply from right hepatic artery, left hepatic artery, cystic artery and posterior superior pancreaticoduodenal artery. The marginal arteries through intricate network of vessels form two plexuses, one around the bile duct (paracholedochal plexus) and another on the surface of bile duct (epicholedochal plexus). Several branches perforate the bile duct wall (intramural plexus) and reach under the bile duct epithelium (subepithelial plexus). The marginal arteries intercommunicate at hilum forming hilar plexus. The

Table 1 Reported studies of portal biliopathy

Ref.	Year of publication	City Country	Number of patients	Age (mean)	Male: Female	Biliary changes	Symptomatic disease
Khuroo *et al*[3]	1993	Kashmir India	21	14.0	13:8	81%	38%
Dilawari *et al*[6]	1992	Chandigarh India	20	22.0	16:4	100%	5%
Sarin *et al*[7]	1992	Delhi India	20	-	16:4	90%	15%
Bayraktar *et al*[8]	1995	Ankara Turkey	44	31.5	24:20	94%	30%
Malkan *et al*[9]	1999	Mumbai India	20	23.0	12:08	85%	10%
Nagi *et al*[10]	2000	Chandigarh India	43	-	25:18	100%	19%
Condat *et al*[11]	2003	Paris France	25	49.5	15:1	84%	28%
Sezgin *et al*[12]	2003	Mersin Turkey	36	-		94%	10%
Khare *et al*[13]	2005	Lucknow India	13	-	9:4	100%	100%
Belhadjbrik *et al*[14]	2006	Tunis Tunisia	17	-		100%	82%
Chevallier *et al*[15]	2006	Nice Cedex 3 France	10	-		90%	40%
Dhiman *et al*[16]	2007	Chandigarh India	53	24.5	36:17	100%	24.5%
Vibert *et al*[17]	2007	Villejuif France	64	-		100%	30%
Oo *et al*[18]	2009	Birmingham UK	13	-		100%	100%
Llop *et al*[19]	2011	Barcelona Spain	67	47.0	41:26	78%	21%
Agarwal *et al*[20]	2011	New Delhi India	39	29.6	27:11	100%	100%
Aguirre *et al*[21]	2012	Bogota Columbia	18	-		100%	100%
Aguilar-Olivas *et al*[22]	2014	DF Mexico	4	-		100%	100%

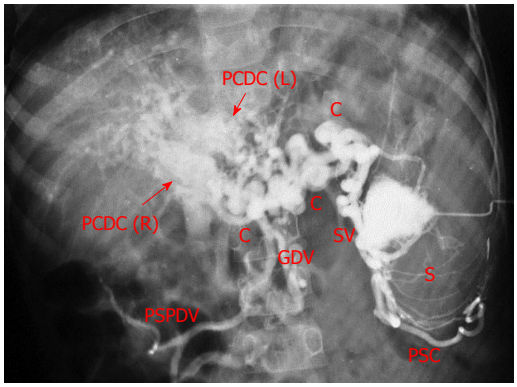

Figure 1 Splenoportovenography in a patient with extrahepatic portal venous obstruction and portal cavernoma. Contrast within splenic pulp (S) is drained by multiple splenic vein channels (SV) into large peripancreatic collaterals (C). There are two broad parallel conglomerate of veins in the porta hepatis (arrows) formed by right and left paracholedochal collaterals [PCDC (R) and PCDC (L) respectively], forming the portal cavernoma. Main portal vein is not seen (thrombosed). Retrograde filling of perisplenic collaterals (PSC), gastroduodenal vein (GDV) and posterior-superior pancreaticoduodenal vein (PSPDV) is seen.

right and left hepatic arteries are connected with each other by communicating arcade. The intrahepatic bile ducts are accompanied by corresponding arteries and form peribiliary plexus. These arteries communicate with venules through arterioportal channels.

The venous drainage of bile duct is accomplished by epicholedochal venous plexus of Saint and paracholedochal plexus of Petren. Theses drain in to 3 o'clock and 9 o'clock marginal veins. An additional 6 o'clock marginal vein may be present posterior to bile duct. The marginal veins drain in to gastric veins, posterior superior pancreaticoduodenal vein and gastrocolic trunk[26].

PORTAL CAVERNOMA

Portal cavernoma is formed by serpiginous, tortuous and dilated 3 o'clock and 9 o'clock marginal veins and venous of plexus of Petren, that bridge thrombosed portal vein. The portal vein, following thrombosis may be atretic or re-canalized. In addition, the epicholedochal plexus of Saint on the surface of bile ducts and cholecystic veins surrounding and within gallbladder wall also dilate and become tortuous (Figure 1)[28]. Portal cavernoma formation may take from a week to a year. Over time, the portal cavernoma may turn in to a "solid tumor-like cavernoma" compromising of fibrous hilar mass containing multiple collateral veins[1,3].

Portal cavernoma in developing countries occurs in children and results from portal vein thrombosis secondary to neonatal umbilical sepsis and dehydration. In adults, portal vein thrombosis with resultant portal cavernoma is caused by a number of conditions including hypercoagulable states, myeloproliferative disorders, Bechet's syndrome, pancreatitis and pylephlebitis. No underlying cause is detectable in around 30% patients. Portal cavernoma formation is rare in portal vein thrombosis with underlying cirrhosis and portal hypertension, as stasis in portal venous system in such patients prevents formation of collaterals.

The diagnosis of portal cavernoma is easily made at grey scale and color Doppler sonography[29]. Portal vein is not visualized or may be atretic or recanalized. Liver hilum reveals multiple dilated serpiginous channels. Flow pattern within the tortuous channels on color and duplex Doppler show portal venous type of flow with absent respiratory or cardiac variation. Patients with solid tumor cavernoma depict an echogenic irregular mass of varying size with dilated tortuous channels passing through the mass. Hepatic arteries demonstrate increased flow to compensate for reduced portal flow. Multiphase computed tomography (CT) and magnetic resonance imaging (MRI) reveal thrombosed portal vein which may be atretic or recanalized with

dilated tortuous channels, enhancing during portal venous phase and not during arterial phase. This helps to exclude arteriovenous malformation. In solid tumor cavernoma, CT and MRI show an echogenic mass with numerous venous channels. In some patients, linear areas of calcification within the previously thrombosed portal vein may be seen, indicating chronic venous thrombosis.

PATHOGENESIS

The pathogenesis of cholangiographic abnormalities in EHPVO and portal cavernoma are multifactorial[3]. Broadly the cholangiographic abnormalities have reversible and fixed component (Figure 2). Reversible component includes those changes which are likely to resolve following portal venous decompression [shunt surgery or transjugular intrahepatic portosystemic shunt (TIPS)]. These include shallow bile duct impressions and indentations causing wall irregularity, smooth strictures with upstream dilatation and luminal filling defects. These changes are as a result of dilatation of veins of the plexus of Saint, causing bile duct impingement. Bile duct strictures with upstream dilatation may be as a result of compression by dilated tortuous collaterals[30,31]. In addition, bile duct filling defects occur due to dilatation of perforators and subepithelial veins in the ducts. This hypothesis is based on findings at MRCP and MR portography which demonstrate signal void-defects of dilated veins around the bile duct lumen[32]. Rarely, bile duct wall shows localized thickening and luminal narrowing as a result of dilated tortuous choledochal vessels. Further shunt surgery or TIPS results in partial or complete resolution of cholangiographic abnormalities. In some cases, these changes of biliopathy may persist after portal decompression. This is related to persistence of portosystemic collaterals and does not necessarily imply ischemia as a cause of bile duct changes. Left hepatic duct is involved more often and shows more severe changes as umbilical vein enters left portal vein, leading to formation of prominent collaterals.

Fixed component cholangiographic abnormalities include those changes which do not regress after portal decompression. These include rigid stricture, angulation, and ductal ectasia. Rigid strictures are usually due to ischemia, which occurs as the time of portal vein thrombosis. As bile duct are supplied by hepatic arteries, ischemic necrosis may result from concomitant hepatic artery thrombosis. In addition, portal vein thrombosis per se may cause bile duct ischemia due to damage to the microvascular bile duct flow. Strictures can also form as a result of prolonged compression by dilated tortuous collaterals. Angulation and rigid strictures result from encasement of bile ducts by solid tumor-like cavernoma[1,3]. Gallstones formation results from several pathogenic mechanisms which include: (1) bile stasis due to strictures; (2) increased lithogenicity of bile; (3) reduced contractility of gallbladder due to collateral in the gallbladder wall; and (4) decreased bile flow due to liver atrophy, resulting from reduced portal venous flow[33].

CLINICAL DISEASE

Majority of patients with portal biliopathy have no biliary symptoms (asymptomatic stage). Such patients have early cholangiographic abnormalities which include duct irregularity; serration, undulation, scalloping of the duct wall; and smooth extrinsic nodular, spiral or stenotic impressions on the ducts and filling defects in the bile and hepatic ducts. This asymptomatic phase of portal biliopathy may last for years and patients develop clinical biliary disease only if they survive to adulthood. Patients with clinical disease are older and have longer duration of portal hypertension. There is sparse data on the other factors like extent of thrombosis and the presence of portosystemic shunts affecting the occurrence of symptomatic biliopathy. During the asymptomatic phase, patients often show elevated serum alkaline phosphatase, the significance of which vis-à-vis biliary disease is difficult to interpret in children. The first detectable clinical disease starts with isolated elevated serum bilirubin and/or detectable icterus[34]. The occurrence of jaundice usually points to presence of slow onset subtle biliary obstruction or hemolysis related to large spleen and is often detected incidentally while evaluating the patients for other symptoms of portal hypertension. Patients with symptomatic biliary disease have advanced cholangiographic abnormalities which include ectasia of the ducts; angulation, displacement and strictures of the bile and hepatic ducts and aneurysmal dilatation of the intrahepatic biliary tree. The patients present with episodes of biliary pain or cholangitis and/or biliary obstruction. Biliary pain may be related to gallstones or bile duct stones or to cholangitis. Biliary obstruction presents with cholestasis with or without episodes of cholangitis[34-37]. There is considerable variation in clinical presentation of biliary obstruction. However, most of patients have recurrent and progressive disease and often need to be hospitalized repeatedly to control symptoms[34,38]. Portal biliopathy late in the clinical course cause marked bile duct abnormalities which include long (> 2 cm) or multifocal strictures, strictures associated with choledochal or intrahepatic calculi and biliopancreatic complications. Therapeutic options in such patients are limited due extensive venous thrombosis and advanced liver disease.

NATURAL COURSE

Data on the natural course of biliopathy following portal vein thrombosis and portal cavernoma are scarce[18,19,39]. Only one study has reported on follow up in 22 patients of portal vein thrombosis and 45 patients with established portal cavernoma[19]. At initial assessment, majority of patients in either group

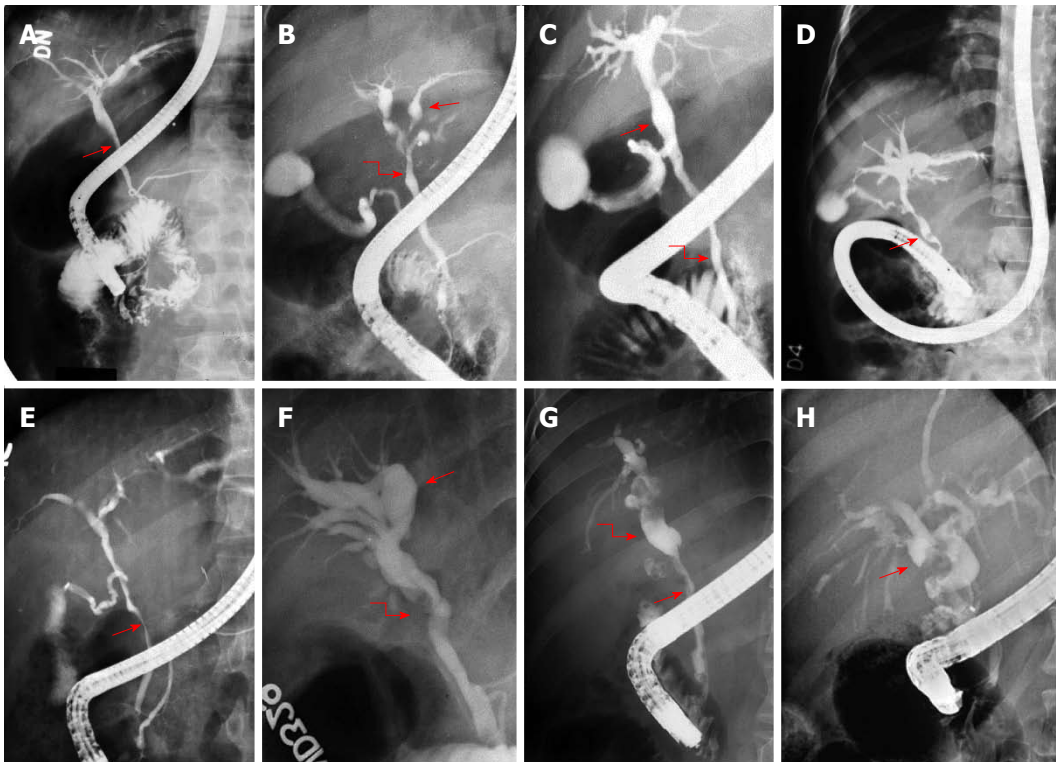

Figure 2 Endoscopic retrograde cholangiograms in 8 patients of portal biliopathy depicting spectrum of cholangiographic abnormalities of portal biliopathy. A: Extrinsic impression on common bile duct (arrow); B: Ectasia of left hepatic duct (arrow) and caliber irregularity of common hepatic duct (curved arrow); C: Ectasia of common hepatic duct (arrow) and caliber irregularity of common bile duct (curved arrow); D: Angulation (arrow) of common bile duct; E: Large smooth impression (arrow) on common bile duct; F: Angulation common hepatic duct (curved arrow) and gross ectasia of intrahepatic ducts (arrow); G: Long smooth stricture of common bile duct (arrow) with upstream dilatation (curved arrow); H: dilated bile ducts with multiple filling defects (arrow). Adapted and modified from Khuroo *et al*[3].

had developed evidence of biliopathy. Symptomatic disease was limited to patients with severe biliary abnormalities. Over a follow up period extending up to 4 years, disease was non-progressive. Authors believed the disease to be a one-time event after acute portal vein thrombosis. However, patients with symptomatic biliopathy have established long-term disease extending for over 8 to 14 years. This suggests that natural course of portal biliopathy is progressive and long term portal hypertension and significant biliary abnormalities leading to symptomatic biliopathy[1].

DIAGNOSIS

Ultrasonography

Ultrasound with color Doppler is an excellent imaging modality in evaluation of portal cavernoma[29] (Figure 3). Gray scale and color Doppler imaging show atretic or racanalized portal vein with a mass of multiple serpentine channels, seen in the porta hepatis. These collaterals demonstrate portal venous type flow[40]. An increased flow in hepatic artery may be seen, representing a compensatory mechanism to the reduced portal flow. Color Doppler has advantage of showing varices around and in the wall of gallbladder. Bile duct wall may show collaterals within the thickened bile ducts. Ultrasound can detect bile duct dilatation with associated cholelithiasis and choledocholithiasis. In addition, hepatic parenchyma can be well seen on ultrasound with associated portosystemic collaterals and splenomegaly. However, ultrasound has limitation in visualization of common bile duct especially in retroduodenal segment and cannot differentiate between extrinsic pressure from collaterals and ischemic stricturing.

Computed tomography or magnetic resonance imaging

Contrast enhanced computed tomography (CECT) or magnetic resonance imaging (MRI) have advantage over ultrasound in delineating the anatomy of portal venous system (portovenography), help to find cause of portal venous thrombosis like pancreatitis and exclude biliary malignancy[21]. MRI is a better imaging tool in this setting as it does not expose incumbent to radiation and delineates better biliary anatomy. MRI can differentiate epicholedochal collaterals, which appear as signal void-defects from paracholedochal collaterals which appear as low signal channels on T2-weighted images[41]. Typical biliary findings of biliopathy are well seen on MR cholangiography[15,42]. Hence MRI is the modality of choice for mapping of the biliary and vascular abnormalities[43] (Figure 4).

Endoscopic ultrasound

Role of endoscopic ultrasound (EUS) in the diagnosis of portal biliopathy is evolving[44-47]. Portal cavernoma

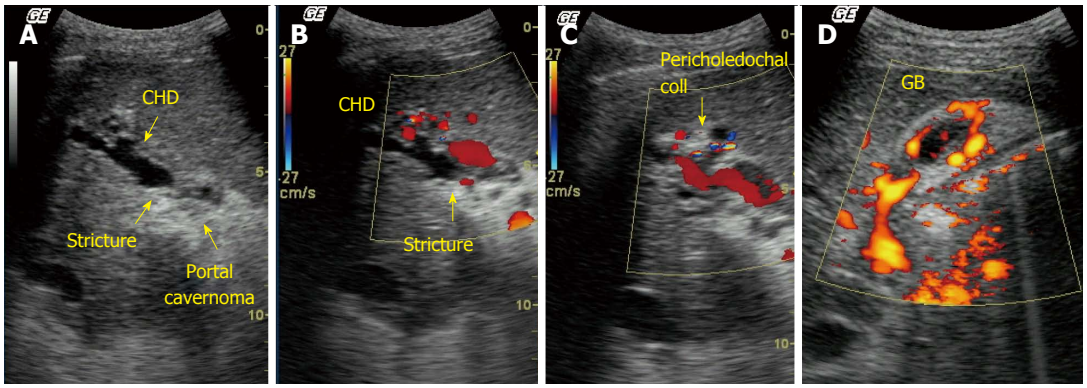

Figure 3 Ultrasound with Doppler in a patient with extrahepatic portal venous obstruction and portal biliopathy. A: Gray scale image shows an echogenic mass in hilum (portal cavernoma). Common hepatic duct (CHD) shows dilatation and luminal irregularity with a stricture at the level of the cavernoma; B: Color Doppler image showed multiple pericholedochal collaterals around the strictured bile duct (arrow); C: Color Doppler image shows pericholedochal collaterals and recanalized irregular portal vein; D: Power Doppler images of gall bladder. Gall bladder wall is thickened with multiple collaterals within the gall bladder wall.

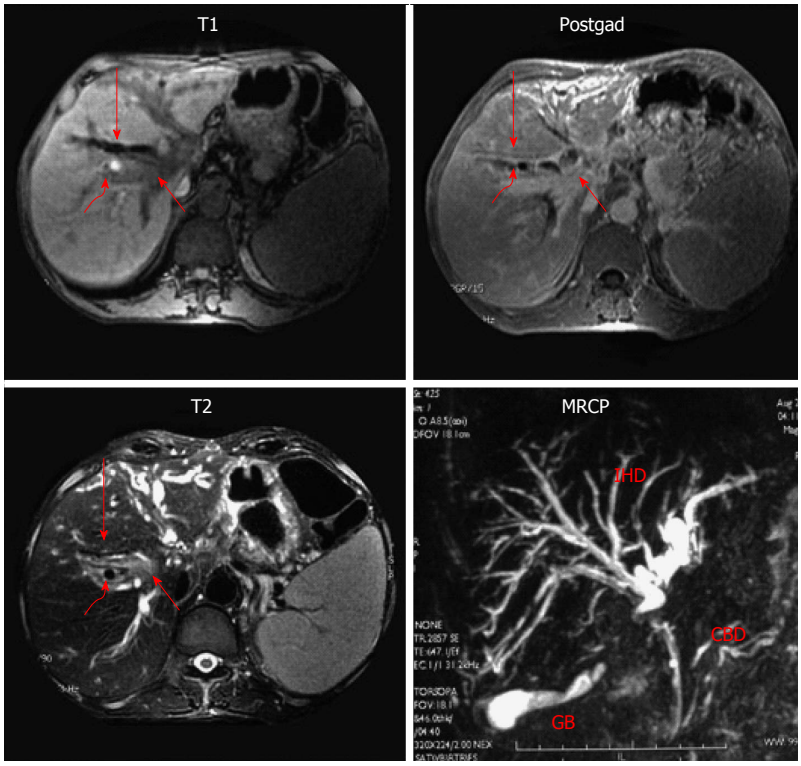

Figure 4 Magnetic resonance imaging with Magnetic retrograde cholangiogram in a patient with portal biliopathy. T1 weighted, T2 weighted and post-gadolinium (postgad) images. Mass lesion (short arrow) is seen at the level of porta hepatis with dilated bile ducts (long arrow) and signal void (curved arrow), depicting collateral channels compressing bile ducts, MRCP. Common bile duct (CBD) shows luminal irregularity. There is a localized irregular stricture in common hepatic duct with upstream gross dilatation of intrahepatic ducts (IHD).

and its anatomy can be well defined. These collaterals appear as multiple vascular channels in and/or around the extrahepatic biliary tract. Paracholedochal, epicholedochal, intracholedochal and subepithelial varices can be well seen and differentiated[48]. Visualization of choledochal and subepithelial venous plexuses is important as biliary endotherapy may increase risk of hemobilia.

Endoscopic retrograde cholangiography

Endoscopic retrograde cholangiography (ERC) is the gold standard for defining the biliary changes of portal biliopathy (Figure 2). In fact, ERC has been employed to define bile duct changes, study their distribution in the biliary tract and assess the severity and grading of abnormalities. ERC is being replaced by ultrasound and MRCP. ERC is invasive, has risk of complications and cannot be employed on repeated occasions for follow up examinations. In contrast ultrasound and MRCP are non-invasive, give comparable images of biliary tract and have advantages to further visualize portal venous system.

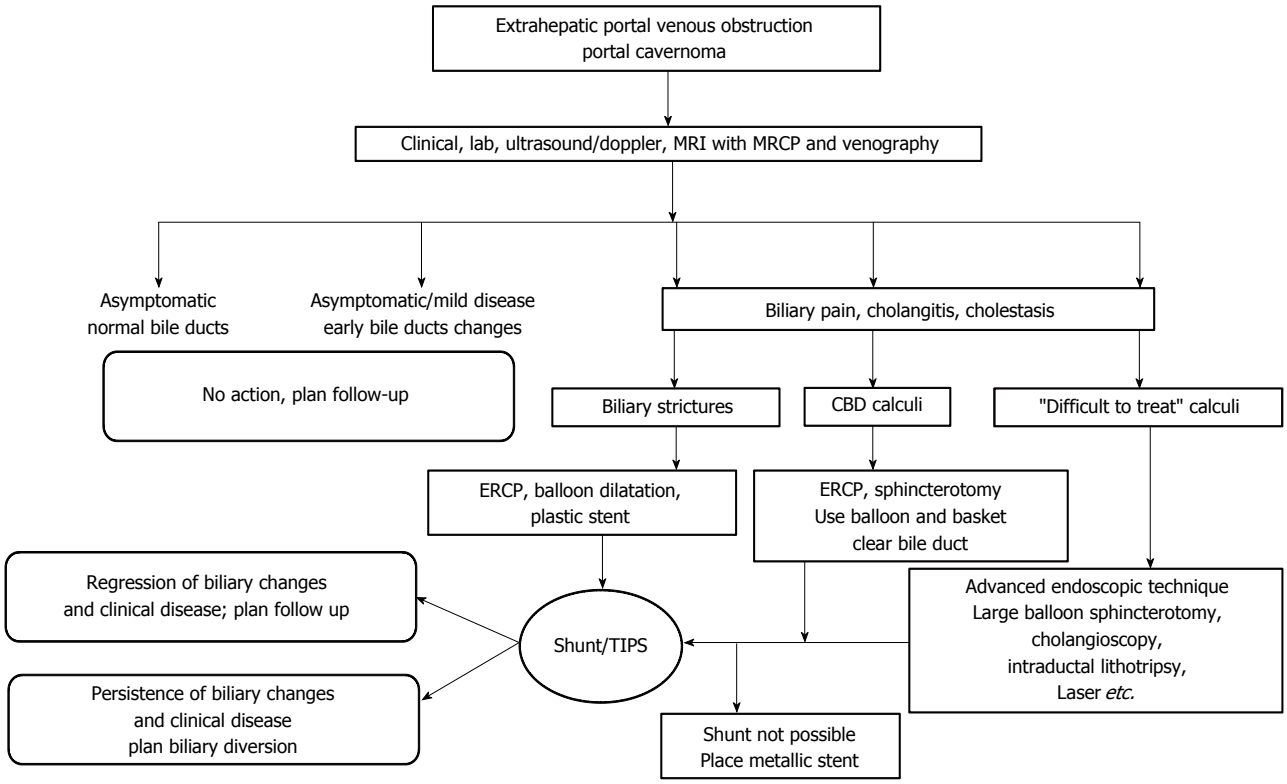

Figure 5 Management algorithm in patients of extrahepatic portal venous obstruction and portal biliopathy. See text under "Management" for details. TIPS: Transjugular intrahepatic portosystemic shunt.

Cholangiographic abnormalities on ERC known to occur in portal biliopathy include: (1) shallow extrinsic impressions/indentations of the bile and hepatic ducts; (2) deep extrinsic impressions/indentations of the bile and hepatic ducts; (3) irregularity in ductal contour; (4) strictures with upstream dilatation; (5) filling defects which may be round, oval, or elongated and are caused by stones, intra-luminal varices, or blood clots; (6) bile duct angulation causing an angle of $\leqslant$ 145° between lower and upper ductal alignment; and (7) ectasia which cause dilated segment of biliary tree without any evident downstream obstruction. The cholangiographic abnormalities may occur in bile ducts alone, hepatic ducts alone, bile ducts with unilateral hepatic ducts and bile ducts bilateral hepatic ducts.

MANAGEMENT

Asymptomatic patients

Asymptomatic patients with portal biliopathy do not need any active treatment. Such patients have early cholangiographic abnormalities. As these changes are progressive in nature, it is worthwhile to have serial assessment of type, extent and severity of these changes on a long term follow up. Ultrasound with color Doppler is ideal imaging tool to evaluate portal cavernoma and biliary tract for strictures and stones. Patients with mild abnormalities in liver tests without clinical disease should be included in asymptomatic disease and need no active intervention.

Endotherapy

Treatment of symptomatic biliopathy can be approached in a stepwise fashion. Initial treatment is aimed at managing biliary strictures and stones. Next portal decompression should be done to reverse biliary abnormalities. Lastly biliary obstruction should be relieved by biliary diversion at surgery[17,22,49,50] (Figure 5). Patients presenting with cholestasis and/or cholangitis should be primarily managed by biliary endotherapy[2,51]. Common bile stones are managed by endoscopic sphincterotomy with stone extraction[33]. Patients with cholangitis or cholestasis need endoscopic biliary drainage with plastic stents or nasobiliary drainage[52]. Biliary strictures can be treated by balloon dilatation and stent placement[53].

Advanced biliary endoscopic procedures can be employed in patients with difficult biliary calculi. Such patients need large balloon sphincteroplasty, cholangioscopy with intraductal lithotripsy using laser and electrohydraulic probes. As of now, even intractable biliary calculi in biliopathy are amenable to endoscopic therapy. Many patients need repeated plastic stents and is a problem for patients who come from far flung regions and cannot reach tertiary care centers regularly. Such patients are candidates for placement of self-expanding metal stent (Figure 6)[54]. Placement of metallic stents in a benign disease may cause difficulty in retrieval of stents if needed and intraoperative difficulty in case these patients require surgical management for persistent biliary

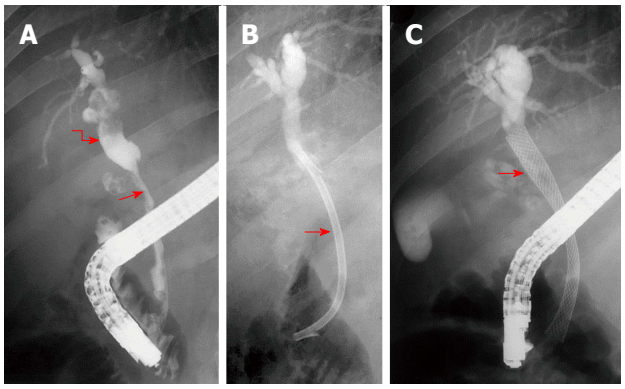

Figure 6 Endoscopic retrograde cholangiogram and metallic biliary stenting in a patient with portal biliopathy. A: Long smooth stricture common bile duct (arrow) with upstream dilatation of intrahepatic ducts (curved arrow); B: A 12 Fr plastic stent (arrow) is placed across the stricture. Patient needed repeated stent replacement in the follow up and was considered for shunt surgery. Shunt surgery was not possible due unavailability of a shuntable portal vein; C: Self-expanding nitinol biliary stent (arrow) placed across the stricture. Patient stays asymptomatic over long-term follow up.

symptoms[18,55]. Endoscopic sphincterotomy along with use of Dormia basket and balloon extraction of stones in portal biliopathy has been shown to be safe[56]. Intraductal and subepithelial collaterals are risk factors for hemobilia. Such varices can be demonstrated on EUS and it may be worth doing this procedure once biliary endotherapy is envisaged. Hemobilia, if occurs, is usually mild and needs conservative treatment and settles in most cases[55,57].

Biliary surgery namely surgical removal of CBD calculi or bilio-enteric anastomosis without prior portal decompression carries higher mortality and should be avoided. Laparoscopic cholecystectomy for gallstones in two patients with pericholecystic collaterals and non-obstructive biliopathy has been performed safely with careful operative strategy and diligent hemostasis[58]. The use of concomitant ursodeoxycholic acid (UDCA) is recommended as it has been reported to be beneficial by some authors[59].

Shunt surgery

With advances in endotherapy, patients with biliopathy are increasingly being managed with multiple stents for prolonged periods of time. Such patients should be evaluated for surgical porto-systemic shunts or TIPS[24,25,60-63] (Figure 7). One of the strong arguments in favor of operative management has been the fact that surgery is a onetime procedure, does not require repeated hospital visits, which is particularly relevant for the majority of EHPVO patients who come from areas where specialized medical help with access to endotherapy may not be available. Shunt procedures cause regression in collaterals and as a result cause improvement in the cholangiographic abnormalities of portal biliopathy and significant clinical improvement. Follow up studies have shown that around two-thirds of patients remain asymptomatic and complete

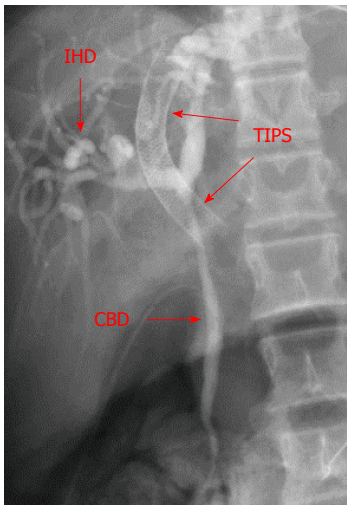

Figure 7 Transjugular intrahepatic porto-systemic shunt in a patient with extrahepatic portal venous obstruction and portal biliopathy. Common bile duct shows a long smooth stricture (CBD) with upstream dilatation of intrahepatic ducts (IHD). Transjugular intrahepatic porto-systemic shunt (TIPS) (arrows) was placed to decompress the portal venous circulation. Follow-up showed significant improvement in cholangiographic appearances.

reversal of cholangiographic abnormalities.

Even if the regression of cholangiographic changes is incomplete, majority of the patients remain asymptomatic probably due to slowing-down of progression of biliopathy after shunt. Around one third of patients continue to have cholangiographic abnormalities and may suffer from repeat biliary symptoms. TIPS, though effective in portal decompression has issues of wider accessibility and high occlusion rate at one year[64]. In patients with extensive thrombosis and non-shuntable vein or blocked shunt, patients must be managed with continued endoscopic stents. Such patients may be candidates for placement of self-expanding metal stents.

Biliary drainage

The third phase is to manage patients who continue to be symptomatic with cholangiographic changes despite portal decompression. Some patients may develop symptoms due to blocked portosystemic shunt. Biliary drainage procedures like hepaticojejunostomy or choledochoduodenostomy are performed with good clinical results[20,65]. Liver transplantation is indicated in patients with secondary biliary cirrhosis and end stage liver disease[66,67].

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REVIEW

Transanal surgery for obstructed defecation syndrome: Literature review and a single-center experience

Wei-Cheng Liu, Song-Lin Wan, SM Yaseen, Xiang-Hai Ren, Cui-Ping Tian, Zhao Ding, Ken-Yan Zheng, Yun-Hua Wu, Cong-Qing Jiang, Qun Qian

Wei-Cheng Liu, Song-Lin Wan, SM Yaseen, Xiang-Hai Ren, Cui-Ping Tian, Zhao Ding, Ken-Yan Zheng, Yun-Hua Wu, Cong-Qing Jiang, Qun Qian, Department of Colorectal and Anal Surgery of Zhongnan Hospital of Wuhan University, Key Laboratory of Intestinal and Colorectal Diseases of Hubei Province, Quality Control Center of Colorectal and Anal Surgery of Health and Family Planning Commission of Hubei Province, Clinical Center of Intestinal and Colorectal Diseases of Hubei Province, Wuhan 430071, Hubei Province, China

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Correspondence to: Qun Qian, MD, PhD, Professor, Chief, Department of Colorectal and Anal Surgery of Zhongnan Hospital of Wuhan University, Key Laboratory of Intestinal and Colorectal Diseases of Hubei Province, Quality Control Center of Colorectal and Anal Surgery of Health and Family Planning Commission of Hubei Province, Clinical Center of Intestinal and Colorectal Diseases of Hubei Province, Wuhan 430071, Hubei Province, China. qunqian2007@163.com
Telephone: +86-27-67812778
Fax: +86-27-67812778

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Abstract

Obstructed defecation syndrome (ODS) is a functional disorder commonly encountered by colorectal surgeons and gastroenterologists, and greatly affects the quality of life of patients from both societal and psychological aspects. The underlying anatomical and pathophysiological changes of ODS are complex. However, intra-rectal intussusception and rectocele are frequently found in patients with ODS and both are thought to play an important role in the pathogenesis of ODS. With the development of evaluation methods in anorectal physiology laboratories and radiology studies, a great variety of new operative procedures, especially transanal procedures, have been invented to treat ODS. However, no procedure has been proved to be superior to others at present. Each operation has its own merits and defects. Thus, choosing appropriate transanal surgical procedures for the treatment of

ODS remains a challenge for all surgeons. This review provides an introduction of the current problems and options for treatment of ODS and a detailed summary of the essential assessments needed for patient evaluation before carrying out transanal surgery. Besides, an overview of the benefits and problems of current transanal surgical procedures for treatment of ODS is summarized in this review. A report of clinical experience of some transanal surgical techniques used in the authors' center is also presented.

Key words: Obstructive defecation syndrome; Transanal surgery; Transanal manual technique; Transanal stapling procedure; Medical assessment; Clinical outcome; Clinical experience

Core tip: Transanal surgery for obstructed defecation syndrome (ODS) remains a challenge for colorectal surgeons. Possible reasons are that reported clinical outcomes of current transanal surgical procedures are controversial and the patient selection criteria for different procedures are usually deficiently described in the current literature. This article reviews the literature regarding transanal surgery, introduces current problems and options for treatment of ODS and summarizes essential assessments needed for patient evaluation and the benefits and problems of each procedure. The aim of this article is to improve the understanding of selective strategies of transanal operations and increase patient satisfaction.

Liu WC, Wan SL, Yaseen SM, Ren XH, Tian CP, Ding Z, Zheng KY, Wu YH, Jiang CQ, Qian Q. Transanal surgery for obstructed defecation syndrome: Literature review and a single-center experience. *World J Gastroenterol* 2016; 22(35): 7983-7998 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i35/7983.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i35.7983

INTRODUCTION

Obstructed defecation syndrome (ODS) is defined as paradoxical contraction or inappropriate relaxation of the pelvic floor muscles during attempted defecation with symptoms such as excessive straining, incomplete or fragmented evacuation, and/or inappropriate propulsive forces during attempted defecation with symptoms such as need for perineal or digital facilitation of defecation, tenesmus, urgency and pelvic heaviness with the normal desire to defecate[1-3].

Rectocele and internal rectal intussusception are anatomic disorders that have frequently been associated with ODS[4-8]. To date, there have been various surgical methods used for the treatment of symptomatic ODS through transanal approaches[9-13]. However, these transanal surgeries for ODS are challenges for all colorectal surgeons. One reason may be that ODS should be described as an "iceberg syndrome" characterized by "emerging rocks", such as internal rectal intussusception and rectocele that may benefit from operation, and also by "underwater rocks" or occult diseases such as rectal hyposensation that may affect the postoperative result of transanal operation[14,15]. Another reason is that few standards of selection criteria for transanal surgical procedures for treatment of ODS have been made yet[4,5,7,8]. Besides, anatomical and physiological disturbances underlying ODS are complex and remain incompletely understood.

These reasons may also explain why reported clinical outcomes of different transanal surgical procedures for treatment of ODS are controversial and remain debatable. None of these procedures has shown superior advantages and each technique has its benefits and disadvantages[16-20]. This article will review the literature regarding current transanal operative procedures for ODS to summarize the problems and alternatives for treatment of ODS and to conclude essential assessments needed for patient evaluation before carrying out transanal surgery for ODS patients. The benefits and disadvantages of each transanal surgical procedure and some experiences of transanal surgery for ODS in a single clinical center will also be presented.

CURRENT OPTIONS FOR ODS TREATMENT

Medical treatments

The basic medical treatments for patients with ODS may be a change of lifestyle, such as drinking plenty of water and eating a fiber diet every day[16,21]. A short period of using retrograde rectal irrigation or large bowel irrigation with warm normal saline can also be an alternative basic medical treatment for patients with ODS[22-24].

For patients with ODS induced by anismus, they may firstly be given 50 units of botulinum toxin A through trananal injection into the puborectalis muscle[25]. Such patients may also benefit from yoga exercises. Patients are trained to relax themselves and control the pelvic floor muscles, which may change the activity of the pelvic floor muscles during incompleted defecation[26,27]. For patients with ODS induced by anismus and rectal hyposensation, biofeedback therapy should be advised[16,28]. If symptoms of the patient are related to pudendal neuropathy and rectal hyposensation, an alternative therapy might be transanal electrostimulation, which is carried out by inserting a small probe connected with a portable electrostimulator into the anus[7].

Psychological counselling plays an important role in the treatment of patients with severe psychological pressure, such as depression and anxiety[29]. Besides, simple relaxation exercises of abdominal and pelvic floor muscles may also be helpful for these patients[30]. Furthermore, the latest psycho-echo-biofeedback therapy combined ultrasound-guided biofeedback with guided imagery and relaxation techniques. It might be a good option for ODS patients with both anismus and server psychological pressure[31]. Another new technology called biofeedback therapy plus transanal electrostimulation might also be an alternative for medical treatment of ODS[32,33].

Alternative transanal surgical methods

The clinical outcomes of non-surgical treatments for anismus induced ODS were conflicting, and the effects are not significant. That may be the reason why partial division of the puborectalis procedure was proposed[34]. This transanal manual procedure was thought to be effective in treating anismus induced ODS by partially dividing the puborectalis to relax the tension of hypertrophic puborectalis muscle[34-38].

As a most commonly used transanal manual procedure, internal Delorme's transanal excision was supposed to be a relatively cheap and pathophysiologically appropriate procedure for many patients with ODS[39].

Based on the stapled hemorrhoidopexy procedure, Corman *et al*[40] introduced an alternative minimally invasive transanal stapling procedure for patients with rectocele and internal intussusception induced ODS. This novel technique could restore the anatomical abnormality (rectocele and rectal intussusception) in the rectum through stapled transanal rectal resection (STARR) by sequentially using double circular stapling devices for the procedure for prolapse and hemorrhoids (PPH). That is why it was named PPH-STARR procedure. By resecting a full-thickness part of the rectum and subsequently strengthening the recto-vaginal septum and resecting redundant rectum, this procedure may provide promising results for ODS treatment.

PPH-STARR technique was confined to patients with large internal rectal intussusception and/or rectocele due to its limitation of resection of a large volume of prolapsed tissue and difficulty in visualizing the procedure. Patients with large internal rectal intussusception and/or rectocele may be treated by using a curved headed stapler device called the Contour-Transtar procedure[41]. For patients with larger prolapses (> 5.0 cm), PPH-STARR has the disadvantage of resecting bands of rectal mucosal prolapses with a maximal width of approximately 4.5 cm, and a better choice might be the "transanal repair of rectocele and rectal mucosectomy with a single circular stapler (TRREMS)" or tissue selecting therapy-stapled transanal rectal resection (TST-STARR) procedure[11,42-44]. When treating patients with ODS induced by rectocele and rectocele with relatively minor rectal intussusception (depth of rectocele more than 4.5 cm), the Bresler procedure or improved linear stapling procedure combined with a Bioabsorbable Seamguard (BSG) should be a smart choice[9,45]. For patients with ODS induced by symptomatic very high take-off internal rectal intussusception which is limited to reach the apex of the prolapse by above procedures, a transanal procedure called the TransAnal Endoscopic (internal) Rectal Prolapse" (TERP) may be advised[46].

ESSENTIAL ASSESSMENTS NEEDED BEFORE TRANSANAL SURGERY FOR ODS

Clinical questionnaires

After hospitalization, all patients should be evaluated with a standardized questionnaire - the Cleveland Clinic Florida constipation score (Wexner score) for the assessment of constipation. The fecal incontinence score questionnaires including St. Marks incontinence score and Cleveland Clinic incontinence score should also be carried out. Moreover, quality of life should be assessed for all patients through the use of the gastrointestinal quality of life index and the Italian version of the Short-Form 12[11,12,34,37,39].

Clinical examinations

All candidates should have prior screening with diagnostic examinations before the transanal operation as follows: (1) gastrointestinal transit time (GITT) assay with 20 radiopaque markers, and repeated abdominal X-ray tests on days 1, 3 and 5; (2) defecography or simultaneous pelvicography and colpocystodefecography (PCCD), including defecography, voiding cystography, vaginal opacification and pelvicography; (3) colonoscopy; (4) endorectal and endoanal ultraosonography; (5) anorectal manometry test; and (6) measurement of pudendal nerve terminal motor latency, anal surface electromyography and balloon expulsion[9,46-49].

Clinical tests

Before surgery, all routine inspections should be completed, including a routine blood test, liver and kidney function tests, chest radiography, and electrocardiograpy[10,36,47-49].

INCLUSION CRITERIA AND EXCLUSION CRITERIA FOR TRANSANAL SURGERY

Inclusion criteria (ODS induced by rectocele and/or rectal intussusception)

Symptoms of patients: Patients[3,9,11,50,51] should fulfill at least two of the following symptoms in the

Table 1 Overview and summary of pros of each transanal operative procedure

Procedure	Pros	Ref.
Partial division of puborectalis	Good short-term follow-up outcome	[35-37]
	More effective compared with common non-surgical procedures	[35]
Internal Delorme's procedure	Good long-term follow-up outcome with advantages as low recurrence rate and without complications such as postoperative constipation	[50,53,58,59]
	Suitable for patients with ODS and postoperative risk of fecal incontinence	[50,53]
PPH-STARR procedure	Overall satisfaction during postoperative long-term follow-up	[52,56,62-65,83-86]
	Without damage to the anal sphincters	[47]
Contour-Transtar procedure	High percent of patient satisfaction during long-term follow-up with advantages such as visualizing the procedure and being suitable for resection of a large volume of prolapsed tissue and without severe complications such as recto-vaginal fistula and fecal incontinence	[41,87,88,91-96]
	With superiority over PPH-STARR procedure	[91]
Bresler procedure and liner stapler and bioabsobable stapler line reinforcement material	High percent of patient satisfaction during long-term follow-up with advantages such as being suitable for resection of a large rectocele with a depth more than 4.5 cm, simple procedure and without severe complications such as recto-vaginal fistula and peritoneal perforation	[9,45,48,99,100]
TRREMS procedure	High percent of patient satisfaction during long-term follow-up with advantages such as being suitable for large prolapses of more than 5.0 cm, a short learning curve and without severe complications	[42-44,101]
TST-STARR procedure	High percent of patient satisfaction during long-term follow-up with advantages such as being suitable for large prolapses of more than 5.0 cm, a short learning curve, direct visualization during surgery and without severe complications	[11]
TERP procedure	Good short-term follow-up outcome	[46]

TRREMS: Transanal repair of rectocele and rectal mucosectomy with a single circular stapler; TST: Tissue selecting therapy; PPH: Procedure for prolapse and hemorrhoids; STARR: Stapled transanal rectal resection.

past 3 mo, with these symptoms appearing at least 6 mo prior to diagnosis: (1) A feeling of attempted defecation during ⩾ 25% of defecations; (2) Frequent feeling to defecate with failed attempts and a feeling of anorectal obstruction-blockage with long periods of time during ⩾ 25% of defecations; (3) Hard or lumpy stools during ⩾ 25% of defecations; (4) Facilitating ⩾ 25% of defecations by using at least one method as follows: digital assistance, perineal support, enema and odd posture; (5) Excessive straining and prolonged painful effort during ⩾ 25% of defecations; and (6) Defecation ⩽ 3 times per week. What's more, patients should satisfy conditions including: (1) Seldom having loose stools without using of laxatives and deficient standards for diagnosis of irritable bowel syndrome; and (2) Impaired defecation proved by using balloon expulsion test or anorectal manometry test.

Clinical history of patients: (1) Unresponsiveness to current intensive and appropriate medical treatment for at least 6 mo, such as basic medical therapy (drinking ⩾ 1.5 L water and taking 10 g lactulose per day and eating high-fiber diet), stimulants, osmotic laxatives and enemas; and (2) the absence of severe psychiatric diseases[45,46,52-54].

Radiological findings and Cleveland Clinic Florida constipation score: (1) The depth of rectocele ⩾ 3 cm and/or rectal intussusception into the anal canal ⩾ 1 cm on straining or defecography after defecation; and (2) a Cleveland Clinic Florida constipation score (Wexner score) ⩾ 12[11,39,41,55,56].

Inclusion criteria (ODS induced by anismus)

The inclusion criteria[3,34,37,38,57] were: (1) Patients with the following symptoms in the past 3 mo, with these symptoms appearing at least 6 mo before diagnosis; (2) Proof of appropriate propulsive forces during defecation (rectal pressure > 45 mmHg); (3) Evidence for loss to rest the pelvic floor muscles or improper contraction through medical examination of the pelvic floor muscles during evacuation combined with defecography, anorectal manometry test, electromyography and balloon expulsion; and (4) A permanent need of digital assistance, enema and laxatives to facilitate evacuation.

Exclusion criteria (ODS induced by rectocele and/or rectal intussusception)

(1) Patients with cystocele or genital prolapse needing transvaginal surgery; (2) Patients with fecal incontinence or ODS induced by anismus or pelvic floor dyssynergia; (3) Patients with anastomotic stoma or foreign material or chronic inflammatory lesions in the rectum or the anal canal; (4) Patients with colonic inertia, neoplasia or anorectal stenosis; and (5) Patients with mental disorders or patients refusing to accept surgery[3,9,11,50,51].

Exclusion criteria (ODS induced by anismus)

(1) Patients with colonic inertia or sphincter defect; (2) Patients with not only anismus but also other defecographic abnormalities; and (3) Patients with former pelvic operation[3,34,37,38,57].

Table 2 Overview and summary of cons of each transanal operative procedure

Procedure	Cons	Ref.
Partial division of puborectalis	Disappointing short-term follow-up outcome	[34,38,57]
	Increased risk of postoperative fecal incontinence	[34,38,57]
Internal Delorme's procedure	Unsatisfactory long-term follow-up outcome with disadvantages such as high recurrence rate, long operative time and complications such as constipation, fissure-in-ano, and transient incontinence	[39,53,55,60,61]
	Unsuitable for patients with ODS and diarrhea	[53]
	Requiring additional sphincteroplasty for patients with ODS and severe fecal incontinence	[60]
	Without superiority to stapling procedures in treatment of rectocele induced ODS	[39,55]
PPH-STARR procedure	Disappointing long-term follow-up outcome with disadvantages such as a long learning curve and complications such as bleeding, puborectalis dyssynergia, urinary retention, granuloma of anastomotic stoma and recurrent ODS	[41,63,67-70,79,80]
	With some severe postoperative complications such as severe proctalgia, fecal incontinence and rectovaginal fistula	[75-78]
	With rare complications such as rectal diverticulum and sigmoid volvulus	[81,82]
	Unsuitable for patients with previous pelvic floor surgery or sphincter weakness	[66,68-70,76-80]
	Limitation of resection of a large volume of prolapsed tissue and difficulty in visualizing the procedure	[41]
Contour-Transtar procedure	Disappointing long-term follow-up outcome with disadvantages such as a long learning curve , relatively complicated procedure, high cost and complications such as bleeding, puborectalis dyssynergia, urinary retention, granuloma of anastomotic stoma and recurrent ODS	[65,87,89,90,97]
	With some severe complications such as recto-vaginal fistula, fecal urgency, fecal incontinence and anorectal pain	[87,89,90]
	Unsuitable for patients with previous pelvic floor surgery or sphincter weakness	[65,87,89,90,97]
	Without superiority over PPH-STARR procedure	[65,97]
Bresler procedure and liner stapler and bioabsobable stapler line reinforcement material	Limited effect on rectal intussusception and unsuitable for patients with sphincter weakness	[45,48,99,100]
TRREMS procedure	Limited effect on severe rectocele	[44]
	Unsuitable for patients with sphincter weakness	[42-44,101]
TST-STARR procedure	Unsuitable for patients with sphincter weakness	[11]

TRREMS: Transanal repair of rectocele and rectal mucosectomy with a single circular stapler; TST: Tissue selecting therapy; PPH: Procedure for prolapse and hemorrhoids; STARR: Stapled transanal rectal resection.

ALTERNATIVE TRANSANAL SURGICAL PROCEDURES FOR ODS

There have been a great variety of transanal surgical techniques to treat patients with ODS and each technique has its benefits and problems. The tabular format of the diverse transanal operative procedures highlighting the pros and cons of each technique is summarized in Table 1 (pros) and Table 2 (cons).

Partial division of puborectalis

As Wallace *et al*[35] and Wasserman[36] reported, patients had good responses after partial division of the puborectalis muscle. Moreover, a comparative study investigated by Faried *et al*[37] showed that partial division of puborectalis was more effective than non-surgical treatment such as biofeedback retraining with a BTX-A injection. However, some studies reported that the outcome of treatment for patients with anismus through lateral or posterior midline division of the puborectalis muscle was disappointing[34,38,57]. Division of the puborectalis muscle failed to improve the ODS symptoms among the majority of patients, but might increase the risk of subsequent incontinence after surgery[34,38,57].

Internal Delorme's procedure

Berman *et al*[50] demonstrated that 21 patients who had undergone this procedure obtained a satisfaction rate of 71% at the 3-year follow-up without any major complications. Ganio *et al*[53] found that 45.7% of the incontinent patients had normal fecal continence after undergoing the internal Delorme's procedure. Tsunoda *et al*[58] and Liberman *et al*[59] also proved that internal Delorme's procedure had a reasonably low recurrence rate and a low morbidity. And this procedure did not lead to constipation post operation. However, this procedure also has limitations such as not being suitable for patients with diarrhea. Additionally, postoperative complications, including fissure-in-ano, suture-line dehiscence and anastomotic stoma stenosis, were observed[53]. As Ohazuruike *et al*[39] reported, 8.6% of patients in their study had felt transient incontinence to gas and fluids, respectively. For ODS patient with severe fecal incontinence, this procedure should be combined with sphincteroplasty to improve postoperative anal continence[60]. However, Ohazuruike *et al*[39] thought that there were no

statistically significant differences between internal Delorme's procedure and PPH-STARR procedure in treatment of ODS induced by rectocele and rectal intussusceptions. Roman *et al*[61] also reported that the long-term outcome of internal Delorme's procedure was less encouraging with a high recurrence rate. Besides, Mahmoud *et al*[55] demonstrated that this manual procedure had disadvantages of longer operative time and hospital stay and more complications, including constipation and fecal incontinence compared with stapling procedures, especially in the treatment of rectocele in ODS.

PPH-STARR procedure

As described by Boccasanta *et al*[62], there was an overall satisfaction rate of nearly 88% for a period of 20 mo of postoperative follow-up after transananl surgery performed with PPH-STARR procedure. Similarly, in another study by Gagliardi *et al*[63], a 65% satisfaction rate was achieved in patients suffering from rectocele and rectal intussusception. Another study also reported that the frequency of defecatory urgency decreased dramatically from 10% at the 3rd mo to 2% by the end of 12 mo of postoperative follow-up[56]. Additionally, radiological and clinical modifications of intussusception and rectocele were observed in 94.6% of patients during the follow-up investigated by Arroyo *et al*[52]. Similar results were also reported by Ding *et al*[64] and Naldini *et al*[65]. Because a circular anal dilator (CAD) was introduced into the anus to provide a better view for surgeons, possible damage to anal sphincters due to the introduction of the CAD was considered by some investigators. For this reason, Boccasanta *et al*[47] suggested in their study that if the stapler was correctly used, there would not be any direct damage to the anal sphincters.

However, the procedure has the advantages of being simple, easy and fast. It requires extensive experience to avoid further postoperative complications and to carefully resect the rectocele and prolapsed tissue. Besides, it may not be suitable for patients with previous pelvic floor surgery[66]. And there were some controversial points of view regarding the complication and poor postoperative outcome of this procedure[67,68]. For instance, the primary and most common perioperative and postoperative complication is "bleeding", which should be repaired intraoperatively by a few anastomotic sutures with absorbable thread[41,63]. Another complication found in several studies was puborectalis dyssynergia[41,69,70]. However, van Dam *et al*[71] demonstrated that the clinical outcome of transanal surgery for ODS was not influenced by the presence of puborectalis dyssynergia. Persistent pain in the anus was another complication appearing frequently after transanal surgery with PPH-STARR. This symptom might result from the staple line rupturing induced by postoperative sphincter spasm in the anus, extreme tension on the anoderm and excessive resection of smooth muscle[72-74]. Postoperative fecal urgency and fecal incontinence were also reported as postoperative complications of PPH-STARR procedure, which may be improved by sacral nerve stimulation or other medical treatments after surgery[75,76]. Besides, Pescatori *et al*[77] and Pescatori *et al*[78] summarized complications of PPH-STARR, including recurrent ODS, severe proctalgia, fecal incontinence and rectovaginal fistula. Furthermore, Asteria *et al*[79] stated that they had observed transient fecal urgency in 18% of cases and urinary retention in 12% of patients. And in a long-term follow-up investigated by Zhang *et al*[80], 14.7% of patients were reported to have similar complications related to granuloma of anastomotic stoma. Early postoperative urgency might result from traumatic inflammation at the staple line, but it could not explain why it lasts so long after surgery. In addition, rare complications such as rectal diverticulum and sigmoid volvulus were also reported[81,82]. However, minor postoperative complications have been mentioned in several studies, while neither major morbidity nor threatening mortality has been observed with PPH-STARR procedure[83-86].

Contour-Transtar procedure

As demonstrated by Renzi *et al*[87], 87% of cases with a prolonged history of constipation had symptom-free defecation after operation. This surgical procedure with a curved head was shown to be good and have a great effect on the development of symptoms in patients suffering from ODS. However, a percentage of patients may develop anal stenosis and may have a risk of spiraling due to the longitudinal and circumferential resection of the prolapsed tissue. On account of these complications, Brescia *et al*[88] modified the technique using an electric scalpel instead of a linear endoscopic stapler for initial longitudinal resection and stated that it could reduce the risk of spiraling. However, with resection of a large circumferential volume of the rectal wall, this new technique was considered to cause potentially severe complications such as recto-vaginal fistula, recto-enteric fistula, fecal urgency and fecal incontinence, which affected the quality of life of patients after surgery[89,90]. Nonetheless, further investigation indicated that clinical outcome using this technique was good if the surgeons carefully resected the anterior rectal wall prolapse without the posterior vaginal wall and corrected the defect using a recto-vaginal flap between the recto-vaginal septum[91,92]. As showed by Martellucci *et al*[91], only 1.5% of patients who underwent Contour-Transtar procedure required further surgery because of anal pain caused by retained staples, and only one patient had rectal perforation rectified by colostomy and closed after 6 mo. They demonstrated that the early

complication rate of this procedure was low and this new procedure had superiority over PPH-STARR procedure. What's more, some following studies reported similar results[93-96]. However, there were also some opposing views. Wadhawan *et al*[97] and Naldini *et al*[65] indicated that there were no statistically significant differences in postoperative clinical outcome, early complications, postoperative pain or hospital stay between PPH-STARR procedure and Contour-Transtar procedure. Similarly, Boccasanta *et al*[98] demonstrated that no improvements in symptoms and defecographic parameters were observed postoperatively in patients who underwent operation using Contour-Transtar procedure compared with PPH-STARR procedure. In addition, the cost of the stapler device used in Contour-Transtar procedure was higher.

Bresler procedure

As showed by Ayav *et al*[45], at least 90% of patients were satisfied and had no symptoms postoperatively in the 3 years of follow-up. And 76% of symptom-free cases were observed after a median-term follow-up. Another study investigated by Jiang *et al*[99] found that the mean constipation score improved significantly from 13.56 preoperatively to 5.07 postoperatively without severe complications as recto-vaginal fistula and peritoneal perforation. Moreover, Zhang *et al*[100] proved that the efficacy of this procedure can be highly appreciable with 72% of patients cured clinically. In addition, a study conducted by D'Avolio *et al*[48] indicated that defecography post operation proved complete correction of the anterior rectal wall defect in all 15 cases, and only a few cases had minor bleeding after surgery.

TRREMS procedure

As described by Regadas *et al*[42], all eight patients with ODS caused by rectocele had a good clinical outcome after TRREMS procedure. Additionally, a complete correction of the rectocele was demonstrated by anal-vaginal digitation and postoperative defecography. Cruz *et al*[43] investigated the outcomes of 75 patients with anorectocele related to rectal intussusception or mucosal prolapse in a prospective multicenter study. Mean Cleveland Clinic Florida constipation score (Wexner score) of these patients decreased meaningfully after the TRREMS procedure, which indicated that this operation is effective and safe. Besides, Leal *et al*[101] showed that TRREMS procedure could significantly reduce the mean constipation and obstructed defecation scores with lowered costs, even in treatment of grades Ⅱ and Ⅲ rectocele. What's more, as reported by Regadas *et al*[44], all 45 patients who underwent a modified TRREMS procedure had complete disappearance of rectal intussusception observed by imaging examination after surgery. Only 13.3% of the cases had a small residual mucosal prolapse.

TST-STARR procedure

Naldini *et al*[11] reported that only three patients had anastomotic bleeding with only one patient needing surgical intervention in the 76 patients who underwent TST-STARR Plus procedure. And only 7.6% of patients with fecal urgency were observed in the 14.5-mo follow-up.

Transanal rectocele repair using liner stapler and bioabsobable stapler line reinforcement material

As described by de la Portilla *et al*[9], a remarkable reduction in rectocele size on defecography and a significant improvement of symptoms as vaginal prolapse during evacuation, rectorrhagia, vaginal prolapse or digitation with bulge/mass, pruritus ani, pain and tenesmus were observed after surgery. No patient exhibited severe complications during the follow-up period and only two cases exhibited fecal urgency.

TERP procedure

As described by Bloemendaal *et al*[46], two of three patients had a significant improvement of ODS symptoms after surgery. Whereas another patient had a left hemicolectomy 1 year after the operation due to an anterior recurrence of internal rectal prolapse with a redundant loop of transverse colon near anastomotic stoma and an ulcer on the anterior wall of the rectum.

OUR EXPERIENCE

Forty-three female patients with ODS induced by rectocele and/or minor rectal intussusception underwent transanal operation through Bresler procedure in three Chinese hospitals led by our center from November 2008 to December 2010. The surgical procedure (Figure 1 and Video 1) is similar to Ayav *et al*[45]'s procedure. There were not any severe postoperative complications. Moreover, the mean constipation score improved significantly post operation. In addition, postoperative defecography also showed a great improvement with complete disappearance of the rectocele in 15 of 28 patients[99].

Our center registered a retrospective study in the Chinese Clinical Trial Registry (No. ChiCTR-ORN-16007696) to compare the clinical outcome between PPH-STARR and Bresler procedures in the treatment of ODS induced by rectocele and rectocele (depth of rectocele < 4.5 cm) with relatively minor rectal intussusception. Our PPH-STARR procedure (Figure 2 and Video 2) is similar to the traditional PPH-STARR procedure[40]. We investigated 30 female ODS patients who underwent Bresler surgery and 30 female ODS patients who underwent STARR surgery at our center from October 2011 to November 2012. However, there were no statistically significant differences ($P > 0.05$) between the two surgical procedures in mean operative time, blood loss or mean postoperative hospital stay (Table 3). Additionally,

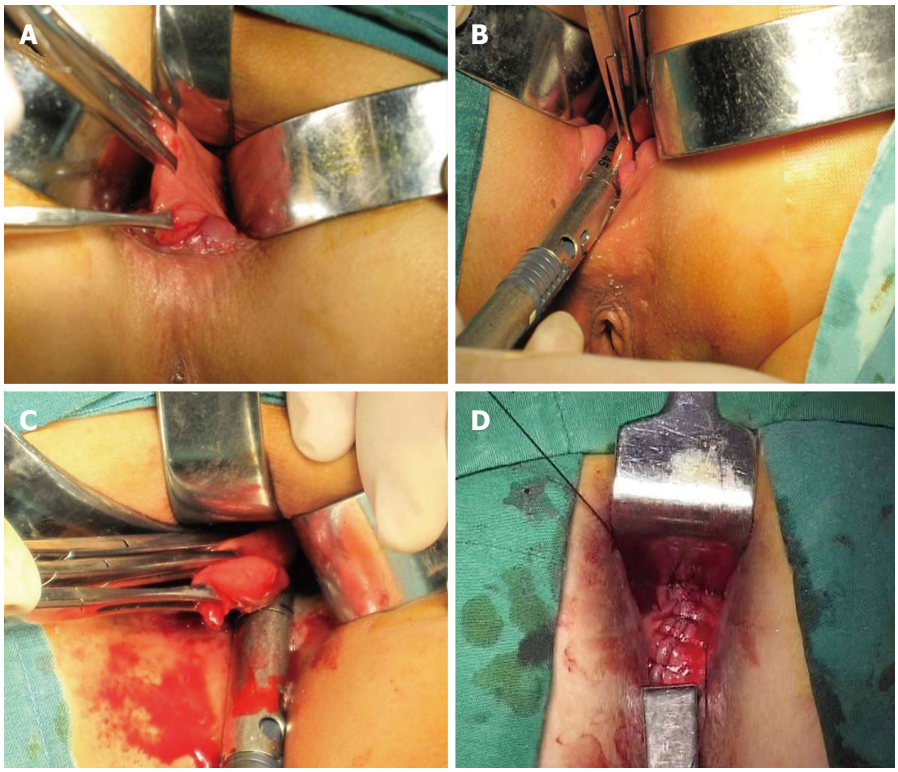

Figure 1 Surgical technique of Bresler procedure. A: The anterior wall of the defect in the rectum should be raised with two or three Allis clamps, and it should be ensured every time that it does not involve the posterior wall of the vagina to avoid further complications; B, C: A single use, reloadable endoscopic linear cutter is introduced, and one or two firings might be necessary depending on the extent of prolapse seen in the rectocele; D: A longitudinal locked running suture, including rectal mucosa, submucosa, and muscle, was made with 2-0 absorbable Vicryl suture along the staple line for the plication of the repaired anterior rectal wall to strengthen the stapled region.

Table 3 Comparison of mean operative time, blood loss and mean postoperative hospital stay between the procedure for prolapse and hemorrhoids-stapled transanal rectal resection and Bresler procedures

	STARR procedure	Bresler procedure	*P* value
Mean operative time (min)	21.5 ± 4.5	21.0 ± 4.0	0.26
Blood loss (mL)	10.0 ± 2.5	9.0 ± 2.0	0.35
Mean postoperative hospital stay (d)	5	5	0.19

STARR: Stapled transanal rectal resection.

Table 4 Comparison of the incidence of postoperative complications between the procedure for prolapse and hemorrhoids-stapled transanal rectal resection and Bresler procedures

	Pain	Fecal incontinence	Bleeding	Total number	Incidence	*P* value
STARR procedure	2	5	1	30	26.7%	0.774
Bresler procedure	3	4	2	30	30.0%	

STARR: Stapled transanal rectal resection.

there were no statistically significant differences ($P > 0.05$) between the two surgical procedures in the incidence of postoperative complications (STARR procedure group *vs* Bresler procedure group = 26.7% *vs* 30%, Table 4). Moreover, evaluation of patient satisfaction in the STARR procedure group was excellent (50%), good (16.7%), fair (10%) and poor (23.3%). The same assessment was excellent (46.7%), good (20%), fair (6.7%) and poor (26.6%) in Bresler procedure group. Moreover, in the short-term follow-up period, postoperative satisfaction rate (patients who felt excellent or good after surgery) of the two surgical procedures was same with 66.7% (Figure 3).

From April 2013 to September 2014, 50 patients (43 females and 7 males) with ODS were treated with TST-STARR procedure at our center. Our surgical procedure (Figure 4 and Video 3) was the same as Naldini *et al*[11]'s procedure. The average time of surgery was 21 ± 4 min (range: 12-35 min), blood loss was 12 ± 2 mL (range: 6-16 mL) and the average hospital stay was 5 d (range: 4-8 d). What's more, there were a few postoperative complications with only one patient with transient fecal urgency and only one patient suffering anastomotic bleeding. Besides, there was only one patient with fecal incontinence and one patient with rectal anastomotic stenosis. What's more, the postoperative Wexner constipation score improved

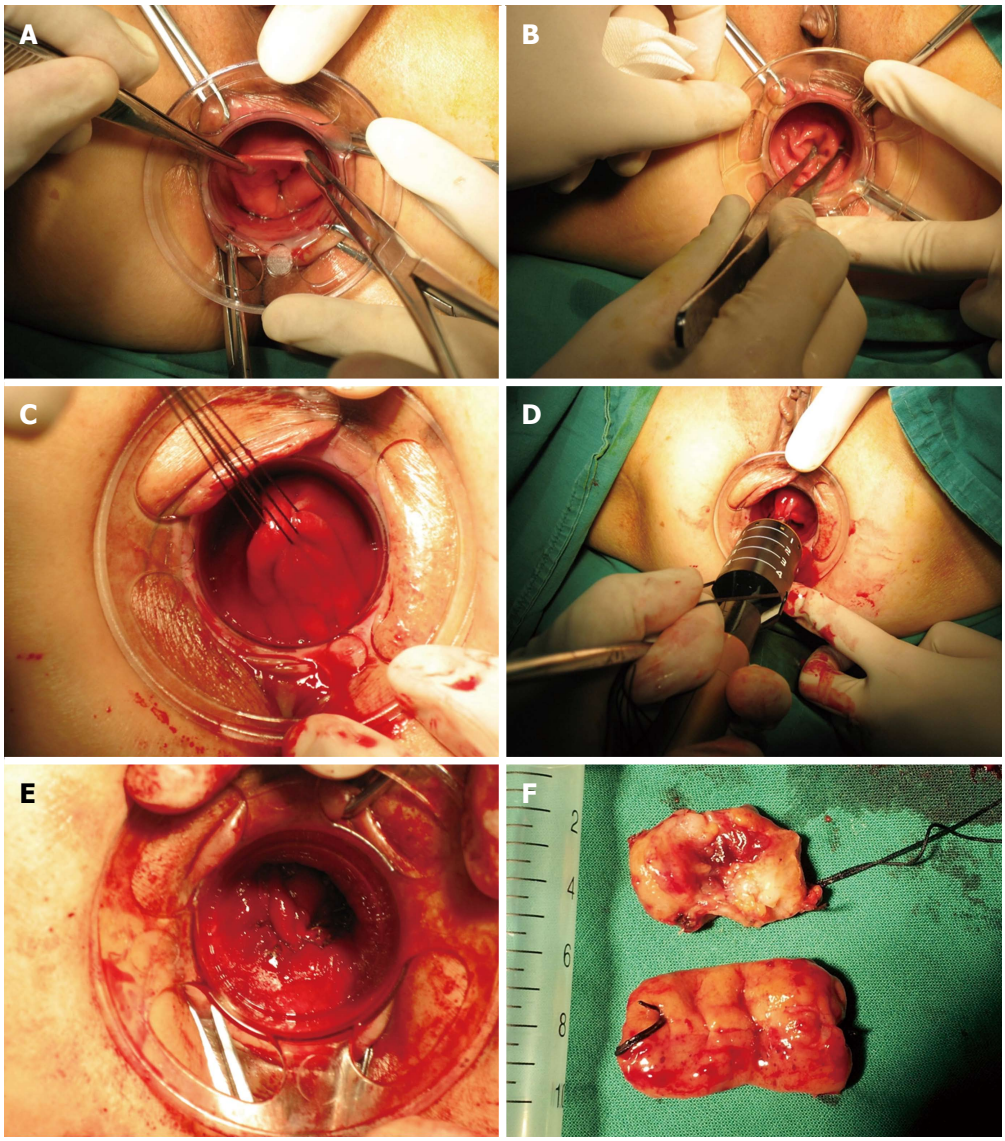

Figure 2 Surgical technique of prolapse and hemorrhoids-stapled transanal rectal resection procedure. A, B: A CAD was introduced into the anal canal, and a sterile betadine gauze hold with a pincer should be used to draw the prolapsed tissue inside the dilator; C: Three purse-string sutures in all of the layers of the rectum were made at 1 cm intervals using Prolene 2-0 in the anterior area of the rectum at 4 cm above the dentate line and from the 9 o'clock direction to the 3 o'clock direction including the apex of the anterior rectocele; D: A PPH device was inserted into the anal canal and closed and fired to perform the rectal anastomosis, and the staple line was reinforced using a 3-0 absorbable Vicryl suture; E: The same procedure was repeated on the posterior rectal wall; F: The resected sample. PPH: Procedure for prolapse and hemorrhoids; STARR: Stapled transanal rectal resection; CAD: Circular anal dilator.

significantly, and the overall satisfaction rates were approximately 84%, 84% and 82% at 3, 6, and 12 mo, respectively.

From January 2015 to December 2015, 45 patients (16 females and 29 males) with anismus induced ODS underwent transanal partial excision of puborectalis at our center. The surgical procedure is shown in Figure 5. And a retrospective study of postoperative outcome through transanal partial excision of puborectalis for treatment of anismus induced ODS had been registered in the Chinese Clinical Trial Registry with No. ChiCTR-ORB- 16007695. Part of the short-time follow-up showed a satisfactory outcome, and the full collection of clinical data and long-term follow-up are in progress. To date, none of these patients had complications such as fecal incontinence after surgery, and only 20% of patients had the recurrent ODS symptom.

DISCUSSION

Although a plenty of studies about the transanal surgical management of ODS have been published, treatment strategies for ODS remain poorly understood. This may be because of a lack of strict patient selection criteria for ODS operation, which is essential for surgeons to define and evaluate the roles of each operative procedure. Additionally, randomized controlled trials and controlled clinical trials with long-term follow-up and review articles based upon these investigations are not enough

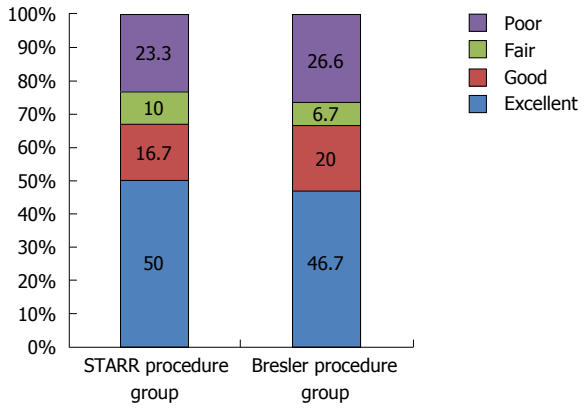

Figure 3 Short-term follow-up of postoperative satisfaction grade of both prolapse and hemorrhoids-stapled transanal rectal resection procedure and Bresler procedure. In 30 patients who underwent PPH-STARR procedure, there were 15 (50%) persons who felt excellent and five (16.7%) persons who felt good post operation. And there were three (10%) persons who just said fair and seven (23.3%) persons complaining about poor outcome after operation. In 30 patients who underwent Bresler procedure, there were 14 (46.7%) persons who felt excellent and six (20%) persons who felt good post operation. And there were two (6.7%) persons who just said fair and eight (26.6%) persons complaining about poor outcome after operation. PPH: Procedure for prolapse and hemorrhoids; STARR: Stapled transanal rectal resection.

for surgeons to evaluate and compare the clinical applications and outcomes of these transanal surgical procedures for the treatment of ODS.

Internal Delorme's procedure was supposed to be an appropriate operation for patients with ODS and internal prolapse with severe symptoms. However, this manual procedure has disadvantages of longer operative time and hospital stay. It also has more complications such as higher postoperative recurrence rate, constipation and recto-vaginal fistula compared with transanal stapling procedures, especially in the treatment of ODS induced by rectocele. Thus, the internal Delorme's procedure should be considered an alternative for patients with ODS induced by intra-rectal intussusception or internal rectal prolapse with relatively minor rectocele, and with supposed postoperative risk of fecal incontinence due to sphincter weakness. A partial division of puborectalis and partial excision of the puborectalis might be alternative to treat patients with anismus induced ODS. However, more long-term clinical controlled trials should be carried out to further investigate the effect of these manual procedures for the treatment of ODS.

PPH-STARR procedure contributed tremendously to treat patients with intra-rectal intussusception and rectocele induced ODS. However, it also has some postoperative complications, including bleeding, recurrence of ODS, fecal incontinence and so on. In addition, surgeons have a long learning curve to master this technique compared with other stapling procedures. What's more, it remains limited to patients with large intra-rectal intussusception with a length more than 4.5 cm and rectocele with a depth more than 4.5 cm. Therefore, it should be advised to apply by surgeons with extensive maintenance experience for the treatment of patients with ODS induced by internal rectal prolapse (length of prolapse < 4.5 cm) and/or rectocele (depth of rectocele < 4.5 cm). The Contour Transtar procedure is technically demanding for treatment of ODS induced by large internal rectal prolapse and/or rectocele and its functional results may be as good as those of the PPH-STARR. However, its cost is relatively high and it may cause severe complications such as recto-vaginal fistula, fecal urgency, fecal incontinence and anorectal pain after surgery. Furthermore, the surgical procedure is relatively complicated. So, it also needs a long learning curve and should be advised to be carried out by surgeons with abundant experience for the treatment of patients with ODS induced by large intra-rectal intussusception and/or rectocele. When treating patients with ODS induced by large rectocele (depth of rectocele more than > 4.5 cm) and large rectocele with relatively minor rectal intussusception, the Bresler technique and a combination of the Bresler technique with bioabsorbable seamguard may be simple and effective choices. Both procedures remove the rectocele completely, but they should be selectively applied to rectocele and rectocele with relatively minor rectal intussusception on account of their limited effect on rectal intussusception. Moreover, all the above transanal stapling procedure should not be advised to be carried out in treatment of patients with ODS and supposed postoperative risk of fecal incontinence due to sphincter weakness.

For patients with large internal rectal intussusception (more than 5.0 cm) and/or rectocele induced ODS, a better choice might be the TRREMS or TST-STARR procedure. Both techniques have advantages such as a shorter learning curve, fewer complications, more space to accommodate the resected tissue and a large volume of tissue resected. Moreover, TST-STARR procedure also provides surgeons with direct visualization during surgery. These two techniques are also suitable for treatment of ODS induced by internal rectal intussusception (less than 5.0 cm) and/or rectocele. Nonetheless, the TRREMS procedure should not be used in rectocele with a depth of more than > 4.5 cm due to its limited effect on severe rectocele. Furthermore, both the TRREMS and TST-STARR procedures should not be considered an alternative for treatment of patients with ODS and sphincter weakness. For patients with ODS induced by symptomatic very high take-off internal rectal prolapse, which is limited to reach the apex by other transanal procedures, TERP procedure should be an alternative choice. However, as a result of the small scale of patients who underwent the above three latest techniques, their clinical outcomes need further investigation and multicenter and randomized controlled trials with large-scale patient and long-term

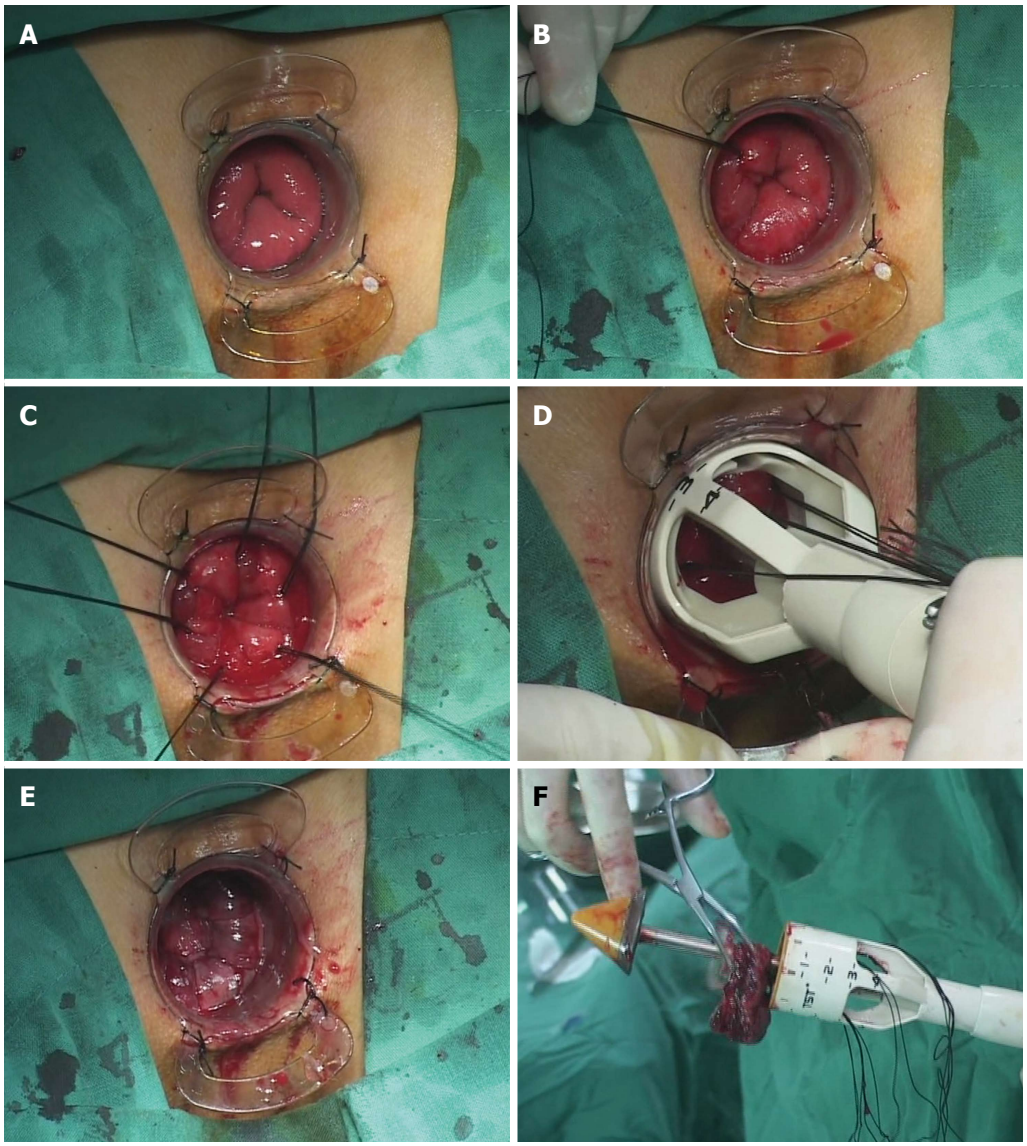

Figure 4 Surgical technique of tissue selecting therapy-stapled transanal rectal resection procedure. A: A CAD was gently introduced and fixed to the perianal skin after digital anal dilatation to assess the scope and degree of prolapse and rectocele; B, C: The parachute technique with six figure-eight sutures was used to pull out the rectocele and prolapsed tissues, and the depth of each suture should reach the rectal muscular layer; D: A 36-mm TST stapler was placed through the CAD, and all traction lines were pulled out through the mega windows; E: The stapler was closed and fired to perform the rectal anastomosis, and the staple line was reinforced using a 3-0 absorbable Vicryl suture; F: The resected sample. TST: Tissue selecting therapy; STARR: Stapled transanal rectal resection; CAD: Circular anal dilator.

follow-up should be carried out.

CONCLUSION

Although transanal surgery for ODS has been presented as a relatively simple, effective and safe treatment in short-term follow-up, the clinical outcomes in long-term follow-up are controversial and remain debatable. Possible reasons are still in need of further investigation. First of all, essential assessments before transanal surgery for ODS, especially the inclusion criteria and exclusion criteria and objective validated measurements for selection of patients, were deficiently described and summarized in most published articles. Additionally, there are few well designed randomized controlled trials comparing outcomes among different transanal surgical procedures for the treatment of ODS. Third, patients may not be strictly selected adhering to the current inclusion and exclusion criteria of these transanal procedures. What's more, the "underwater rocks" or occult diseases such as the psychosomatic component of ODS might be neglected by some colorectal surgeons. Last but not the least, supplementary therapies such as a high-fiber diet, conservative treatment with drugs and even a movement promoting defecation may be considered unnecessary to introduce to patients after surgery by some surgeons. From our experience, to get better clinical outcomes and patient satisfaction, the priority is to strictly select of proper transanal surgical procedure for each patient according to the inclusion and exclusion criteria for each transanal

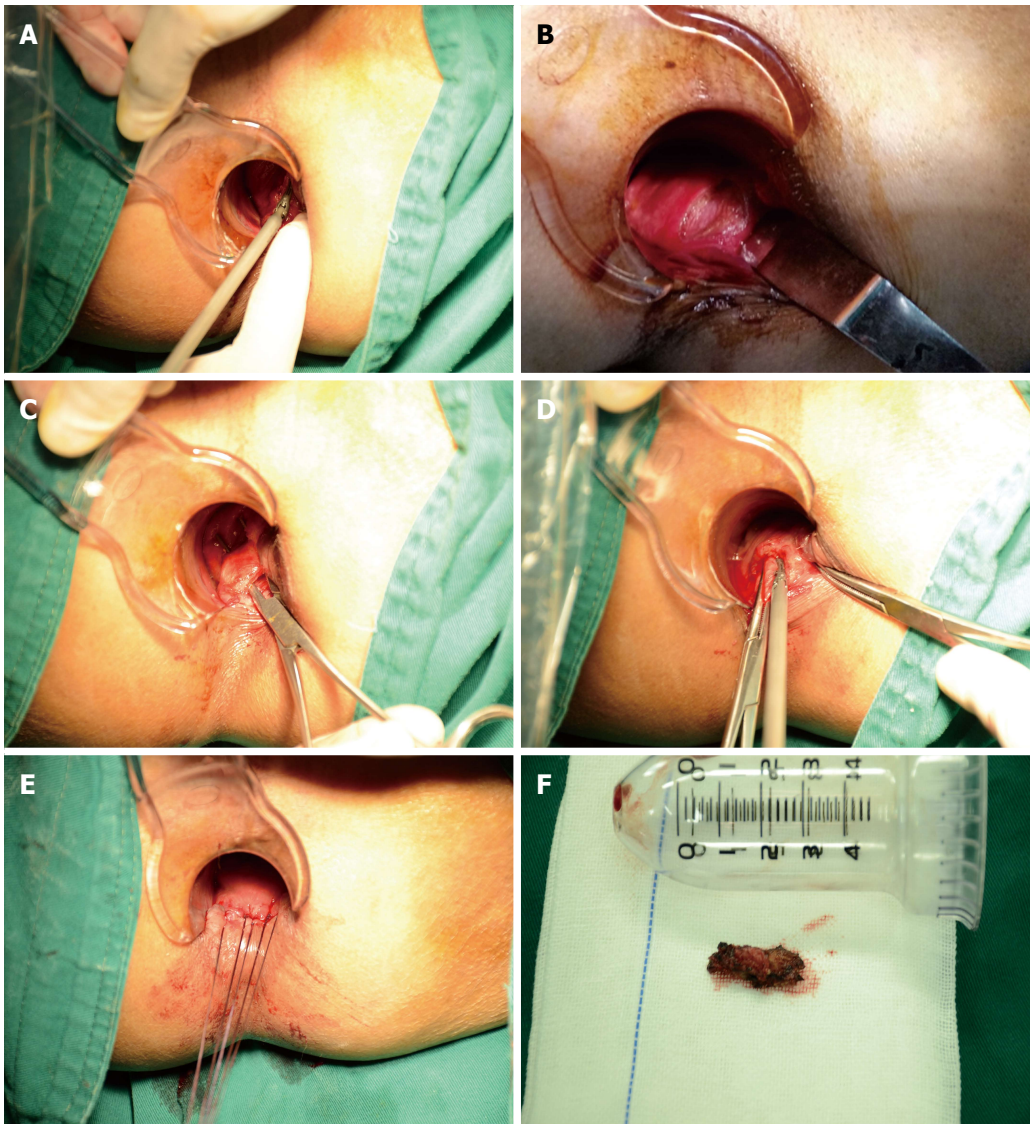

Figure 5 Technique of transanal partial excision of the puborectalis. A: Making a lateral incision of approximately 3 cm located 1 cm up on the dentate line on the rectal mucosa from the 3 o'clock direction to the 5 o'clock direction using an ultrasound knife; B: The rectal postero-lateral wall was dissected to the puborectalis; C, D: The puborectalis muscle was lifted up and approximately 2 cm was removed with an ultrasound knife; E: A full-thickness suture of the rectal wall was carried out; F: The resected sample.

procedure and the actual situation of the patient. In addition, surgeons should not only pay attention to surgery itself but also conservative treatments such as a change of lifestyle, psychotherapy, pelvic floor and abdominal muscle relaxation exercises and so on in order to improve patient satisfaction. Unquestionably, more large-scale, long-term prospective, multicentric and randomized controlled trials are needed to validate these preliminary findings and provide us with a better understanding of transanal surgery and stricter selection criteria for choosing proper transanal surgical procedure for each ODS patient in the future.

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REVIEW

Oncolytic viruses against cancer stem cells: A promising approach for gastrointestinal cancer

Fang Huang, Bin-Rong Wang, Ye-Qing Wu, Fan-Chao Wang, Jian Zhang, Yi-Gang Wang

Fang Huang, Bin-Rong Wang, Ye-Qing Wu, Fan-Chao Wang, Jian Zhang, Yi-Gang Wang, Xinyuan Institute of Medicine and Biotechnology, School of Life Sciences, Zhejiang Sci-Tech University, Hangzhou 310018, Zhejiang Province, China

Fang Huang, School of Public Health, Zhejiang University, Hangzhou 310058, Zhejiang Province, China

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Correspondence to: Dr. Yi-Gang Wang, Xinyuan Institute of Medicine and Biotechnology, School of Life Sciences, Zhejiang Sci-Tech University, No. 5, Road 2, Xiasha District, Hangzhou 310018, Zhejiang Province, China. wangyigang43@163.com
Telephone: +86-571-86843187
Fax: +86-571-86843185

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Abstract

Gastrointestinal cancer has been one of the five most commonly diagnosed and leading causes of cancer mortality over the past few decades. Great progress in traditional therapies has been made, which prolonged survival in patients with early cancer, yet tumor relapse and drug resistance still occurred, which is explained by the cancer stem cell (CSC) theory. Oncolytic virotherapy has attracted increasing interest in cancer because of its ability to infect and lyse CSCs. This paper reviews the basic knowledge, CSC markers and therapeutics of gastrointestinal cancer (liver, gastric, colon and pancreatic cancer), as well as research advances and possible molecular mechanisms of various oncolytic viruses against gastrointestinal CSCs. This paper also summarizes the existing obstacles to oncolytic virotherapy and proposes several alternative suggestions to overcome the therapeutic limitations.

Key words: Cancer stem cells; Gastrointestinal cancer; Oncolytic virotherapy; Molecular mechanism

Core tip: Cancer stem cells (CSCs) are derived from tumor cells, which are responsible for tumor relapse and drug resistance. The high incidence, lethality, relapse and drug resistance of gastrointestinal cancer requires a novel therapeutic strategy against CSCs. Oncolytic viruses hold much promise because they kill tumor cells but are minimally toxic to normal cells. Isolation and identification of CSC markers for

treatment of gastrointestinal cancer will benefit the engineering of oncolytic viruses and targeting anti-tumor effects. This paper reviews research on oncolytic viruses against gastrointestinal CSCs, and toxicity and immunological barriers to oncolytic virotherapy, and proposes alternative strategies.

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INTRODUCTION

Cancers of the genital, digestive and respiratory systems have the highest incidence and mortality[1]. Stomach, liver and colon cancers have been among the five most commonly diagnosed and leading causes of cancer mortality over the past few decades[1]. However, since the early 1990s, the cancer mortality rate has declined due to improvements in health care[2].

Traditional therapies for tumors (surgery, chemotherapy and radiotherapy) have made great progress in most patients with early cancer. Especially in recent years, novel targeted anti-cancer agents have been utilized clinically and have largely improved the survival rate of many cancer patients. Unfortunately, relapse still occurs months or years later when cancer patients are treated with the above approaches and they cannot be treated successfully again. Apart from suboptimal surgical debulking, drug resistance of tumor cells, and inability of chemotherapy or radiotherapy to target all cancer cells within a given patient[3], the cancer stem cell (CSC) theory can explain cancer relapse, which has been confirmed by many studies and accepted by an increasing number of scientists.

CSCs, also named cancer-initiating cells, are a small population of tumor cells and a subclass of stem-cell-like tumor cells. The term CSC was originally coined to describe features of these cells that are similar to *bona fide* normal stem cells that share basic properties of self-renewal and pluripotency[4]. Since a subclass of CSCs, $CD34^{+}CD38^{-}$ cells, derived from the blood of patients with acute myeloid leukemia, was reported in 1994, the presence of CSCs has been verified in a variety of primary tumor tissues and tumor cell lines, including gastrointestinal tract cancer[5]. The hypothesis that CSCs originate from normal stem cells is still uncertain, but their origin is likely to differ among human cancers. CSCs are tumorigenic and responsible for cancer relapse and metastasis, which implies that their role in producing daughter cells that constitute a new tumor bulk is similar to the role of normal stem cells in generating a bulk organ, such as blood from bone marrow stem cells. Moreover, both normal stem cells and CSCs express drug resistance genes, such as the ATP-binding cassette protein efflux pump ABCG2, which endows these cells with resistance to environmental toxins and chemotherapy or radiotherapy[6]. Nevertheless, CSCs also have many other features dissimilar to normal stem cells as well as their different or uncertain origin. Thus, it is urgent to isolate and characterize the CSCs, and exploit targeting treatment to reduce relapse and improve survival rate in patients with gastrointestinal tract cancer[7].

In the past two decades, researches have discovered a promising biological therapy for cancer, namely, oncolytic virotherapy. Oncolytic viruses are natural or modified viruses that can effectively and specifically infect cancer cells and kill them in preclinical models and clinical trials[8]. Oncolytic virotherapy has attracted increasing attention in cancer research as an emerging therapeutic approach because of its multiple anti-cancer pathways. For example, oncolytic viruses can infect highly proliferative cells (non-CSCs) and quiescent cells (CSCs), and directly lyse them, but they are not pumped out of infected cells by ABCG2 like chemotherapeutic drugs[9-11]. Other mechanisms include indirect killing of uninfected cancer cells, such as destruction of tumor vessels, and induction of anti-tumor immunity[12]. More importantly, oncolytic viruses exhibit targeted anti-tumor activity against CSCs, which is responsible for resistance to traditional treatments and tumor recurrence[11].

This review focuses on recent studies using oncolytic viruses against gastrointestinal cancer and highlights the novel approach to eradicate CSCs.

GASTROINTESTINAL CANCER, CSCs AND THERAPY

Gastric cancer

Gastric cancer (GC) is a heterogeneous chronic disease characterized by epidemiological and histopathological differences among countries. GC is one of the leading causes of cancer-related death worldwide. The origin of gastric carcinogenesis is still controversial. The past most popular model involved several initiators and continuator agents that provide a multifactorial and multistep pathogenesis for GC formation[13]. *Helicobacter pylori* (*H. pylori*) infection is recognized as a necessary but insufficient cause of GC[14].

Recent advances consider that GC essentially is a stem cell disease and GC stem cells (GCSCs) are the basis for gastric carcinogenesis[15]. GCSCs may be derived from resident stem cells in gastric tissues with a chronically inflamed environment in the case of *H. pylori*-induced gastritis[16]. Alternatively, due to exhaustion of the native gastric stem cells from their niches induced by chronic inflammatory stress, GCSCs are recruited from bone-marrow-derived stem

cells into the gastric epithelium. Further studies have found high expression of drug-resistance genes such as *ALDH* and *MDR* and specific molecular markers such as CD44, CD133, leucine-rich repeat-containing G-protein coupled receptor (Lgr)5, signal transducer and activator of transcription 3, and aquaporin 3[15,17,18]. These form the basis of drug resistance in GC and provide a molecular target for identification and isolation of GCSCs, and GCSC-targeted therapy. Treatment for GC patients is currently suboptimal, due to patients being commonly treated in a uniform fashion irrespective of disease subtype[19].

Liver cancer

Liver cancer is the sixth most common cancer and third leading cause of cancer mortality worldwide. Liver cancer mainly falls into three types: hepatocellular carcinoma (HCC) (90%), intrahepatic cholangiocarcinoma, and mixed cell carcinoma[20]. Besides, there are many metastatic liver cancers from other malignant diseases, such as lung cancer. In Asia, especially in China, liver cancer is common; mainly because of the escalating epidemic of chronic hepatitis B or C infections[21]. Therefore, exploring optimal therapy for liver cancer is an important area of research. Liver cancer stem cells (LCSCs) have been isolated from liver cancer tissues. This has resulted in progress in liver cancer diagnosis and evaluation of prognosis and pathogenesis, despite constant debate about the new surface markers of LCSCs[22]. The reported major LCSC markers include CD133, CD90, epithelial cell adhesion molecule (EpCAM), OV6, CD44 and Nanog[22]. Although some of the markers are also expressed on the surface of other CSCs and normal stem cells, detection of LCSC-specific molecules is beneficial for diagnosis and evaluating and monitoring treatment of liver cancer.

Pancreatic cancer

Pancreatic cancer (PC) is considered to be one of the deadliest cancers, with almost uniform lethality despite aggressive treatment[23]. However, resistance to conventional therapy and early distant metastasis are still cause for unsatisfactory prognosis of PC patients, even though there has been important progress in the molecular, pathological and biological understanding of PC. Thus, there is an urgency to explore the mechanisms of pancreatic carcinogenesis and tumor recurrence and metastasis, and develop novel, targeted therapeutic strategies. Recently, a small population of tumor-initiating cancer cells, termed PC stem cells (PCSCs), has been identified in many PC patients and cell lines, and is responsible for tumor initiation, progression and metastasis[24]. Identification of PCSC surface markers is crucial to isolate and characterize PCSCs. So far, the identified molecular markers for PCSCs include CD133, CD24, CD44, EpCAM, epithelial specific antigen, c-Met, aldehyde dehydrogenase (ALDH)1, and more recently, doublecortin-like kinase 1 and Lgr5[24,25], which are well recognized in xenograft models and some PC tissues. Further studies have shown that these markers are often co-expressed at metastatic sites or invading margins of PCSCs and pancreatic ductal cancers[23], such as CD133/CXCR4 receptor, CD24/CD44/EpCAM and CD133. Although the populations of PCSCs account for $\leqslant$ 1% of all PC cells, they are involved in cancer relapse and resistance to chemo- or radiotherapy[26]. Thus, our ultimate goal is to understand PCSCs further and explore potential therapeutic targets for PC.

Colorectal cancer

Colorectal cancer (CRC) is one of the most common cancers worldwide, and affects > 1 million people, resulting in about 715000 deaths in 2010[27,28]. In China, CRC is the fifth most common form. Incidence of CRC in China is lower than that in western countries, but it has increased in recent years to become a substantial burden, particularly in more-developed areas[29]. Treatment options for CRC are based largely on cancer stage. Patients without distant metastasis usually receive surgery as initial treatment. In patients with advanced disease, CRC is rarely cured completely due to drug resistance and recurrence, and patients are not eligible for surgery[30]. Therefore, understanding of CRC formation and progression is urgently needed. In addition to accumulation of genetic abnormalities and dysregulation of gene expression, CRC stem cells (CCSCs) also play important roles in CRC carcinogenesis, promotion, metastasis and recurrence. CCSCs share the same molecular signaling features with normal stem cells, such as Wnt, Notch and transforming growth factor-β, and differ in tumorigenic potential[31]. More importantly, isolation of CCSCs can be achieved by screening subpopulations of CRC cells based on one or more cell surface markers, including CD133, CD166, CD44, CD24, β1 integrin-CD29, Lgr5, EpCAM, ALDH1, Musashi RNA binding protein-1, doublecortin-like and CAM kinase-like 1 (DCAMLK1) or ephrin B receptors[32-34] (Table 1), which largely contribute to the better stratification of prognosis and treatment response, as well as the development of new targeting strategies.

ONCOLYTIC VIROTHERAPY

The issue of which oncolytic viruses are to be engineered to eliminate CSCs, and their mechanisms of action have begun to be addressed. Current viruses have a broad range of sources and categories, including wild-type animal viruses, live virus vaccines, and human virus mutants in which critical genes for virus replication that are dispensable in cancer cells have been deleted or mutated. These attenuated live virus vaccines or modified viruses hold much promise because they have been proved to be efficient against

Table 1 Cancer stem cell markers of different gastrointestinal cancers

Cancer types	CSC markers	Ref.
Gastric cancer	CD44, CD133, Lgr5, STAT3, Aquaporin 3	[15,17,18]
Liver cancer	CD133, CD90, EpCAM, OV6, CD44, Nanog	[22]
Pancreatic cancer	CD133, CD24, CD44, EpCAM, ESA, c-Met, Aldh1, DclK1, Lgr5	[24,25]
Colorectal cancer	CD133, CD166, CD44, CD24, b1 integrin-CD29, Lgr5, EpCAM, ALDH1, Msi-1, EphB	[32-34]

CSC: Cancer stem cell; Lgr5: Leucine-rich repeat-containing G-protein coupled receptor 5; STAT3: Signal transducer and activator of transcription 3; EpCAM: Epithelial cell adhesion molecule; ESA: Epithelial-specific antigen; Alkh1: Aldehyde dehydrogenase 1; DclK1: Doublecortin-like kinase-1; Msi-1: Musashi-1; EphB: Ephrin-B.

tumor tissues, yet minimally toxic to normal cells and tissues[3]. Oncolytic viruses have also been armed to deliver anti-cancer genes with different functions, thereby increasing their anti-tumor effects.

Oncolytic adenovirus

In the past two decades, oncolytic adenovirus (OncoAd) has become a promising agent for treatment of many cancers including gastrointestinal cancer. The cancer targeting gene-virotherapy (CTGVT) strategy, which was proposed by our group through combining virotherapy and gene therapy, showed greater anti-tumor effects when compared with monotherapy[35,36]. The representative modified mutant adenovirus, ZD55, was designed by deleting the immediate-early protein E1B (55 kDa) based on the CTGVT strategy to target the p53 dysfunction pathway or nuclear export of viral RNA in tumor cells[10]. Other than E1B detection, another common mutant of adenoviruses is the 24 bp deletion of the E1A retinoblastoma (Rb) binding site (Δ24). Mutant adenoviruses show obvious tumor selectivity because viral replication is promoted in tumor cells with a defective Rb/p16 pathway and abolished in normal cells with intact Rb/p16[37]. Most cancer cells and CSCs harbor defects in the Rb and/or p53 pathway, which makes it possible to use OncoAd to eradicate the gastrointestinal cancer cells. Besides, the transcription-targeted strategy has been a common approach through using cancer or tissue-specific promoter to control the expression of viral early essential genes for replication.

Recent studies have shown that the Golgi glycoprotein GOLPH2, usually named GP73, is an excellent HCC marker candidate, and even its promoter activity and specificity are better than the most common liver cancer marker α-fetoprotein[38,39]. Our group constructed a novel dual-regulated oncolytic adenovirus GD55 targeting HCC, using the GP73 promoter to regulate E1A expression and deletion of E1B based on the CTGVT strategy[10]. The novel GOLPH2-regulated GD55 conferred higher adenovirus replication and infectivity for liver cancer cells than did ZD55. We also confirmed that ZD55 eliminated LCSCs (data unpublished). The LCSC-like cells were enriched with suspension culture and the properties of acquired LCSCs were validated through detecting expression of CSC-related transcription factors and receptors (*e.g.*, Nanog, octamer-binding transcription factor 4, EpCAM and DR5). Oncolytic virus ZD55 resulted in obvious cytotoxicity and killing (the minimum cell viability for Huh7 spheres is 26.7%) of LCSC-like cells, and induced significant apoptosis (the maximum apoptosis rate for Huh7 spheres is 60%)[40]. We proceeded to verify whether GD55 could also destroy LCSCs as well as non-CSCs. Our results indicated that GD55 significantly elicited cytotoxicity and oncolysis in LCSC-like cells enriched in suspension culture, and exhibited more obvious killing than ZD55. GD55 induced marked apoptosis of LCSC-like cells *in vitro* and *in vivo*, and inhibited propagation of cells and angiogenesis in xenograft tumor tissues[40]. Thus, GD55 may represent an attractive therapeutic agent for targeting LCSCs with better clinical outcomes for HCC patients.

Studies of other targeting strategies for OncoAd have also been pursued. Adenovirus tropism modification by constructing chimeric virus capsid has been used to overcome the lack of the host cell surface coxsackie-adenovirus receptor (CAR) in tumor cells, because most common adenovirus serotypes such as Ad5 infect and enter cells through the fiber knob of the viral capsid binding to CAR[41]. Yu *et al*[42] reported that a new OncoAd, Ad5PTDf35, which is an Ad5 vector with Tat-PTD modified hexon and 35 serotype fiber, showed greatly enhanced transduction of primary human cell cultures, including pancreatic islets and tumor-initiating cells, compared to unmodified Ad5. Therefore, this modified Ad5PTDf35 may be further developed as an oncolytic agent for targeted CSC therapy.

Xu *et al*[43] reported that oncolytic adenovirus ZD55-mediated acetylcholinesterase (AChE) overexpression exhibited a potent anti-tumor effect on GC. The results showed that the constructed adenoviral vector ZD55-AChE inhibited GC cell and GCSC growth, and low doses of ZD55-AChE induced the mitochondrial pathway of apoptosis. ZD55-AChE repressed tumor growth *in vivo*, and the anti-tumor efficacy was greater than that of the replication-deficient adenoviral vector (Ad-AChE). ZD55-AChE represents a potential therapeutic agent for human GC. Yano *et al*[44] investigated the efficacy of a genetically engineered, telomerase-specific oncolytic adenovirus, OBP-301, to mobilize the cell cycle and kill quiescent $CD133^+$ CSC-like cells in human GC cells. They found that OBP-301 efficiently killed $CD133^+$ GCSCs resistant to chemoradiotherapy. OBP-301 induced cell-cycle mobilization from G0/G1 to S/G2/M phases and subsequent cell death in quiescent GCSCs by mobilizing cell-cycle-related proteins. OBP-301

mobilized quiescent CSC-like cells in tumor spheres and xenografts into S/G2/M phases where they lost viability and CSC-like cell properties and became chemosensitive.

Oncolytic herpes simplex viruses

As a neurotropic virus, oncolytic herpes simplex virus (OncoHSV) has been investigated widely in preclinical and clinical trials for patients with neurological malignancies like glioblastoma and neuroblastoma, and melanoma. In particular, the first-in-class oncolytic virus agent talimogene laherparepvec (T-VEC) is a genetically modified, attenuated recombinant HSV expressing granulocyte-macrophage colony-stimulating factor (GM-CSF). It was authorized by the US Food and Drug Administration and European Food Safety Authority because T-VEC improved durable response rate in patients with advanced melanoma in a phase Ⅲ trial[45]. T-VEC is also being tested in several other cancers, such as digestive tract cancers, alone and in combination with standard cancer therapeutics and other immunotherapy agents[46].

Although OncoHSV was broadly utilized in clinical trials on nervous system tumors, some studies have shown its potential for killing gastrointestinal cancer cells and CSCs. Yang *et al*[47] reported that OncoHSV is an effective agent for colon cancer and exhibits significant killing efficacy in colon cancer cells and colon CSC-like cells *in vitro* and *in vivo*. Miao *et al*[48] designed a transcriptionally regulated OncoHSV, YE-PC8, in which a cell-cycle-regulatable luciferase transgene cassette was replaced with the infected cell protein (ICP)6 coding region of the HSV-1 genome, and found that intratumoral injection of YE-PC8 resulted in 77% and 80% tumor regression in human glioma and human HCC xenografts, respectively. Thus, YE-PC8 viruses confer tumor selectivity in proliferating cells and may be developed further as a feasible approach to treat human cancers. A report by Fong *et al*[49] showed a phase Ⅰ trial of another multimutated OncoHSV, NV1020, in patients with metastatic CRC who had failed first-line chemotherapy *via* hepatic arterial administration. The tumor size decreased, median survival rate was prolonged, and levels of the tumor marker carcinoembryonic antigen decreased in patients after HSV infection, which suggested that genetically engineered HSV can be delivered safely into the human bloodstream to produce selective infection of tumor tissues and biological effects. In an earlier phase Ⅰ clinical trial, OncoVEXGM-CSF, a second-generation OncoHSV, was administered by intratumoral injection in patients with gastrointestinal cancer who had failed prior therapy. The results showed that OncoVEXGM-CSF was well tolerated, with the main adverse effects being local inflammation, erythema and febrile responses, and exhibited an anti-tumor effect after delivery *via* a safe protocol[50].

Efficacy of OncoHSV has been demonstrated preclinically and clinically in LC, CRC and glioma. A recent study has examined the ability of OncoHSV to kill CSCs mainly from neural tumors. Kambara *et al*[51] developed an oncolytic HSV-1 mutant rQNestin34.5 which expresses ICP-34.5 under control of a synthetic nestin promoter. Nestin is expressed in embryonic neuroglial cells and has been used as a CSC marker in several cancers including brain tumors, and rQNestin34.5 showed significantly more potent inhibition of tumor growth compared with control virus *in vivo*[52]. Further studies found that rQNestin34.5 can infect and kill neuroblastoma CSCs[9], implying that OncoHSV efficiently targets CSCs from gastrointestinal cancer.

Oncolytic vaccinia virus

Vaccinia virus (VV) belongs to the poxvirus family, and is famous because it was first utilized as a vaccine against smallpox until its eradication worldwide. Recently, oncolytic vaccinia virus (OncoVV) showed potential as it was attenuated for use as a transfer vector for therapy of human cancers. Two main mutated OncoVVs were designed by deleting the thymidine kinase (TK) gene or B18R gene[53]. The TK-deleted OncoVV undergoes preferential replication in dividing cells and shows tumor cell specificity, and the DNA synthesis of mutant virus requires TTP, which is only provided by dividing cells. The B18R-deleted mutant virus has oncolytic capacity because it causes interferon (IFN)-mediated enhanced virus inactivation in normal cells, based on the effect of B18R gene against type Ⅰ IFNs[53]. Our group previously constructed a tumor-targeted VV carrying *SMAC/DIABLO* gene, which was knocked out in the region of the TK gene (VV-SMAC). We found that VV-SMAC efficiently infected and destroyed HCC cells *via* triggering both caspase-dependent apoptosis and necroptosis[54]. Our data suggest that VV-SMAC is a potential candidate, and combination of VV-SMAC and vinblastine may provide a new avenue for treatment of HCC[54]. To date, several genetically modified OncoVVs delivering various therapeutic genes have exerted obvious anti-tumor effects in clinical trials, through targeting cancer-specific antigens and inducing anti-tumor immunity[55].

Recently, Yoo *et al*[56] reported that a cancer-favoring OncoVV (CVV) shows enhanced suppression of stem cell-like colon cancer (SCC). The engineered CVV is an evolved Wyeth strain of VV lacking TK, and can successfully override drug resistance and suppress SCC, with improved survival rates and complete eradication of tumor mass. This can be synergistically enhanced by simultaneous treatment with the anticancer drug 5-fluorouracil[56]. Chard *et al*[57] investigated the anti-tumor efficacy of interleukin (IL)-10-armed VV (VVLΔTK-IL10) in PC cell lines, mice bearing PC xenografts, and a PC transgenic mouse model. They found that VVLΔTK-IL10 has strong potential as an anti-tumor therapeutic agent for PC.

Table 2 Oncolytic viruses against gastrointestinal cancer stem cell

Oncolytic viruses	Cancer types	CSC source	Effect	Description	Ref.
Adenovirus	Liver	CL	Susceptible	GP73 needed	[40]
	Pancrease, prostate	PC	Susceptible	Tat-PTD modified hexon and Ad5/35	[42]
	Gastric	CL	Mixed	AChE	[43]
	Gastric	CL	Susceptible	OBP-301, telomerase-specific	[44]
HSV2	Colon	CL	Susceptible	No virus modification or co-therapies	[47]
Vaccinia	Colon	CL	Mixed	Viral TK deficiency	[56]
Measles virus	HCC, colon	PX	Susceptible	Retargeted to CD133	[68]

CL: Cell line-derived spheres or cell lines sorted by marker expression; GP73: Golgi protein 73; HCC: Hepatocellular carcinoma; HSV: Herpes simplex virus; PX: Primary xenograft; PC: Primary cancer cells sorted by marker expression; Tat-PTD: Protein transduction domain (PTD) from the HIV-1 Tat protein; AChE: Acetylcholinesterase; TK: Thymidine kinase; NDV: Newcastle disease virus.

Another preclinical trial was performed using hamsters as a PC model to assess the anti-tumor immunity of another OncoVV armed with human GM-CSF, which was selective for epidermal growth factor receptor pathway mutation and tumor-associated hypermetabolism[58]. However, their cytocidal effect on PCSCs needs further study. Although there are few reports about OncoVV inhibiting gastrointestinal CSCs, many OncoVV constructs exhibit excellent anti-tumor effects targeting CSCs from ovarian cancer, glioma and lymphoma[59].

OncoVV JX-594, derived from Wyeth strain VV and genetically modified to delete the TK gene and express human GM-CSF gene, has entered into phase Ⅲ clinical trials because of its excellent anti-tumor efficacy. JX-594 was first modified to augment the intrinsic targeting and oncolytic potential of VV and to enhance anti-tumor immunity by GM-CSF expression[60]. Several clinical trials using JX-594 have shown functional anti-cancer immunity, tumor necrosis, and improved survival through multiple mechanisms and injection pathways in primary HCC and other metastatic gastrointestinal cancers[61,62].

Newcastle disease virus

Newcastle disease virus (NDV), an avian paramyxovirus type 1, is an attractive oncolytic agent for cancer virotherapy. The mechanisms of NDV-mediated cytotoxicity in cancer cells include the dominant role of apoptosis induction by caspase pathway activation, and indirect anti-cancer activity by activation of both innate and adaptive immune responses[63]. Although no specific studies have reported the effect of NDV on CSCs, there are several completed and ongoing clinical trials using NDV-based tumor vaccines and direct administration of naturally occurring NDV to patients with gastrointestinal tumors[64]. For most treatment of CRC, attenuated NDV Ulster strain exerted obvious prolonged survival with 97.9% 2-year survival compared to 73.8% in the control group[65]. Liang *et al*[66] reported a clinical study of an autologous NDV-modified tumor cell vaccine in a phase Ⅲ study of stage Ⅰ-Ⅳ CRC patients and found significant improvement of median overall survival in the vaccine group. In addition, the clinical benefit was shown in patients with unresectable colorectal, stomach, liver and pancreatic cancers after treatment with NDV vaccine, suggesting its promising future.

Measles virus

The attenuated strains of measles virus (MV) have been shown to infect and kill a large variety of tumor cells specifically but not normal cells in phase Ⅰ clinical trials. The most common Edmonston strain of MV has shown clinical benefits for treating diverse solid cancer types, including lymphoma and myeloma[67]. Bach *et al*[68] designed oncolytic MV retargeted to CD133, termed MV-141.7 and MV-AC133, which infected and selectively eliminated $CD133^+$ cells from tumor tissue, and showed strong anti-tumor effects and prolonged survival in mouse models of human HCC and colon cancer. This virus is currently being assessed as an oncolytic agent in clinical trials (Table 2). Another study armed and retargeted MV through the prostate stem-cell antigen expressed on PC but not on non-neoplastic tissue, and obtained beneficial therapeutic effects in a PC xenograft model and PC cells, including gemcitabine-resistant pancreatic adenocarcinoma cells[69].

Myxoma virus

Similar to VV, myxoma virus (MYXV) is a double-stranded DNA virus from the Poxviridae family. The natural host of MYXV is rabbits, which makes MYXV only pathogenic to European rabbits and it does not cause any human diseases. MYXV has been shown to infect human cancer cells and result in cytotoxicity through Akt activation *via* interaction with a viral ankyrin-repeat host range factor[70]. Akt is the key factor of the PI3K/Akt pathway, which plays a critical role in cancer development and regulating the survival of CSCs in medulloblastoma following radiation[71], suggesting that MYXV is a potential therapeutic agent for eradication of CSCs. To date, several preclinical studies have proven that MYXV is an attractive candidate oncolytic virus that could be developed as a promising oncolytic agent for PC, where activated Akt signaling is often up-regulated[72,73]. In PC cell lines and

disseminated PC models, MYXV was shown to inhibit tumor growth, prolong survival and act synergistically with gemcitabine therapy[74,75]. However, further evaluation of MYXV in other gastrointestinal cancers and CSCs is warranted.

Reovirus

Reovirus is a double-stranded RNA virus, and is considered an orphan virus due to its ubiquitous nature and absence of severe pathophysiology. Reovirus infects the respiratory or gastrointestinal tract, but infection is asymptomatic and considered benign, implying that reovirus exhibits cytopathic effects and oncolytic potential in cancer cells[76]. Furthermore, activated Ras signaling contributes to tumor-specific viral replication and oncolysis of reovirus[77]. Currently, oncolytic reovirus is used widely to treat Ras-activated gastrointestinal cancers *in vitro* and *in vivo*, which causes apoptosis of TRAIL-resistant GC cells by down-regulation of Akt and inhibition of peritoneal metastasis[78,79]. In particular, reolysin, a novel reovirus-based agent, induced endoplasmic reticulum stress-mediated apoptosis in PC[80] and prolonged overall survival in a phase I trial of recurrent glioma[81,82]. Although there are few reports of reovirus in gastrointestinal CSCs, research in breast CSCs and glioblastoma stem-like cells has yielded promising results[83,84].

Vesicular stomatitis virus

Vesicular stomatitis virus (VSV) is a negative-sense, single-stranded RNA rhabdovirus. VSV mainly infects livestock as an animal pathogen and is usually asymptomatic in humans and only rarely causes a flu-like syndrome. VSV is highly sensitive to IFN response, which makes VSV as an ideal naturally oncolytic agent for cancer cells with a deregulated IFN response, but having no effect in normal cells[59,85]. Another study reported that VSV exhibits effective oncolytic activity and apoptosis induction in tumor cells with aberrant p53, Ras, or myc function[86]. This indicates that VSV is an optimal candidate as an oncolytic agent because most gastrointestinal cancers have the above aberrant signaling pathways.

Metastasis of CRC is incurable with currently available treatments. Recombinant VSV-GFP is able to replicate extensively in CRC cells and lyse hepatic metastasis of CRC in immunocompetent mice[87]. Recombinant VSV (rVSV) vectors expressing a mutant (L289A) NDV fusion protein, rVSV-NDV/F(L289A), was effective in treating a multifocal CRC liver metastasis model through repeated hepatic arterial administration[88]. The results indicate that VSV can be an effective and safe oncolytic agent against hepatic CRC metastasis and may be developed for the treatment of cancer patients in the future. However, oncolytic VSV is toxic in animals when administered systemically at high doses. Its safety can be improved by an MΔ51 deletion in the viral genome. A mutant attenuated form of the virus, rVSV(MΔ51), which has a single amino acid deletion of methionine-51 of the matrix protein to provide additional protection for normal cells by restoring IFN-mediated responses, exerts robust cellular inflammatory responses and cytotoxicity in HCC lesions[89]. The safety of oncolytic VSV delivering IFN β gene was further demonstrated by intrahepatic or intratumoral injection in rodents and non-human primates[90]. This implies that VSV can be developed as an effective and safe oncolytic agent to treat advanced HCC patients in the future. Although numerous studies have convincingly shown the ability of rVSV to inhibit tumor growth in CRC, HCC and PC[91], whether rVSV is able to target and kill CSCs remains unknown. Reports that an engineered VSV variant could target Her2/neu-overexpressing breast CSCs[3,92] bring greater understanding of the biology and molecular mechanisms of VSV.

Alphavirus M1

Alphavirus M1 virus is a naturally occurring alphavirus and an arthropod-borne togavirus, which was isolated from culicine mosquitoes by the Yan group on Hainan Island, China[93]. The novel alphavirus M1 possesses the features of oncolytic viruses and can induce apoptosis of malignant glioma cells *via* down-regulation and nucleolar translocation of p21WAF1/cyclin-dependent kinase inhibitor 1 or CDK-interacting protein 1 (CIP1)[93]. It was recently found that M1 can target cancer cells deficient in zinc-finger antiviral protein and has potent oncolytic efficacy and high tumor tropism in LC *in vitro*, *in vivo* and *ex vivo*[94]. The studies provide a novel insight into potentially unknown oncolytic viruses for further cancer therapy.

IMMUNOGENIC EFFECTS OF VIROTHERAPY AND POTENTIAL FOR COMBINATION WITH IMMUNOTHERAPY

Although the mechanisms of carcinogenesis and cancer development and their relationship with CSCs have not been clarified, the CSC theory in diverse cancer types, including gastrointestinal cancer, is supported by increasing evidence. Studies have testified that a few subpopulations of CSCs derived from tumor tissues are tumorigenic and able to generate the bulk of non-tumorigenic tumor cells. With the isolation, identification and characterization of CSCs, many new targeting therapy strategies have been shown to target CSCs to prevent cancer recurrence and metastasis to secondary organs[3]. Oncolytic viruses are considered to have therapeutic potential because they can eradicate CSCs through broadening the permissiveness for viral replication to CSCs, and their unique molecular mechanisms.

There are some limitations hampering the efficacy of oncolytic virotherapy for CSCs when it is performed by

intravenous administration. These drawbacks include liver or spleen trapping, clearance of viral particles by neutralizing antibodies, impact of tumor microenvironment or niche on viral replication, activated cellular immune response against viral infection, and virus-induced inflammatory response. To overcome these obstacles, recent efforts have been made towards: (1) isolation and identification of gastrointestinal CSC-specific markers and design of new engineered viruses to enhance potency; (2) PEGylation of oncolytic viruses, and use of cells or nanoparticles as potent vectors for oncolytic virus delivery[95]; (3) modification and disruption of the tumor vasculature to suppress the pernicious environmental conditions[96]; (4) combinatorial strategies with viruses, therapeutic genes and chemo- or radiotherapy with a mechanistic rationale[97]; (5) transient immunosuppression to improve the efficacy of oncolytic virotherapy[98]; and (6) use of gastrointestinal CSC-derived models in oncolytic virus evaluation[97].

With the advent of a new era of cancer immunotherapy, the checkpoint inhibitors such as cytotoxic T-lymphocyte antigen (CTLA)-4, programmed cell death protein-1 and programmed death-ligand (PD-L)1 have shown promising results in gastrointestinal cancer patients with tumor regression, and have prolonged survival[99]. In particular, chimeric antigen receptor therapy, a personalized therapeutic approach that involves genetically modifying patients' T cells with tumor antigen receptor to target tumor cells, has yielded encouraging results in leukemia, up to complete remission[100]. Besides tumor cytolysis and growth inhibition, oncolytic virotherapy also promotes an immune response against distant niduses due to production of cytokines and release of tumor antigens[101]. Therefore, the combinatorial strategy of oncolytic virotherapy and cancer immunotherapy may synergistically boost the anti-tumor response as well. Actually, the practice of combining oncolytic viruses and immunotherapy is still ongoing in the following two aspects. The common strategy is the design of oncolytic virus vectors encoding immuno-related genes such as antibodies against CTLA-4, PD-L1 and GM-CSF, which show therapeutic benefits[102]. Another hopeful approach is combined therapy with oncolytic viruses and immune cells such as cytokine-induced killer cells and dendritic cells[103].

Current preliminary data support the rationale that oncolytic virotherapy has outstanding potential in targeting CSCs in patients with gastrointestinal cancer. The clinical benefit of the first OncoHSV T-VEC in melanoma has spread to other oncolytic viruses, such as OncoVV JX-594, and other types of cancer, including gastrointestinal cancers[104]. With the discovery of new tumor antigens and CSC markers, new engineered viruses can be developed to target entry receptors specific to tumors and limit CSC function. The goals of combination of oncolytic viruses and other therapeutic methods (chemotherapy, radiotherapy, and especially immunotherapy) are to eradicate tumor progress and CSCs and avoid systemic side effects in cancer patients.

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Liver grafts from hepatitis B surface antigen-positive donors: A review of the literature

Elisabetta Loggi, Fabio Conti, Alessandro Cucchetti, Giorgio Ercolani, Antonio Daniele Pinna, Pietro Andreone

Elisabetta Loggi, Fabio Conti, Alessandro Cucchetti, Giorgio Ercolani, Antonio Daniele Pinna, Pietro Andreone, Dipartimento di Scienze Mediche e Chirurgiche, Università degli Studi di Bologna, 40138 Bologna, Italy

Pietro Andreone, Centro di Ricerca per lo Studio delle Epatiti, Università degli Studi di Bologna, 40138 Bologna, Italy

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Correspondence to: Pietro Andreone, MD, Professor of Internal Medicine, Dipartimento di Scienze Mediche e Chirurgiche, Università degli Studi di Bologna, Via Massarenti, 9, 40138 Bologna, Italy. pietro.andreone@unibo.it
Telephone: +39-51-2143618
Fax: +39-51-345806

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Abstract

The scarcity of available organs and the gap between supply and demand continue to be the main limitations of liver transplantation. To relieve the organ shortage, current transplant strategies have implemented extended criteria, which include the use of liver from patients with signs of past or present hepatitis B virus (HBV) infection. While the use of liver grafts from donors with evidence of past HBV infection is quite limited, some data have been collected regarding the feasibility of transplanting a liver graft from a hepatitis B surface antigen (HBsAg) positive donor. The aim of the present work was to review the literature regarding liver transplants from HBsAg-positive donors. A total of 17 studies were identified by a search in Medline. To date, HBsAg positive grafts have preferentially been allocated to HBsAg positive recipients. The large majority of these patients continue to be HBsAg positive despite the use of immunoglobulin, and infection prevention can only be guaranteed by using antiviral prophylaxis. Although serological persistence is evident, no significant HBV-related disease has been observed, except in patients coinfected with delta virus. Consistently less data are available for HBsAg negative recipients, although they are mostly promising. HBsAg-positive grafts could be an additional organ source for liver transplantation, provided that the risk of reinfection/reactivation is properly prevented.

Key words: Liver transplantation; Hepatitis B; Marginal grafts; Hepatitis B positive graft; Hepatitis B surface antigen positive donor

Core tip: Organ shortage is the main problem of liver transplantation, and the use of marginal grafts could

increase the donor pool. Data accumulated to date show that hepatitis B surface antigen-positive grafts could be an additional organ source for liver transplantation. The requirements that have to be fulfilled are the lack of a significant hepatitis B virus-disease of the graft, and the use of a proper prophylactic regimen, which is now largely available.

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INTRODUCTION

Since the first liver transplant operation, performed by Thomas Starzl in 1963, significant advances have been made in liver transplantation (LT), which has become a standard procedure for clinical conditions in which LT is the only therapeutic option[1,2]. These remarkable efforts have resulted in excellent survival rates, which currently exceed 80% at 1 year, and outcomes continue to improve[3]. Over the past decade, improvements in the treatment of viral hepatitis and modifications in the life styles have produced substantial changes in the indications for LT. While hepatitis C (CHC)-related disease remains the leading indication, a significant reduction has been observed in hepatitis B (CHB)-related cirrhosis, even in hyperendemic areas[4]. In contrast, the number of metabolic end stage liver disease cases requiring LT is progressively increasing[5,6]. Globally, the need for liver grafts is still high, and since the early 90's, it has been increasing progressively, which in turn has widened the gap between organ supply and demand[7]. According to the last UNOS report in 2014, the number of recipients on the waiting list is more than twice the number of performed transplants (data available on https://optn.transplant.hrsa.gov/data/).

The organ shortage is forcing the search for additional sources, in particular the use of so-called marginal grafts, namely organs carrying a risk of impaired function, or at risk to transmit infections and malignancy[8,9]. This latter category includes grafts from donors with serological evidence of hepatitis B virus (HBV) infection. Based on the worldwide prevalence of HBV "past" and present infection (2 billion and 350 million subjects, respectively), this procedure may significantly relieve organ scarcity, especially in highly-endemic countries. While the use of liver grafts from donors with past HBV infection (HBcAb-positive only) is a relatively common procedure[10,11], the use of grafts from donors with chronic HBV infection (HBsAg-positive) is much more limited.

This work aims to review the literature regarding liver transplants from HBsAg-positive donors to assess the risk of the procedure for patients and graft survival based on donor-recipient features as well as the peculiarities of management.

HBsAg-POSITIVE DONOR AND LIVER TRANSPLANTATION

Our search was performed on Medline/PubMed using the search terms "HBsAg positive liver donor", "HBsAg-positive" "graft" and "liver transplantation," and only papers published in English were selected. Two authors (Elisabetta Loggi and Fabio Conti) reviewed the literature independently. The search was carried out in February 2016 without a lower limit on the search and was restricted to peer-reviewed, full-text English language publications. The described search retrieved 315 abstracts. From them, 17 original research articles about liver transplantation using grafts from HBsAg positive donor were identified and selected.

The first pioneering experience was performed in the pre-antiviral era and involved a HBV-seronegative paediatric recipient who was transplanted with a liver from an HBsAg-positive donor due to an urgent need for re-transplant[12]. The choice was made because the patient's condition was life threatening. In the absence of prophylaxis, the patient tested positive for HBsAg after the LT, and the HBV infection rebounded clinically seven months later. The condition was managed by reducing immunosuppressive therapy and administering ciprofloxacin. Despite the difficult situation, the last observation reported loss of serum HBsAg and stable condition 2 years after LT.

After the introduction of effective combined prophylaxis with Lamivudine and Hepatitis B Immunoglobulin (HBIg), the first use of HBsAg-positive grafts was described from an Italian group that allocated three organs to three HBV-infected recipients, 2 of them with Hepatitis Delta virus (HDV) coinfection[13]. Post-LT, the HBsAg persisted in all recipients, despite HBIg administration. The HBsAg persistence appeared to favour HDV superinfection, which in turn caused rapidly progressive liver disease, requiring retransplantation in one case. Only the HBV mono-infected recipient experienced an uneventful post-LT course[13]. This preliminary experience, although performed on a small case series and largely unsuccessful, revealed some critical observations useful for the subsequent management of these types of organs. First, HDV coinfection in the recipient should represent a primary contraindication for the use of an HBsAg-positive liver. The persistence of HBsAg coupled with the viral suppression mediated by Lamivudine likely acts synergistically to give HDV the opportunity to replicate and rapidly cause liver injury. Second, HBIg fails to control HBsAg when it's derived from a double source (donor and recipient). Moreover, the report

underlines the importance of transplanting a liver free of significant disease. Although the histological assessment was suggestive of minimal disease, it should be noted that all three donors exhibited a slight elevation of the international normalized ratio and mild thrombocytopenia, raising some doubts on the presence of an inactive carrier status. To confirm these findings, a subsequent case report describes a very similar clinical outcome with a HDV/HBV coinfected recipient after transplant with an HBsAg-positive graft. In this case, the antiviral prophylaxis was also Lamivudine plus Adefovir (switched to Tenofovir later) coupled with a 48-wk course of Pegylated interferon. This treatment failed to prevent HDV reinfection, resulting in decompensated liver disease and the need for retransplantation[14].

A completely different scenario emerged when HBsAg-positive grafts were allocated to recipients without HDV. Several case reports describe the transplantation of grafts from HBsAg-positive donors into recipients with HBsAg-positive cirrhosis[15,16] as well as in one case of HCV coinfection[17]. Interestingly, in two of these cases, living donor liver transplantation was performed[15,16]. This procedure is prominent in East Asia, where brain-dead donors were largely unavailable, and where the chance of finding a HBV-infected donor is relatively high[18]. In both cases, the donors had an inactive HBV infection and combined antiviral prophylaxis (HBIg and Lamivudine) was started at transplant. The post-transplantation course was completely uneventful in one case[15]. The other case was complicated by HBV reactivation that required the reinforcement of antiviral treatment. At the last follow-up, performed at 4 and 5 years after LT, both recipients were reported to be in stable clinical and virological conditions, despite the persistence of HBsAg. Moreover, neither donor experienced exacerbation of their liver disease at the last available follow-up[16].

By learning the key findings from the broader experiences in the setting of HBcAb-positive transplantation, the general suggestion is to allocate these organs to recipients with HBV-related liver disease, also for issue of management: indeed, the HBsAg positive patients require life-long anti-HBV treatment anyway, regardless of type of graft[11,19-21].

However, some evidence suggests that the setting of an HBsAg-positive graft defines a different situation. In a recent case series, we described a clear dichotomy between HBsAg-positive and HBsAg-negative recipients in terms of viral control[22,23]. In particular, patients with chronic HBV infection before LT, even when treated with nucleos(t)ide analogues and HBIg, were unable to clear the virus. Conversely, patients with a past HBV infection (HBcAb-positive only) even cases of HBsAg reappearance, were able to re-evoke their effective specific immune response, leading to spontaneous HBsAb production and HBsAg-loss. In these cases, passive prophylaxis was not required, and antiviral treatment could be discontinued. The re-use of memory response in these cases could be assumed indirectly by the fact that only response restricted to self or matched HLA alleles were detected in our experimental system[23]. These findings allowed us to conclude that patients with a previous HBV immune control (HBcAb-positive or both HBcAb- and HBsAb-positive) are likely the best candidates to receive an HBsAg-positive liver graft.

The feasibility of using an HBsAg-positive graft was further reinforced by additional data derived from several case series[24-26], where a total of 16 HBsAg-positive recipients received a graft from HBsAg-positive inactive carriers (Table 1). Among them, all but two remained HBsAg positive and, for this reason, HBIg was discontinued within the first month post-LT. In two patients a HBsAg loss was achieved[26]. Some patients experienced HBV reactivation shortly post LT or viral rebound due to tyrosine-methionine-aspartate-aspartate motif mutation (rtM204I and rtM204V); however, all of these events were successfully controlled by switching or adding Adefovir to Lamivudine therapy. Three deaths were reported, but were not ascribed to HBV. However, one of the causes was hepatocellular carcinoma (HCC), so, in our opinion, this may have been related to HBV. The last available follow-up, obtained at a variable time after LT, reported stable clinical conditions and viral suppression, with no evidence of active hepatitis at the histological assessment.

More recent studies report data obtained in larger sample sizes that are not exclusively Asian, which dominated the first anecdotic experiences (Tables 1 and 2). A retrospective analysis of the clinical outcome of 23 HBV-infected patients who received a HBsAg-positive graft led to the conclusion that this procedure is safe, although 3 patients died due to recurrent HCC within 2 years post-LT[27] .

Saidi *et al*[28] reviewed the LT outcome data in United States using the United Network for Organ Sharing (UNOS) database, showing similar survival of both the graft and the patient between the 92 recipients of HBsAg-positive grafts *vs* recipients of HBsAg-negative grafts. The study population consisted largely of patients requiring LT for HBV-related disease (74%). Of note, the authors reported that the HBV infected graft was more frequently used in MELD exceptional cases, as predicted for a marginal graft. Similar approaches, using the UNOS database as a source, collected the outcome data of all consecutive transplant patients in the United States from 1987 to 2010[29,30]. In the study by Li, among the 92157 patients undergoing LT, 78 HBsAg-positive graft recipients were selected[29]. Each of them was then matched with 4 recipients of an HBsAg-negative graft on the basis of demographics (donor sex and recipient sex, as well as age at transplant),

Table 1 Published studies with liver transplantation using hepatitis B surface antigen (+) positive donors in hepatitis B surface antigen (+) recipients

Ref.	Patient No.	Prophylaxis		Outcome at the last FU			Median FU (mo)
		Nucleos(t)ide Analogue	HBIg	HBV disease	HBsAg	HBV-DNA	
Franchello *et al*[13]	3	LMV LMV + ADV (*n* = 1)	Yes	No (*n* = 1) Yes (HDV =2)	Persistence	Negative HDVRNA +	19
Ho *et al*[17]	1	LMV + ADV	No	No	Persistence	Negative	24
Hwang *et al*[16]	1	LMV + ADV	Yes	Mild	Persistence	Negative	64
Soejima *et al*[15]	1	LMV	Yes	No	Persistence	Negative	48
Jiao *et al*[24]	2	LMV	Yes	Mild	Persistence	Negative	48
Jang *et al*[25]	6	LMV + ADV	Yes	No	Persistence	Negative	22.5
Bahde *et al*[14]	1	LMV + ADV	Yes	HDV cirrhosis	Persistence	Negative HDVRNA +	50
Loggi *et al*[23]	6	LMV + ADV LMV + TDF	Yes	No	Persistence	Negative	42
Choi *et al*[26]	8	LMV (*n* = 2) ETV (*n* = 6)	Yes	No	Persistence (*n* = 6) Loss (*n* = 2)	Negative	25.5
Ju *et al*[27]	23	ETV	Yes	No	Persistence (*n* = 17) Loss (*n* = 1)	Negative	NA
Saidi *et al*[28]	68	NA	NA	NA	NA	NA	NA
Li *et al*[29]	15	NA	NA	NA	NA	NA	NA
Yu *et al*[31]	38	Not specified	Yes	No	Persistence	Negative	NA
Jeng *et al*[32]	13	ETV	No	No	Persistence	Negative	46

In HDV-coinfected recipients. HBIg: Hepatitis B immunoglobulins; LMV: Lamivudine; ADV: Adefovir; TDF: Tenofovir; ETV: Entecavir; FU: Follow-up; NA: Not available.

disease stage (MELD and status of urgency), and technical transplant aspects (warm ischemia time). The outcomes comparison suggested similar graft and patient survival rates. In addition, the causes of death were similar in the two groups.

Interestingly, in this study, the patient population was heterogeneous in term of aetiologies of liver disease leading to LT; in contrast to the previously described experiences, HBV infection represented the minority in the group of recipients of HBsAg positive grafts (19%).

Equally promising data were described in two single transplant centres in China and in Taiwan[31,32]. The first[31] compared the post-LT outcomes of a group of 42 patients receiving HBsAg-positive grafts with those of 327 recipients of HBsAg-negative livers. There were no significant differences in the post-LT course between the two groups. Also in this case, the allocation policy for the HBsAg positive grafts affected the preferential allocation of HBsAg grafts to HBsAg-positive recipients (the percentage of HBV-infected patients in HBsAg-positive graft recipients was 90.5%). The study confirmed the persistence of HBsAg after LT, and the uselessness of administrating HBIg either at a high or low dosage. It is noteworthy that the study included 10 HBeAg-positive grafts and that the HBeAg status of the recipient was determined by the donors, regardless of his pre-LT serologic profile. However, no further details are provided in this specific subgroup. The second work[32] , which is the last paper published on this specific issue to date, confirmed the positive data, reporting an uneventful post-LT course without HBV reactivation. The peculiarity of this study population is that, in addition to patients with advanced liver disease, it also included subjects with acute liver failure and variable but positive viremia at the time of transplant.

CONCLUSION

There is accumulating evidence that the use of HBsAg-positive grafts could represent an additional and safe organ source for liver transplantation and that the risk of reinfection/reactivation can be efficiently prevented or managed. To generate uniform recommendations for the management of grafts from HBV-positive donors, consensus guidelines were recently published by the American and Canadian Society of Transplantation[10]. Consequently, the use of these organs can relieve the organ shortage, especially in high-endemic areas.

This general statement is particularly significant in the limited setting of experiences with HBsAg-positive grafts, considering the following points: first, the data generally arise from case reports or small case series, and the large majority of them were obtained in Asian populations; second, the post-LT management was considerably heterogeneous in terms of immunosuppressive or immunoprophylaxis protocols (Tables 1 and 2). Finally, these organs were first used in urgent situations because of a lack of alternatives, and consequently, the trend was to allocate HBsAg-positive grafts to more compromised patients.

To date, HBsAg-positive liver grafts have been preferentially given to HBsAg-positive recipients. HBsAg

Table 2 Published studies with liver transplantation using hepatitis B surface antigen (+) positive donors in hepatitis B surface antigen (-) recipients

Ref.	Patient No.	Etiology of liver disease	Prophylaxis		Outcome at the last FU			Median FU (mo)
			Nucleos(t)ide Analogue	HBIg	HBV disease	HBsAg	HBV-DNA	
Gonzalez *et al*[12]	1	Crypto	NO	Yes	Mild	Negative	Negative	24
Loggi *et al*[22]	1	HBV	LMV	No	No	Negative	Negative	18
Loggi *et al*[23]	4	HCV (n = 3) PBC (n = 1)	LMV LMV + ADV	Yes	Mild	Negative	Negative	42 (12-60)
Saidi *et al*[28]	24	NA	NA	NA	Survival similar to controls	NA	NA	NA
Li *et al*[29]	63	HCV (n = 34) NASH (n = 2) Alcohol (n = 6) Other (n = 17)	NA	NA	Survival similar to controls	NA	NA	NA
Krishnamoorthi *et al*[30]	15	NA	NA	NA	Survival similar to controls	NA	NA	NA
Yu *et al*[31]	4	NA	NA	NA	Survival similar to controls	NA	NA	NA
Jeng *et al*[32]	1	HCV	ETV	No	No	Negative	Negative	12

Crypto: Cryptogenetic HBV cirrhosis; PBC: Primary biliary cirrhosis; NASH: Nonalcoholic steatohepatitis; FU: Follow-up; NA: Not available.

persists long term, and HBIg administration is useless in promoting HBsAg clearance. This fact generated some concern in the first cases, where the prolonged use of Lamivudine was thought to expose patients to the risk of resistance; however, the high genetic barrier of the nucleos(t)ide analogues currently available overcomes this problem. Of note, these drugs have been proven effective in preventing HBV reinfection in LT for HBV-disease in HBIg-free regimens[33].

On the other hand, data regarding the use of HBsAg-positive grafts in HBsAg-negative recipients is lacking. The experience in this specific setting should be reinforced in the near future because HBsAg-negative patients will continue to represent the large majority of subjects on waiting lists that can benefit from receiving these organs.

It should be underlined that additional tools are now available for optimizing risk assessment and monitoring the post-LT outcome. Among them, the quantification of HBsAg levels, recently introduced into regular clinical practice, can improve both the assessment of the HBsAg-positive donor and the outcome of the HBsAg-positive graft recipient. For the donor, the absolute requirement for the HBsAg-positive graft allocation is to utilize a liver without significant disease. In addition to histological examination, quantification of circulating HBsAg can provide higher confidence in the inactive status of donor[34]. For the recipient, the quantification of HBsAg provides information about the "entity" of reactivation, and in the longitudinal assessment, it indicates the efficacy of antiviral control by therapy.

Moreover, tools now available for monitoring the recipient, including the assessment of liver fibrosis by non-invasive techniques, can significantly simplify post-LT monitoring in lieu of a liver biopsy. Furthermore, the potency of antiviral therapy with high-genetic barrier drugs represents a valid prevention strategy. Finally, the availability of an effective hepatitis B vaccine, which can be administered to non-immune sexual partners of HBsAg positive graft recipients, decreases the social impact of this procedure, which has recently been raised as a concern for this kind of transplant[35].

We think that, in addition to the heterogeneity, the main limitation of the presented studies is the lack of a longer follow-up. Even the studies showing UNOS transplant data in a decennial time frame do not report data on the oldest cases, or they cannot be interpreted because they were performed in the pre-prophylaxis era[28-30].

A longer follow-up could help to define the following major points: first, whether to carry a virus-infected graft under immune suppression exposes the recipients of an increased risk of HCC; and second, whether the need to continue the antiviral therapy probably life long poses some safety issues.

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ORIGINAL ARTICLE

Basic Study

May the assessment of baseline mucosal molecular pattern predict the development of gluten related disorders among microscopic enteritis?

Giuseppe Losurdo, Floriana Giorgio, Domenico Piscitelli, Lucia Montenegro, Claudia Covelli, Maria Grazia Fiore, Antonio Giangaspero, Andrea Iannone, Mariabeatrice Principi, Annacinzia Amoruso, Michele Barone, Alfredo Di Leo, Enzo Ierardi

Giuseppe Losurdo, Floriana Giorgio, Lucia Montenegro, Antonio Giangaspero, Andrea Iannone, Mariabeatrice Principi, Annacinzia Amoruso, Michele Barone, Alfredo Di Leo, Enzo Ierardi, Section of Gastroenterology, Department of Emergency and Organ Transplantation, University of Bari, 70124 Bari, Italy

Domenico Piscitelli, Claudia Covelli, Maria Grazia Fiore, Section of Pathology, Department of Emergency and Organ Transplantation, University of Bari, 70124 Bari, Italy

Author contributions: Principi M, Barone M, Di Leo A and Ierardi E conceived the study; Losurdo G, Montenegro L, Giangaspero A, Iannone A and Amoruso A collected the data; Piscitelli D, Covelli C and Fiore MG performed immunohistochemistry and pathological evaluations; Giorgio F performed molecular analysis; Losurdo G and Giorgio F performed statistical analysis; Principi M performed endoscopy; Losurdo G and Ierardi E wrote the manuscript; all authors read and approved the final version of the manuscript.

Institutional review board statement: The study was reviewed and approved after two meetings of all the authors (all affiliated to the same Department) before and after immune-histochemical and molecular analysis.

Institutional animal care and use committee statement: This study was not performed on experimental animals.

Informed consent statement: All study participants provided informed written consent prior to endoscopic investigation. Additional oral consent to perform immunohistochemistry and molecular analysis was obtained. No ethical committee approval was required because all invasive procedures had been performed according to the current clinical patient management.

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Correspondence to: Enzo Ierardi, Professor, Section of Gastroenterology, Department of Emergency and Organ Transplantation, University of Bari, Piazza Giulio Cesare 11, 1, 70124 Bari, Italy. ierardi.enzo@gmail.com
Telephone: +39-805-594034
Fax: +39-805-593088

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Abstract

AIM

To evaluate mucosal baseline mRNA expression of tissue transglutaminase 2 (tTG2), interferon gamma (IFNγ), toll-like receptor 2 (TLR2) and Myeloid Differentiation factor 88 (MyD88) in patients with microscopic enteritis (ME).

METHODS

We retrospectively enrolled 89 patients with ME of different etiology, which was defined within a 2-year mean period of follow-up. Baseline histological examination was performed on Hematoxylin-Eosin stained sections and CD3 lymphocyte immunohistochemistry was used for intraepithelial lymphocyte count (IELs). ME was defined according to the criteria of Bucharest Consensus Conference. For each patient, formalin embedded biopsy samples of the duodenum referred to the period of ME diagnosis were retrieved. Real-time polymerase chain reaction (RT-PCR) was used to detect the amount of mRNA coding for tTG2, IFNγ, TLR2 and MyD88, and the quantity was expressed as fold change compared to controls. Control group was represented by duodenal normal specimens from 15 healthy subjects undergoing endoscopy for functional symptoms. Comparisons among continuous variables were performed by One way analysis of variance (ANOVA) and Bonferroni's test. The χ^2 test was used for categorical variables. Pearson's test was used to evaluate correlations. Receiver operating curves were drawn for all four markers to estimate sensitivity and specificity in discriminating the development of CD and GS.

RESULTS

After a period of follow up of 21.7 ± 11.7 mo, the following diagnoses were achieved: gluten related disorders in 48 subjects (31 CD; 17 GS) and non-gluten related ones in 41 (29 Irritable Bowel Syndrome - IBS; 12 Others). CD patients had the highest tTG2 levels (8.3 ± 4.5). The ANOVA plus Bonferroni analysis showed that CD > Other ME > GS = IBS > negative controls. A cut off value of 2.258 was able to discriminate between CD and GS with a sensitivity of 52.94% and a specificity of 87.1%. Additionally, CD patients had the highest IFNγ levels (8.5 ± 4.1). ANOVA plus Bonferroni demonstrated CD > Other ME > GS = IBS > negative controls. A cut off of 1.853 was able to differentiate CD and GS with a sensitivity of 47.06% and a specificity of 96.77%. Patients with non gluten-related causes of ME exhibited the highest TLR2 levels (6.1 ± 1.9) as follows: Other ME > CD = GS = IBS > negative controls. TLR2 was unable to discriminate CD from GS. Patients with CD overexpressed MyD88 levels similarly to non gluten-related causes of DL (7.8 ± 4.9 and 6.7 ± 2.9), thus CD = Other ME > GS = IBS > negative controls. A cut off of 3.722 was able to differentiate CD from GS with a sensitivity of 52.94% and a specificity of 74.19%. IELs count (15-25 and more than 25/100 enterocytes) strongly correlated with mRNA levels of all tested molecules ($P < 0.0001$).

CONCLUSION

Our results confirm that a single marker is unable to predict a discrimination among ME underlying conditions as well as between CD and GS. Mucosal high levels of tTG and IFNγ mRNA may predict the development of CD more than GS with high specificity, despite an expected low sensitivity. TLR2 does not discriminate the development of CD from GS. MyD88 levels indicate that intestinal permeability is more increased when a severe intestinal damage underlies ME in both gluten related and unrelated conditions. Therefore, the results of the present paper do not seem to show a clear translational value.

Key words: Celiac disease; MyD88; Microscopic enteritis; Gluten sensitivity; Tissue transglutaminase; Interferon gamma; Toll-like receptor 2

Core tip: Microscopic enteritis (ME) is an inflammatory condition, which is characterized by increased intraepithelial CD3 lymphocytes in the duodenum and can be due to both gluten and non-gluten related diseases. It is often difficult to achieve a final diagnosis in cases of ME, therefore the assessment of baseline mucosal molecular pattern may be helpful. In this study, we demonstrated that tissue transglutaminase and interferon gamma may predict the development of Celiac Disease more than Gluten Sensitivity with high specificity, despite an expected low sensitivity.

Losurdo G, Giorgio F, Piscitelli D, Montenegro L, Covelli C, Fiore MG, Giangaspero A, Iannone A, Principi M, Amoruso A, Barone M, Di Leo A, Ierardi E. May the assessment of baseline mucosal molecular pattern predict the development of gluten related disorders among microscopic enteritis? *World J Gastroenterol* 2016; 22(35): 8017-8025 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i35/8017.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i35.8017

INTRODUCTION

Duodenal lymphocytosis (DL) is a condition characterized by a pathologic infiltration of lymphocytes in the epithelium (IELs) of duodenal mucosa[1]. It is not a single entity, since several conditions may underlie this picture. The main associated disorders may be gluten related (Celiac disease - CD; Non celiac gluten sensitivity - NCGS; and Wheat allergy - WA) and non-gluten related (Irritable bowel syndrome - IBS; infectious or parasitic diseases; autoimmune disorders: vasculitides, connective tissue diseases and inflammatory bowel disease; immunoglobulin deficiencies; drug damage)[2-8]. Therefore, DL may be considered as an "umbrella term" rather than a single entity. Recently, the Bucharest Consensus has proposed "Microscopic enteritis (ME)" as an alternative term to DL, and has reviewed and standardized an algorithm for its diagnosis and treatment[9,10].

In the ME scenario, it is well known that CD is an autoimmune enteropathy triggered by the ingestion of gluten and represents the most common cause of

intestinal malabsorption and villous atrophy[11]. NCGS [or simply gluten sensitivity (GS)] is a disorder showing intestinal and extra-intestinal symptoms related to the ingestion of gluten containing food, in subjects not suffering from either CD or WA[12]. It is an emerging gluten related cause of ME and intestinal symptoms, with an increasing rate of diagnosis[13,14]. However, the spectrum of ME encompasses even non gluten-related disorders. For instance, IBS may show increased IELs as well as organic diseases[1,15]. For this reason, it is often difficult to formulate a differential diagnosis in ME, and the diagnostic *iter* may be long and time consuming. This aspect is of relevance especially for GS, whose diagnosis is essentially clinical. Moreover, the clinical manifestations of GS often overlap with IBS, and this is a diagnostic challenge[16,17]. A previous experience of our group found that IELs count of 15-25 IELs/100 enterocytes, autoimmune thyroiditis, folate deficiency and diarrhea may be predictive factors for GS[18], but a reliable marker has not been discovered yet despite the report that GS is characterized by the upregulation of toll-like receptor 2 (TLR2)[19].

In our experience, a IELs infiltrate > 15 per 100 enterocytes paralleled an enhanced expression of pro-inflammatory cytokines, in particular interferon gamma (IFNγ), in subjects with suspected seronegative CD[20]. Therefore, a pro-inflammatory status may underlie CD-related ME and the intestinal assessment of baseline mucosal molecular pattern could likely give useful information about ME underlying conditions. In detail, tissue Transglutaminase 2 (tTG2), IFNγ, TLR2 and Myeloid Differentiation factor 88 (MyD88) have been suggested as potential targets in this field[19]. tTG2 is the main autoantigen involved in the pathogenesis of CD, and it has been demonstrated that it is over-expressed in the mucosa of patients with CD[21,22]. IFNγ is a pro-inflammatory cytokine that is essential for innate and adaptive immunity against infections[23]. Aberrant IFNγ expression is associated with a number of autoinflammatory and autoimmune diseases, including CD[24]. TLR2 is a type of cellular receptor mainly involved in innate immunity, which has been proposed as a mediator of a potential inborn response to gliadin[25]. MyD88 is an adapter protein mediating intracellular pathways triggered by TLRs to activate the transcription factor NF-κB[26]. Indeed, some gliadin peptides may bind TLR2 and drive the production of interleukin 1, a proinflammatory cytokine, through the mediation of MyD88[27]. Moreover, the MyD88 was found to be a key protein mediating the release of zonulin in response to gliadin, thus leading to an increase of mucosal permeability in CD[28]. Therefore, an increase of MyD88 may be considered as a marker of an alteration of intestinal barrier.

The aim of the present study was to investigate the duodenal mucosal transcriptomic expression of these four molecules in the prediction of ME underlying disorders at baseline, before a 2-year follow-up mean period, and to assess their potential accuracy in discriminating the development of CD and GS.

MATERIALS AND METHODS

Patients

We retrospectively enrolled 89 consecutive patients with ME followed up for a mean period of two years until a diagnosis was reached. ME was defined according to the criteria of the Bucharest Consensus Conference[9].

Follow-up strategies which allowed achieving final diagnosis have been described elsewhere[18]. In detail, CD was diagnosed if duodenal biopsy showed a microscopic picture of Marsh 1 or higher, along with the positivity of IgA anti tissue transglutaminase 2 (anti-tTG2) antibodies, according to current guidelines[29]. The diagnosis of GS was made according to the Salerno criteria[30]. Patients with IBS fulfilled the Rome Ⅲ criteria and underwent a series of investigations (serology for CD, full blood count, folate, vitamin B_{12}, serum protein electrophoresis with immunoglobulin subclasses, stool investigations, fecal occult blood test, calprotectin, urea/lactose/glucose breath test and, if necessary, colonoscopy with random biopsy samples) in order to rule out organic diseases[31]. Finally, patients with established non-gluten related cause of ME (*Helicobacter pylori* infection, autoimmune disorders) were included.

We excluded subjects with immunoglobulin deficiencies, which may show possible molecular deregulation of duodenal mucosa. A group of 15 dyspeptic patients, undergoing upper endoscopy and duodenal biopsy without ME, represented the negative control group.

Histology and immunohistochemistry

For each patient, formalin embedded biopsy samples of the duodenum performed at baseline were retrieved. Histological examination had been carried out on Hematoxylin-Eosin stained sections. Immunohistochemistry of CD3 lymphocytes had been performed using monoclonal murine antibody (Novocastra Leica Biosystems Ltd, Newcastle, United Kingdom), according to the manufacturer's instructions. In all subjects, IELs were counted in a field containing at least 1000 enterocytes and expressed as number per 100 enterocytes. We selected biopsy specimens with at least 15 IELs/100 enterocytes to define ME, as established in previous reports[17,19]. The count was executed in the epithelial layer by two observers (DP and MGF) in a blinded fashion. Collection and processing was managed according to BRISQ recommendations[32].

Molecular analysis

Real time polymerase chain reaction (RT-PCR) was used to detect the amount of mRNA coding for tTG2, IFNγ, TLR2 and MyD88 in duodenal mucosa. As well-stated, mRNA levels were expressed as fold-change compared

Table 1 Primers and probes

Tissue transglutaminase 2
Primer Forward:ATAAGTTAGCGCCGCTCTCC
Primer Reverse: CGGTGGCTCCTTCCACTG
Probe: GCCAGCCGCCAGTG
Interferon gamma
Primer Forward: CGCTTTACTTTATAGAAAACCTGGA
Primer Reverse: TCAATGAAGAGAACTTGGTCATTC
Probe: GCTTGAATCTAAA
Toll-like receptor 2
Primer Forward: CAAGATTCAAAGTATTTA
Primer Reverse: CCAGGTG CATTTAAAGA
Probe: TGCCCCTACTCAATCT
MyD88
Primer Forward: CAAGGCCTTGTCCCTGC
Primer Reverse: TCTGCCCTGCCTCCT
Probe: AGGCCCTGGGTGTGTGT

to controls. The relative expression of the studied gene levels was calculated with the $2^{-\Delta\Delta CT}$ method. RNA was extracted from at least 5, 10 μm sections of paraffin block using the RNeasy FFPE Kit (Qiagen, GmbH, Heidelberg, Germany), specifically designed for the purification of total RNA from formalin-fixed paraffin-embedded (FFPE) tissue sections, according to a validated protocol[33]. Five hundred microliters (μL) of xylene were added to the sections to yield a solution that was vortexed for 10s and then incubated for 10 min at room temperature (25 ℃). This step was repeated twice. Subsequently, 500 μL of absolute ethanol was added and the novel solution was again vortexed vigorously for 10 s and centrifuged for 2 min at 11000 rpm in order to remove residual xylene. The supernatant was carefully removed by pipetting without disturbing the pellet. Finally, the mRNA concentrations were estimated by ultraviolet absorbance at 260/280 nm. We performed the agarose formaldehyde gel run to confirm the RNA integrity. Imaging analysis after this procedure was performed with the Bio-Rad Chemidoch Analyzer (Bio-Rad Laboratories S. r. l., Milan, Italy). Aliquots of total mRNA (1 mg) were reverse-transcribed using random hexamers and TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, United States) in a final volume of 50 μL. Two step reverse transcription PCR was performed using the first-strand cDNA with a final concentration of 1 × TaqMan gene expression assay, *i.e.,* the analyzed molecules and glyceraldehyde 3 phosphate dehydrogenase as reference gene (Applied Biosystems, Foster City, CA). The final reaction volume was 25 μL and analyzed in triplicate (all experiments were repeated twice). A non template control (Rnase free water) was included on every plate. Our method was further validated by including in each assay fresh samples from three normal patients, frozen at -90 ℃ until the analysis. These samples were treated with the same technique as the paraffin embedded samples, except for the paraffin removal and rehydration procedures. Specific thermal cycler conditions were employed using a real time PCR System (Applied Biosystems). A standard curve plus validation experiment was performed for each primer/probe set. The reference gene was represented by glyceraldehydes3phosphate dehydrogenase. Primers and probes are reported in Table 1.

Statistical analysis

Comparisons among continuous data obtained in our groups of patients were performed by one way analysis of variance (ANOVA) and Bonferroni's test as post-hoc analysis to compare head-to head each group. The χ^2 test was used for categorical variables. Values of $P < 0.05$ were considered significant. Receiver operating curves (ROC) were drawn to estimate sensitivity and specificity of tTG2, IFNγ, TLR2 and MyD88. Correlations between IELs count, tTG2, IFNγ, TLR2 and MyD88 were assessed by Pearson's test. Diagnostic agreement for the IEL count was tested by calculating the weighted Cohen's *k* coefficient interpreted in accordance with the Landis and Koch benchmarks, whereby a value of more than 0.8 indicated excellent agreement. Statistical analyses were performed using the statistical software GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, United States).

RESULTS

Patients baseline features

We enrolled 89 patients with ME. After the follow up the following diagnoses were observed: 31 CD, 17 GS, 29 IBS and 12 other non gluten-related ME. Among the 12 non gluten-related ME, 4 had small bowel Crohn's disease, 6 *H. pylori* infection, 1 scleroderma and 1 lymphocytic colitis. The most relevant clinical, demographic and histopathological characteristics are summarized in Table 2. A good agreement among pathologists was achieved ($k = 0.86$, 95%CI: 0.75-0.91). The IELs count, DQ 2 or 8 positivity and the presence of weight loss, abdominal pain and diarrhea were the most important discriminating factors between CD, GS and non-gluten-related diseases (Table 2). Villous atrophy (Marsh 3 stage) was found in 14 out of 31 CD patients, Marsh 2 in 3 and Marsh 1 in 14 patients.

tTG2

The mucosal expression of mRNA-tTG2 is represented in Figure 1A. In detail, CD patients had the highest levels (8.3 ± 4.5) compared to GS (3.6 ± 2.7), IBS (3.5 ± 1.8), other ME (5.3 ± 2.3) and negative controls (1.001 ± 0.089). The ANOVA plus Bonferroni analysis showed that CD > Other ME > GS = IBS > negative controls.

The ROC curve analysis, displayed in Figure 1B showed that a cut off of 2.258 was able to discriminate between CD and GS with a low sensitivity (52.94%) and a good specificity (87.1%; AUC = 0.804).

Table 2 Main demographic, clinical and pathologic features of enrolled patients

	Celiac disease (*n* = 31)	Gluten sensitivity (*n* = 17)	Irritable bowel syndrome (*n* = 29)	Other ME (*n* = 12)	Negative controls (*n* = 15)	*P* value
Age	34 ± 12	34.3 ± 6.1	36.2 ± 13.4	34.7 ± 14.2	32.6 ± 9.5	0.89[1]
Sex (F/M)	19/12	15/2	22/7	11/1	9/6	0.06[2]
IELs count	51.6 ± 10.6	18.6 ± 4.9	15.5 ± 5.1	19.0 ± 7.6	5.3 ± 1.5	< 0.001[1]
Weight loss	19 (61.3)	7 (41.2)	4 (13.8)	6 (50)	0	0.002[2]
Abdominal pain	25 (80.6)	16 (94.1)	28 (96.5)	8 (66.6)	0	0.04[2]
Diarrhea	21 (67.7)	6 (35.3)	29 (100)	6 (50)	0	< 0.001[2]
Weakness	14 (45.1)	8 (47.0)	8 (27.6)	3 (25)	0	0.09[2]
Headache	2 (6.5)	7 (41.2)	4 (13.8)	0 (0)	0	0.73[2]
DQ 2-8	29 (93.5)	10 (58.8)	11 (37.9)	0 (0)	NA	< 0.001[2]
Iron deficiency anemia	8 (25.8)	2 (11.8)	2 (6.9)	8 (66.6)	0	0.63[2]

[1]ANOVA test; [2]χ^2 test for trend. NA: Not available.

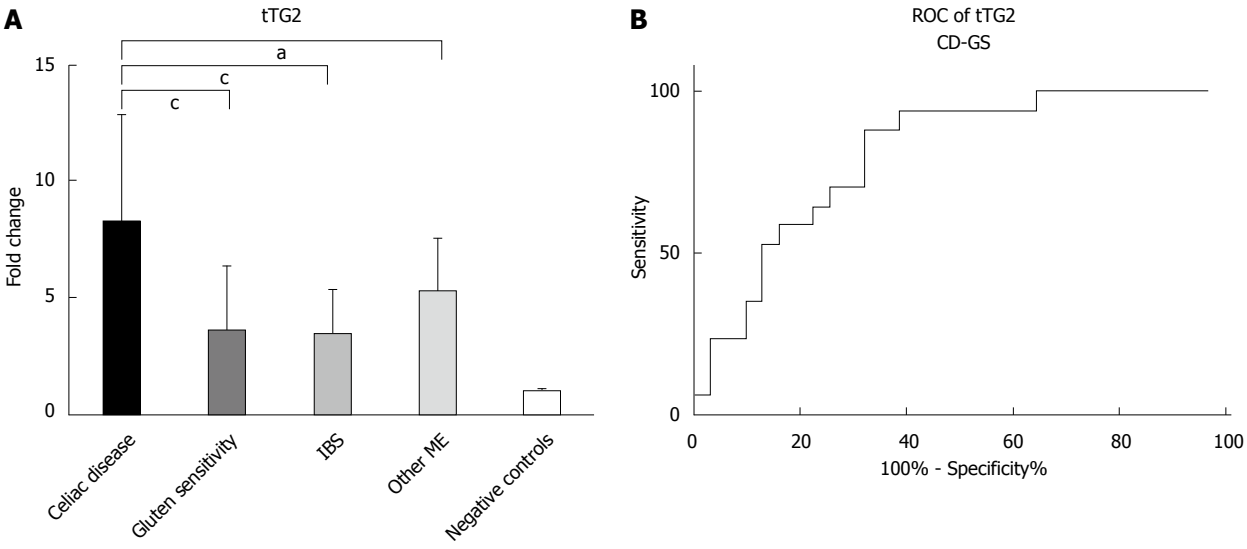

Figure 1 Pattern of mucosal expression of tTG2-mRNA in subjects with different causes of microscopic enteritis. A: ANOVA plus Bonferroni analysis showed that CD > Other ME > GS = IBS > negative controls, $^{a}P < 0.05$, $^{c}P < 0.001$; B: ROC curve of CD *vs* GS comparison is reported. ROC: Receiver operating curves.

IFNγ

The mucosal expression of mRNA-IFNγ is displayed in Figure 2A. We observed that CD patients had the highest levels (8.5 ± 4.1) compared to GS (3.3 ± 2.8), IBS (3.3 ± 2.6), other ME (4.6 ± 2.1) and negative controls (1.001 ± 0.15). The ANOVA plus Bonferroni analysis showed that CD > Other ME > GS = IBS > negative controls.

The analysis of ROC curve (Figure 2B) showed that a cut off of 1.853 was able to differentiate CD and GS with a sensitivity of 47.06% and a specificity of 96.77%, with an AUC of 0.816.

TLR2

The mucosal expression of mRNA-TLR2 is represented in Figure 3. Patients with non gluten-related causes of ME were characterized by the highest levels (6.1 ± 1.9), greater than GS (3.1 ± 1.8), IBS (3.5 ± 2.0), CD (4.1 ± 2.4) and negative controls (1.006 ± 0.18). The ANOVA plus Bonferroni analysis showed that Other ME > CD = GS = IBS > negative controls.

MyD88

The mucosal expression of mRNA-MyD88 is represented in Figure 4A. Patients with CD expressed levels similar as non gluten-related causes of ME (7.8 ± 4.9 and 6.7 ± 2.9), higher than GS (4.2 ± 2.3), IBS (4.3 ± 2.4), and negative controls (0.99 ± 0.17). The ANOVA plus Bonferroni analysis demonstrated that CD = Other ME > GS = IBS > negative controls.

The analysis of ROC curve (Figure 4B) showed that a cut off of 3.722 was able to differentiate CD and GS with a sensitivity of 52.94% and a specificity of 74.19%, with an AUC of 0.712.

Correlation between IELs count and transcriptome analysis

In all cases, the IELs count correlated with mRNA levels with a strong significance ($P < 0.0001$). The expression of tTG2 directly correlated to IELs ($r = 0.66$, 95%CI: 0.53-0.76). IFNγ showed a similar pattern, with an $r = 0.56$, 95%CI: 0.42-0.68. A less relevant, despite significant correlation, was found for TLR2 (r

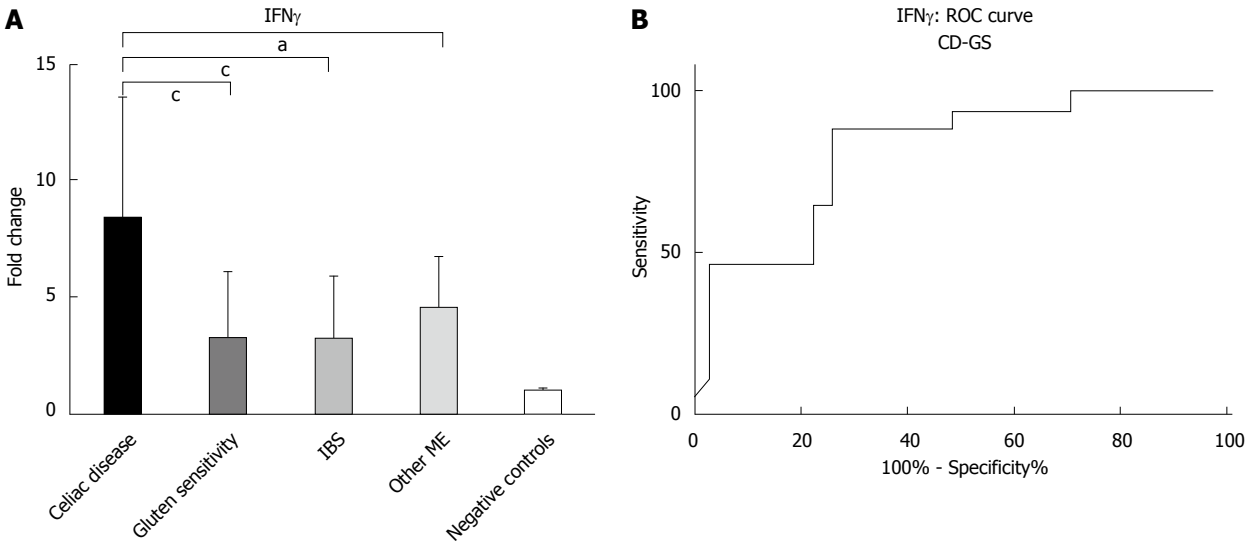

Figure 2 Pattern of mucosal expression of interferon gammaγ-mRNA in subjects with different causes of microscopic enteritis. A: ANOVA plus Bonferroni analysis showed that CD > Other ME > GS = IBS > negative control, $^{a}P < 0.05$, $^{c}P < 0.001$; B: ROC curve of CD *vs* GS comparison is reported. ROC: Receiver operating curves.

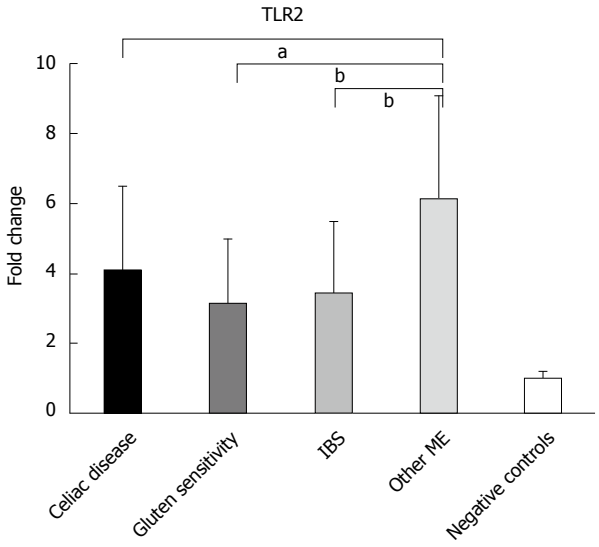

Figure 3 Pattern of mucosal expression of toll-like receptor 2-mRNA in subjects with different causes of microscopic enteritis. ANOVA plus Bonferroni analysis showed that Other ME > CD = GS = IBS > negative controls, $^{a}P < 0.05$, $^{b}P < 0.01$.

= 0.32, 95%CI: 0.13-0.48) and for MyD88 (r = 0.49, 95%CI: 0.33-0.63).

DISCUSSION

ME often represents a diagnostic dilemma, therefore a careful algorithm should be applied in such cases to achieve the final diagnosis[9,18]. IELs infiltrate represents the only common denominator; therefore, in our series we selected only subjects showing IELs levels higher than 15/100 enterocytes, independently from ME etiology. This cut-off value was associated with molecular mucosal changes suggestive of inflammatory damage/repair in our experience[20]. IELs infiltrate is a well known feature of GS and CD[34,35], but it has been described even in patients with duodenal involvement in systemic disorders[36]. IBS may show an increased IELs infiltrate, thus confirming that it cannot be considered only as a functional entity[37]. In a previous experience of our group, we demonstrated a positive correlation between the amount of IELs infiltrate and the mucosal expression of tTG2 and IFNγ in patients with seronegative suspected CD[20,38]. In the present study, moreover, we confirmed that IELs infiltrate parallels mucosal molecular expression, more markedly for tTG2 and IFNγ, but also for TLR2 and MyD88. Therefore, our results demonstrate that ME clearly underlies a local inflammatory status.

tTG2 is the main autoantigen involved in CD pathogenesis[21], however it is an enzyme essential for the process of wound healing and tissue reparation, since it is able to create crosslinks between peptides[39]. For this reason, tTG2 is over-expressed not only in gluten-related disorders, but even in all pathological conditions that induce mucosal injury, as confirmed in the present study. In our analysis, we reported that tTG2 has a good performance in the comparison between CD and GS or IBS. This detail could be useful especially in the subgroup of subjects with IBS who show positivity of anti-tTG antibodies, without histological evidence of CD[40]. Moreover, our result may explain the reason of a four-fold increased risk of CD in subjects with IBS[41]. A pattern similar to tTG was found for IFNγ. Indeed, patients with CD show the upregulation of IELs-secreted IFNγ. This ability has been demonstrated even in peripheral T lymphocytes of CD patients, which are able to produce high IFNγ levels when stimulated by gliadin peptides[42,43]. For these reasons, tTG2 and IFNγ could represent a good diagnostic tool in gluten related-disorders. In summary, we found a high specificity of tTG2 and IFNγ,

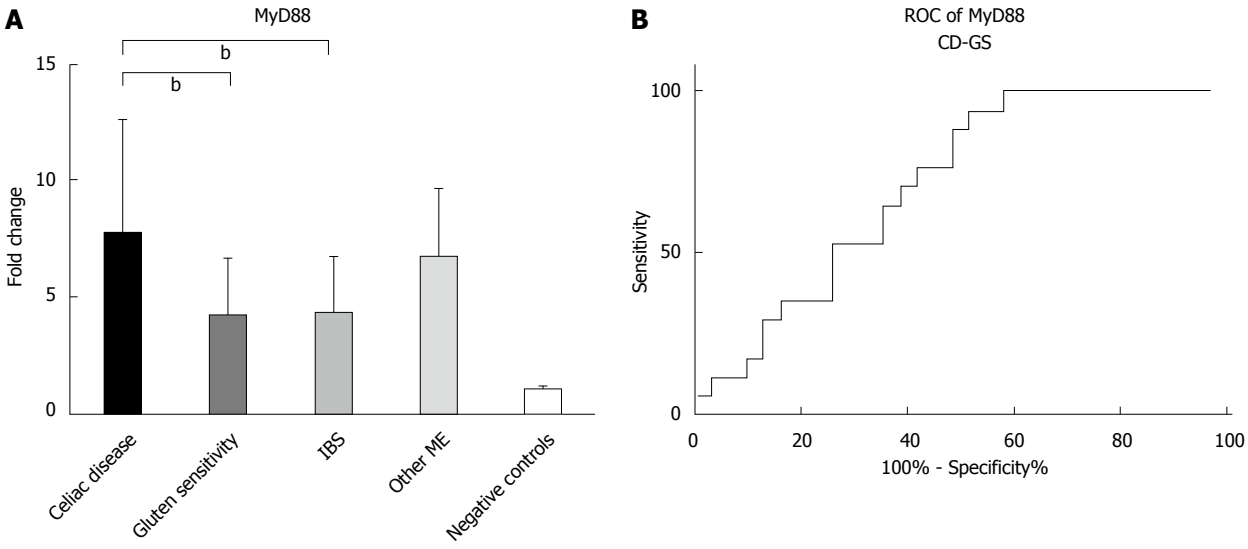

Figure 4 Pattern of mucosal expression of MyD88-mRNA in subjects with different causes of duodenal lymphocytosis. A: ANOVA plus Bonferroni analysis showed that CD = Other ME > GS = IBS > negative controls, $^{b}P < 0.01$; B: ROC curve of CD *vs* GS comparison is reported. ROC: Receiver operating curves.

despite an expected low sensitivity, in discriminating CD from GS development. The expression of these molecules in GS has been poorly explored until now and available data are controversial. Some reports have shown an IFNγ overexpression in GS similar to CD[44], while others described a marked increase only in CD[19]. In the present study, we have found that high levels of IFNγ are more predictive of CD than GS.

TLR2 is a receptor involved in the innate immune response against non-self antigens. It has been demonstrated that some gliadin peptides may bind such receptor and address the production of interleukin 1, a proinflammatory cytokine, trough the mediation of MyD88[27]. Moreover, MyD88 was found to be a key protein mediating the release of zonulin in response to gliadin, thus leading to an increase of mucosal permeability in CD[28]. In a previous report[19], patients with GS expressed higher levels of TLR2 than subjects with CD. However, we found that ME had similar baseline mRNA levels encoding for TLR2, independently from the successive development of CD or GS within a two-year period. Surprisingly, in our series, non gluten related ME had the highest levels of TLR2. This finding could be related to the deep deregulation of TLRs, which has been described in IBD (a possible cause of non gluten related ME). Indeed, such receptors mediate the immune response against the microbiota, a phenomenon that has been claimed as a trigger in IBD pathogenesis[45-47].

In regard to the molecular pattern of MyD88, similarly to TLR2, we found that patients with non gluten related causes of ME showed higher levels than IBS and GS, but comparable with CD. This finding suggests that a potential increase of intestinal permeability may be more marked when a severe intestinal damage underlies ME. On the other hand, MyD88 has been poorly investigated in CD. Eiró *et al*[48] demonstrated that its increased expression paralleled mucosal TLR4 in CD. Therefore, MyD88 overexpression overtaking TLR2 may be explained by a MyD88-independent pathway for TLR2 in CD, as described by Junker *et al*[25].

In conclusion, our results suggest that a single marker is unable to discriminate the development of different ME underlying conditions as well as between CD and GS. High mucosal levels of tTG and IFNγ mRNA may predict the development of CD more than GS with high specificity. TLR2 does not discriminate the development of CD from GS. High MyD88 levels may indicate that intestinal permeability is more increased when a severe intestinal damage underlies ME (CD as well as Crohn's disease). Finally, a reliable marker for GS diagnosis has not yet been found; however, further studies need to be addressed to evaluate whether the combination of different mucosal markers could help the differential diagnosis with CD and support the identification of doubtful cases of GS[49,50]. Therefore, the results of the present paper do not seem to show a clear translational value.

COMMENTS

Background

Microscopic enteritis is an inflammatory condition, which is characterized by increased intraepithelial CD3 lymphocytes in the duodenum and can be due to both gluten and non-gluten related diseases.

Research frontiers

The MyD88 was found to be a key protein mediating the release of zonulin in response to gliadin, thus leading to an increase of mucosal permeability in CD. Therefore, an increase of MyD88 may be considered as a marker of an alteration of intestinal barrier.

Innovations and breakthroughs

The authors demonstrated that tissue transglutaminase and interferon gamma

may predict the development of Celiac Disease more than Gluten Sensitivity with high specificity, despite an expected low sensitivity.

Peer-review

This report seeks to distinguish among 4 causes of duodenal lymphocytosis (celiac disease, non-celiac gluten sensitivity, wheat allergy and irritable bowel syndrome) by retrospectively comparing the mRNA expression of tissue transglutaminase 2, interferon gamma, toll-like receptor 2 and myeloid differentiation factor 88 in duodenal biopsies from 89 patients obtained up to two years previously.

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ORIGINAL ARTICLE

Basic Study

Dietary advanced glycation end-products aggravate non-alcoholic fatty liver disease

Christopher Leung, Chandana B Herath, Zhiyuan Jia, Sof Andrikopoulos, Bronwyn E Brown, Michael J Davies, Leni R Rivera, John B Furness, Josephine M Forbes, Peter W Angus

Christopher Leung, Chandana B Herath, Zhiyuan Jia, Sof Andrikopoulos, Peter W Angus, Department of Medicine, the University of Melbourne, Austin Health, Heidelberg, Melbourne, Victoria 3084, Australia

Christopher Leung, Peter W Angus, Department of Gastroenterology and Hepatology, Austin Health, Austin Hospital, Heidelberg, Melbourne, Victoria 3084, Australia

Bronwyn E Brown, Michael J Davies, Heart Research Institute, Newtown, New South Wales 2042, Australia

Leni R Rivera, John B Furness, University of Melbourne, Victoria 3052, Australia

Leni R Rivera, Metabolic Research Unit, School of Medicine, Deakin University, Victoria 3216, Australia

Josephine M Forbes, Glycation and Diabetes Complications Group, Mater Medical Research Institute, South Brisbane, Queensland 4101, Australia

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Correspondence to: Christopher Leung, MBBS, FRACP, Department of Gastroenterology and Hepatology, Austin Health, Austin Hospital, 145 Studley Rd, Heidelberg, Victoria 3084, Australia. chris.leung@y7mail.com
Telephone: + 61-3-94965000
Fax: +61-3-94963487

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Abstract

AIM

To determine if manipulation of dietary advanced glycation end product (AGE), intake affects non-alcoholic fatty liver disease (NAFLD) progression and

whether these effects are mediated *via* RAGE.

METHODS

Male C57Bl6 mice were fed a high fat, high fructose, high cholesterol (HFHC) diet for 33 wk and compared with animals on normal chow. A third group were given a HFHC diet that was high in AGEs. Another group was given a HFHC diet that was marinated in vinegar to prevent the formation of AGEs. In a second experiment, RAGE KO animals were fed a HFHC diet or a high AGE HFHC diet and compared with wildtype controls. Hepatic biochemistry, histology, picrosirius red morphometry and hepatic mRNA were determined.

RESULTS

Long-term consumption of the HFHC diet generated significant steatohepatitis and fibrosis after 33 wk. In this model, hepatic 4-hydroxynonenal content (a marker of chronic oxidative stress), hepatocyte ballooning, picrosirius red staining, α-smooth muscle actin and collagen type 1A gene expression were all significantly increased. Increasing the AGE content of the HFHC diet by baking further increased these markers of liver damage, but this was abrogated by pre-marination in acetic acid. In response to the HFHC diet, $RAGE^{-/-}$ animals developed NASH of similar severity to $RAGE^{+/+}$ animals but were protected from the additional harmful effects of the high AGE containing diet. Studies in isolated Kupffer cells showed that AGEs increase cell proliferation and oxidative stress, providing a likely mechanism through which these compounds contribute to liver injury.

CONCLUSION

In the HFHC model of NAFLD, manipulation of dietary AGEs modulates liver injury, inflammation, and liver fibrosis *via* a RAGE dependent pathway. This suggests that pharmacological and dietary strategies targeting the AGE/RAGE pathway could slow the progression of NAFLD.

Key words: Advanced glycation end-products; Fructose; Steatohepatitis; Non-alcoholic fatty liver disease; Hepatic fibrosis; Oxidative stress

Core tip: A novel high fructose, high cholesterol diet produces hepatic non-alcoholic steatohepatitis (NASH) with fibrosis in 33 wk and increasing the Advanced glycation end products (AGEs), content of this diet *via* baking increases hepatic fibrosis whilst vinegar marination decreases dietary AGE levels, abrogating the harmful effects of AGEs. $RAGE^{-/-}$ animals appeared to be protected from the additional harmful effects of a high AGE containing diet suggesting the central role of RAGE in progression of NASH. Increased cell proliferation and oxidative stress in isolated primary Kupffer cells with the addition of AGEs suggests they are an important mechanism in which AGEs contribute to liver injury.

Leung C, Herath CB, Jia Z, Andrikopoulos S, Brown BE, Davies MJ, Rivera LR, Furness JB, Forbes JM, Angus PW. Dietary advanced glycation end-products aggravate non-alcoholic fatty liver disease. *World J Gastroenterol* 2016; 22(35): 8026-8040 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i35/8026.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i35.8026

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease in the world and is strongly linked to the burgeoning rates of diabetes and obesity[1]. Although most patients with NAFLD are asymptomatic and do not develop clinically significant liver injury, many progress to non-alcoholic steatohepatitis (NASH), cirrhosis and liver cancer. However, the host and/or environmental risk factors that determine whether patients with simple hepatic steatosis go on to develop NASH and its complications remain unclear[2].

Advanced glycation end products (AGEs), also known as glycotoxins, are a complex group of compounds that are formed when active sugar moieties become bound to proteins causing browning and other irreversible modifications[3]. Foods that are highly processed or dry heated at high temperatures, such as broiled foods, have particularly high AGE content[4]. However, they are also formed endogenously and this occurs at an increased rate in diabetes, most likely because of hyperglycaemia. There is now considerable evidence that accumulation of AGEs are implicated in the pathogenesis of diabetic renal, neurological, retinal and vascular complications[5].

AGEs act on several receptors but the receptor for advanced glycation end products (RAGE), a member of the immunoglobulin superfamily of cell-surface molecules, is the best characterised of these receptors[6]. Engagement with RAGE, which in turn increases inflammation and oxidative stress is thought to be the main way in which AGEs impart these pathogenic effects[7]. This receptor is expressed in a number of cell types, including endothelial cells, vascular smooth muscle cells, peripheral blood mononuclear cells, macrophages (including Kupffer cells) and HSCs[8].

A number of lines of evidence to suggest that, in NAFLD, AGEs may be a "second hit" that contributes to the progression from simple steatosis to NASH and liver fibrosis[9]. Several studies have shown that RAGE plays a role in acute liver injury and that blockade of RAGE can ameliorate toxic, ischaemic and cholestatic liver damage[10-12]. In chronic liver injury, hepatic expression of RAGE is significantly increased[13], and in NAFLD AGE levels correlate with the severity

of fibrosis, leading to speculation that AGEs play a primary role in disease pathogenesis[14]. At a cellular level, AGEs induces ROS *via* oxidative stress[15] and this is a significant mechanism underlying the pathogenesis of NASH[16]. Furthermore, diabetes, which increases AGE formation RAGE expression and oxidative stress, worsens the progression of fibrosis in a number of human liver diseases, including NAFLD[17] and hepatitis C[18].

In a previous study, we showed that in a short term methionine choline deficient (MCD) model of NAFLD, a high AGE diet increased hepatic AGE content and exacerbated liver injury, oxidative stress and liver fibrosis and that AGEs produced RAGE dependent profibrotic effects in activated hepatic stellate cells (HSCs)[19]. However, the relevance of the MCD model to human disease is limited since the metabolic profile of MCD fed animals is generally the converse of human NAFLD[20]. In contrast, long-term murine models based on a high-calorie diet high in fat, fructose and cholesterol [high fructose, high cholesterol (HFHC) diet], yield NASH associated with many of the metabolic features of human NASH[21]. The aim of the current study was to explore whether increasing dietary AGE consumption could precipitate the development of NASH in a novel HFHC model of NAFLD. We also performed complementary studies in RAGE KO animals to determine the role of RAGE signalling in our liver disease model.

MATERIALS AND METHODS

Experimental design

Experiments were approved by the Austin Health Animal Ethics Committee and performed according to the National Health and Medical Research Council (NHMRC) of Australia Guidelines for animal experimentation.

Male C57Bl6 mice (n = 10/group) were fed a high fat, high fructose, high cholesterol (HFHC) diet [Specialty Feeds SF11-109 (21% saturated fat, 10% fructose, 2% cholesterol)] for 33 wk and compared with animals on normal chow (Specialty Feeds AIN93G). This is a novel dietary model combining amounts of saturated fat (especially animal fats such as ghee) and fructose to mimic unhealthy Western diets[22]. This model is different to other high fat diets in that we gave amounts of fat actually consumed by patients for a longer period to induce fibrosis[23]. Two percent cholesterol was incorporated as this has been shown to be a critical driver for fibrosis in murine dietary models of NASH[22] and 10% fructose was added, both due to its prevalence in Western diet as well as its potent ability to induce AGE formation[24] (See Supplementary materials 2). In two further groups, the effects of further increasing dietary AGE content by baking (described below), were studied in animals fed normal chow and in animals fed the HFHC diet. A final group was fed a baked HFHC diet that was marinated in acetic acid (vinegar 4% w/v) prior to heating to prevent the formation of AGEs, as is seen in Mediterranean style diets[25]. In a second experiment, RAGE KO animals were fed a HFHC diet or a high AGE HFHC diet for 33 wk and compared with WT controls. At the completion of each experiment, insulin resistance was measured *via* HOMA-IR and oral glucose tolerance testing (OGTT) performed as previously described[26]. Livers were harvested for assessment of liver injury and serum alanine aminotransaminase (ALT) levels were measured by auto-analyser (Beckman Instruments, Fullerton, CA).

Production of AGEs

Dietary AGE content was increased by baking at 160 ℃ for 1 h which we have previously shown produces an approximately 4 fold increase in AGE levels[12]. CML is the predominant AGE in food and the extent of advanced glycation in the diet was assessed by measuring CML content using high performance liquid chromatography with fluorescence detection against authentic standards of CML[27].

For cell studies, AGEs were prepared *in vitro* by incubating bovine serum albumin (BSA, 50 mg/mL) with 0.5 mol/L glucose in 100 mmol/L sodium phosphate buffer, pH 7.4. This was incubated at 37 ℃ for 6 wk, followed by dialysis against phosphate buffered saline (PBS) for 48 h at 4 ℃ to remove any free glucose. AGE-BSA and BSA were then passed through an endotoxin column (Detoxigel, Pierce, Rockford, IL, United States) to remove any possible endotoxin contaminants. The extent of advanced glycation was assessed by CML levels with ELISA and by isotope dilution, selected ion monitoring gas chromatography-mass spectrometry[12,27].

Liver histology

Paraffin embedded, paraformaldehyde fixed sections of liver (4 μm) were stained with haematoxylin and eosin and picrosirius red (Polysciences Inc. Warrington, PA) for assessment of liver fibrosis and steatohepatitis by an independent pathologist. The NAFLD Activity Score (NAS) system was used to quantify steatohepatitis using ten × 200 light microscopic fields in a blinded fashion as previously described[28]. Each field was scored using the following criteria: For hepatic steatosis: grade 0, no fat; grade 1, steatosis occupying less than 33% of the hepatic parenchyma; grade 2, 34%-66% of the hepatic parenchyma; grade 3, more than 66% of the hepatic parenchyma; for inflammatory cell infiltration: grade 0:none; grade 1, 1-2 foci/field; grade 2, 3-4 foci/field; grade 3, more than 4 foci/field (steatosis 0-3, lobular inflammation 0-2, hepatocellular ballooning 0-3 and fibrosis 0-4)[28]. Collagen content of the liver was quantified histologically using computerized quantification of picrosirius red staining, as described previously[12]. This was assessed at × 100 magnification in a total of ten

Table 1 Primer and probe sequences used for real time qPCR

Gene name	Probe/ primer	Sequence
IL-6	Probe	5-FAM ATTGCCATTGCACAACT-3
	Forward	5-GGGAAATCGTGGAAATGAGAAA-3
	Reverse	5-AAGTGCATCATCGTTGTTCATACA-3
MCP1	Probe	5-FAM-AATGGGTCCAGACATAC-3
	Forward	5-GTCTGTGCTGACCCCAAGAAG-3
	Reverse	5-TGGTTCCGATCCAGGTTTTTA-3
TNFα	Probe	5-FAM-TCACCCACACCGTCAG-3
	Forward	5-GGCTGCCCCGACTACGT-3
	Reverse	5-TTTCTCCTGGTATGAGATAGCAAATC-3
RAGE	Probe	5-FAM-CACAGCCCGGATTG-3
	Forward	5-GCTGTAGCTGGTGGTCAGAACA-3
	Reverse	5-CCCCTTACAGCTTAGCACAAGTG-3
alphaSMA	Probe	5-FAM-TGCCAGATCTTTTCC-3
	Forward	5-GACGCTGAAGTATCCGATAGAACA-3
	Reverse	5-GGCCACACGAAGCTCGTTAT-3
COL1A1	Probe	5-FAM-ATCGACCCTAACCAAG-3
	Forward	5-GACTGGAAGAGCGGAGAGTACTG-3
	Reverse	5-CCTTGATGGCGTCCAGGTT-3
CTGF	Probe	5-FAM-C ACTGCCTGGTCCAGAC-3
	Forward	5-GCTGCCTACCGACTGGAAGA-3
	Reverse	5-CTTAGAACAGGCGCTCCACTCT-3

fields per section (per animal), using computerized quantification and results were expressed as proportion of picrosirius red staining per field.

Assessment of hepatic oxidative stress

Superoxide anion and ROS are key mediators of liver injury and cellular dysfunction associated with the progression of fatty liver disease. Immunohistochemistry for HNE, a marker of lipid peroxidation, was performed on 4 μm sections of paraffin embedded liver as previously described[12]. The primary HNE antibody (1:500 Alpha Diagnostic International, San Antonio, Texas, United States) in 0.1% normal goat serum with PBS was applied and then incubated with biotinylated secondary goat anti-mouse antibody (1/100) followed by incubation with avidin-biotin horseradish peroxidase. Peroxidase conjugates were subsequently localized using diaminobenzidine tetrahydrochloride chromogen (Sigma-Aldrich, Sydney, Australia). The relative positive staining in each group was determined by computerized quantification (MCID; Imaging Research, Ontario, Canada) at × 100 magnification (10 fields per animal).

Quantitative real time PCR

Total RNA was extracted using TRI reagent (Sigma-Aldrich) and reverse transcribed to cDNA using a protocol previously described[29]. Gene expression was normalized to the expression of the endogenous control, ribosomal 18S. Each sample was run and analysed in duplicate. The normalized values from normal chow livers were used as the calibrator with a given value of 1 and the other groups compared with this calibrator. The gene probe, forward and reverse primer sequences are detailed in Table 1.

Kupffer cell experiments

Kupffer cells (KCs) were isolated from rat livers as described previously[30,31]. Further details can be found in Supplementary materials 1. KCs were cultured in M199 medium (Invitrogen), supplemented with 10% (v/v) FCS (foetal calf serum) containing 100 units/mL penicillin and 100 μg/mL streptomycin (Invitrogen), at 37 ℃ in an oxygenated, humidified cabinet containing 5% CO_2.

Twenty thousand KCs per well were plated in black 96 well plates at 37 ℃. The cells were incubated with 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) (10 μmol/L, Sigma-Aldrich, St Louis, MO, United States) for 30 min, and washed twice with PBS. The cells were then treated with either AGEs or vehicle BSA at physiological concentrations of 100 μg/mL. Measurement of intracellular ROS generation was performed using 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) as described previously[32]. Briefly, fluorescence measurements were taken with excitation and emission wavelengths of 485 nm and 520 nm respectively on an Optima Microplate Reader (BMG Labtech, Mornington, Australia).

KCs were assayed for their proliferative response to AGEs using a BrdU cell proliferation assay (Roche Applied Science, IN, United States) as per manufacturer's instructions. KCs were cultured in 96 well plates, with 10000 cells per well and treated with BSA and AGEs at 100 μg/mL.

Statistical analysis

Results are expressed as mean ± SE and analysed by analysis of variance (ANOVA) and Student's two-tail, unpaired *t*-test where appropriate with Prism 5 software (GraphPad Software, San Diego, United States). Data that were not normally distributed with equal variance were log transformed prior to analysis. $P < 0.05$ was considered significant.

RESULTS

Novel 33 week high fat, HFHC dietary model of NAFLD produced both steatohepatitis and significant fibrosis

The HFHC diet was well tolerated and the initial body weights of the experimental groups were similar. All the HFHC groups (HFHC, HFHC baked and HFHC baked + acetic acid) gained more weight, as expected, over the 33 wk compared with normal chow mice, and there was no final difference in weight gain, liver weight or liver to body weight ratio among the HFHC groups (Table 2). The daily weight of chow consumed each day was the same in all the HFHC groups

The diet produced features similar to human NASH with ballooning of hepatocytes and oxidative stress as assessed by HNE accumulation and steatosis grade > 3 (Figure 1A-C), with significant increases in ALT and lobular inflammation (Figure 1D and E). The diet also induced hepatic fibrosis with the typical "river delta" tendrils of lobular fibrosis seen in fibrotic human NASH

Table 2 Animal characteristics and metabolic findings

	Body weight (g)	Epididymal fat (g)	Liver weight (g)	Blood glucose (mmol/L)	OGTT (AUC)
Normal chow	35.4 ± 1.5[a]	1.4 ± 0.2[a]	1.3 ± 0.1[a]	8.0 ± 0.7	654.1 ± 94.4
HFHC	43.4 ± 1.7	2.7 ± 0.2	3.7 ± 0.4	8.2 ± 0.6	528.7 ± 65.2
HFHC baked	45.5 ± 1.2	2.8 ± 0.2	3.4 ± 0.5	7.8 ± 0.3	506.2 ± 70.2
HFHC baked and acetic acid	44.6 ± 0.9	2.6 ± 0.1	3.6 ± 0.2		

Data are mean ± SEM at 33 wk of normal chow or high fat high cholesterol feeding. [a]$P < 0.01$ *vs* HFHC groups. AUC: Area under the curve; OGTT: Oral glucose tolerance test.

(Figure 2A). This was associated with an elevation in profibrotic and proinflammatory cytokine expression in the liver (Figure 2B and C, Supplementary materials 3). Thus using a combination of physiological amounts of fat, fructose and a prolonged duration of feeding, a reliable model of NASH with fibrosis was generated. Importantly this diet did not result in insulin resistance in any of the groups as measured by blood glucose OGTT (Table 2).

Diet high in AGEs did not cause steatosis or steatohepatitis in the normal liver (normal chow animals)

Both normal chow and normal chow baked groups had similar food intake and there was no difference in weight gain, liver weight or liver to body weight ratio between the two groups. In line with previous studies[19], baking the normal chow diet increased AGE content by over 6 fold (Figure 3) but feeding with baked chow did not change liver biochemistry, produce steatosis, oxidative stress or fibrosis (Figures 1 and 2).

Changes in dietary AGE content modulate oxidative stress and hepatocyte ballooning in HFHC induced steatohepatitis

Baking the HFHC diet markedly increased CML content (Figure 3). Other studies have shown that pre-marination of food in vinegar decreases AGE levels to much lower levels comparable to raw food or boiled food. Importantly, the pre-marination of the HFHC baked diet with acetic acid reduced CML levels in the baked diet to non-baked levels (Figure 3). These AGE levels in the HFHC baked diet are similar to the CML levels found in a moderate to high AGE typical Western diet[33].

Steatosis was significantly increased in all HFHC groups but not further increased by baking to increase its AGE content (Figure 1C). However, the ballooning produced by HFHC feeding was further increased by the high AGE/HFHC diet and this effect was inhibited by the reduction in dietary AGE content achieved by vinegar pre-marination (Figure 1A). Oxidative stress is strongly implicated in liver injury and ballooning degeneration in NASH. Higher levels of HNE were detected in the livers of the HFHC high AGE group compared to those fed the HFHC diet alone. Furthermore, hepatic HNE content was reduced to the levels observed in the HFHC group when AGE content of the diet was reduced by acetic acid (Figure 1B). These findings implicate increased generation of oxidative stress in the pathogenesis of AGE mediated injury in this model.

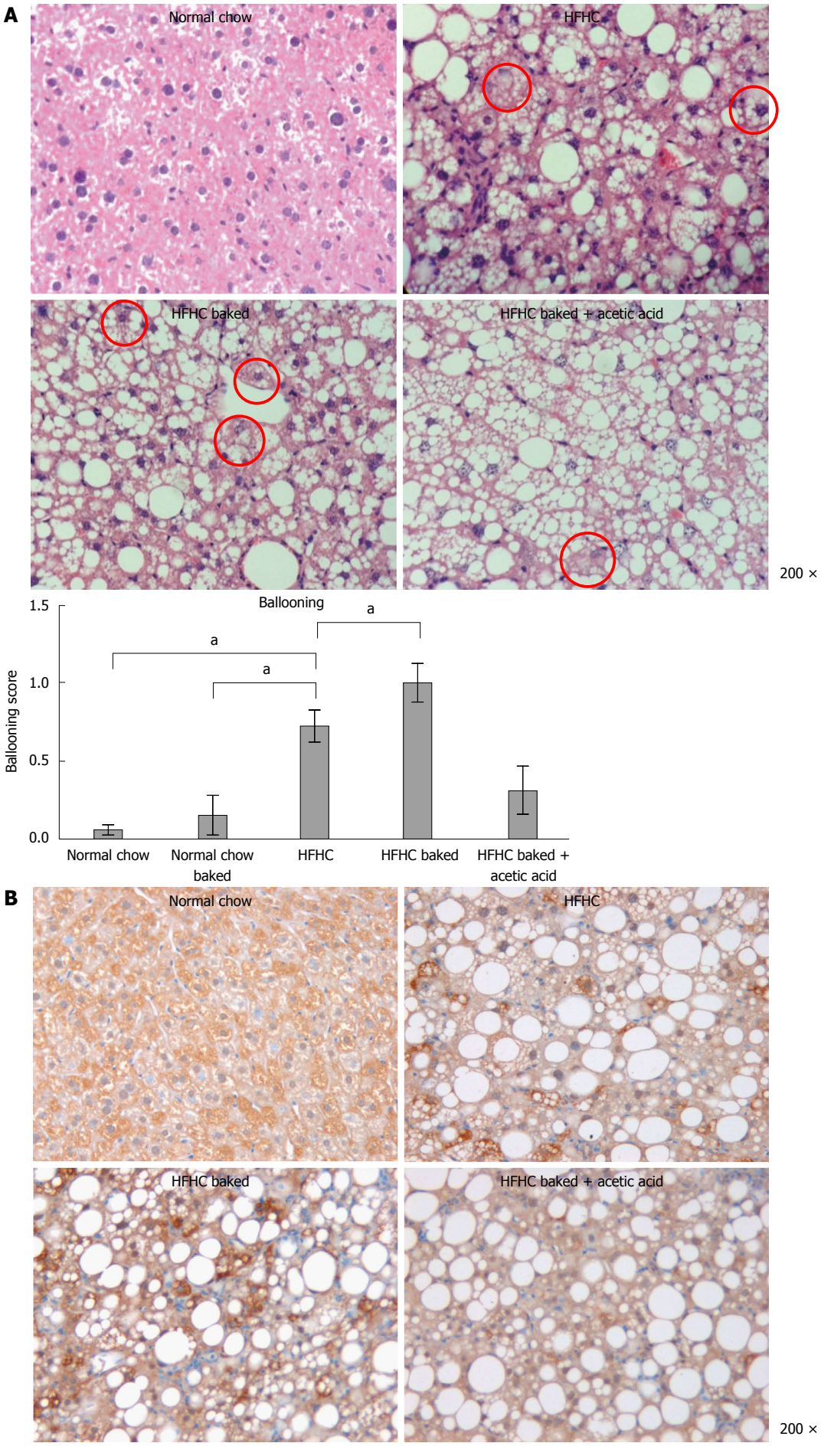
We previously showed that AGEs increase activation and proliferation of hepatic stellate cells and lead to the production of ROS by these cells[19]. However KCs are responsible for hepatic AGE uptake and play a central role in NASH pathogenesis. We therefore isolated primary KCs to determine the effects of AGEs on these key drivers of inflammation and injury in NAFLD. As shown in Figure 4A, the generation of ROS by isolated Kupffer cells, as measured by 2,3-DCFDA, was significantly increased by the addition of AGEs to the medium compared to vehicle. Moreover, Kupffer cell proliferation as assessed by BrDU incorporation was also significantly increased in Kupffer cells exposed to AGEs compared to BSA vehicle alone (Figure 4B).

Lobular inflammation, ALT levels and proinflammatory cytokine expression were all significantly increased in the HFHC model (Figure 1D and E, supplement 3) but were not affected by the dietary AGE content.

Modulation of dietary AGE content increased markers of liver fibrosis in the HFHC model

As outlined above, in this novel HFHC model, fibrosis was largely confined to zone 3 and produced a sinusoidal pericellular fine "river delta" pattern. This is in keeping with human NASH where fibrosis tends to follow a centrizonal pattern[34]. The proportional area stained was further increased when the HFHC diet had been baked to increase AGE content (Figure 2A). In addition to more pronounced zone 3 fibrosis, periportal fibrosis was also observed in HFHC high AGE fed animals following the pattern of fibrosis progression in human NAFLD[34]. However, the portal pattern of inflammation was not affected by changing dietary AGE content.

In keeping with the results of picrosirius red staining, *COL1A* gene expression was significantly increased in both HFHC groups compared with normal chow controls and levels were higher in the mice with a high oral intake of AGEs compared with HFHC alone (Figure 2B). Activation of myofibroblasts, as assessed by hepatic α-SMA gene expression was also significantly increased in the HFHC model and further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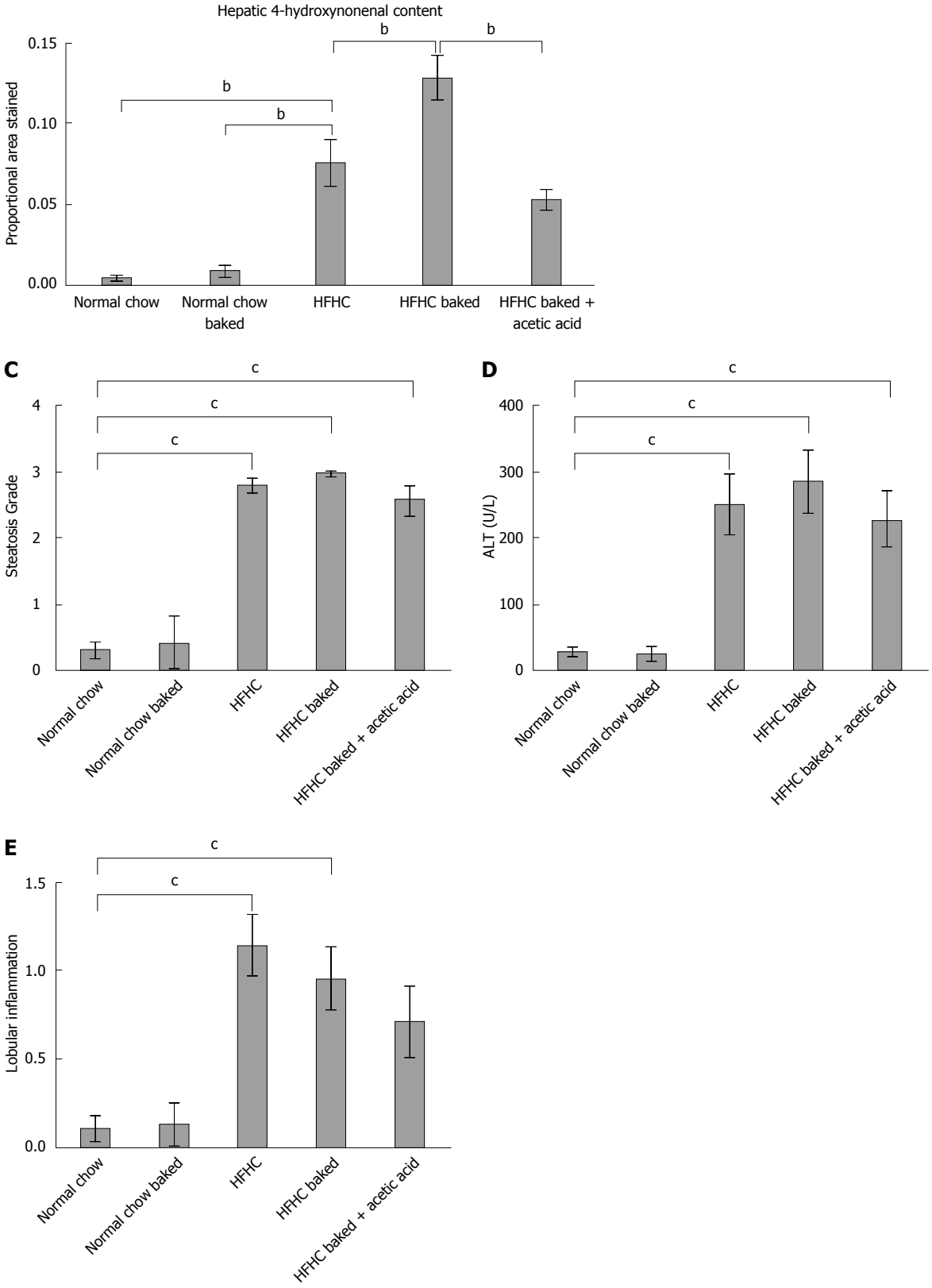
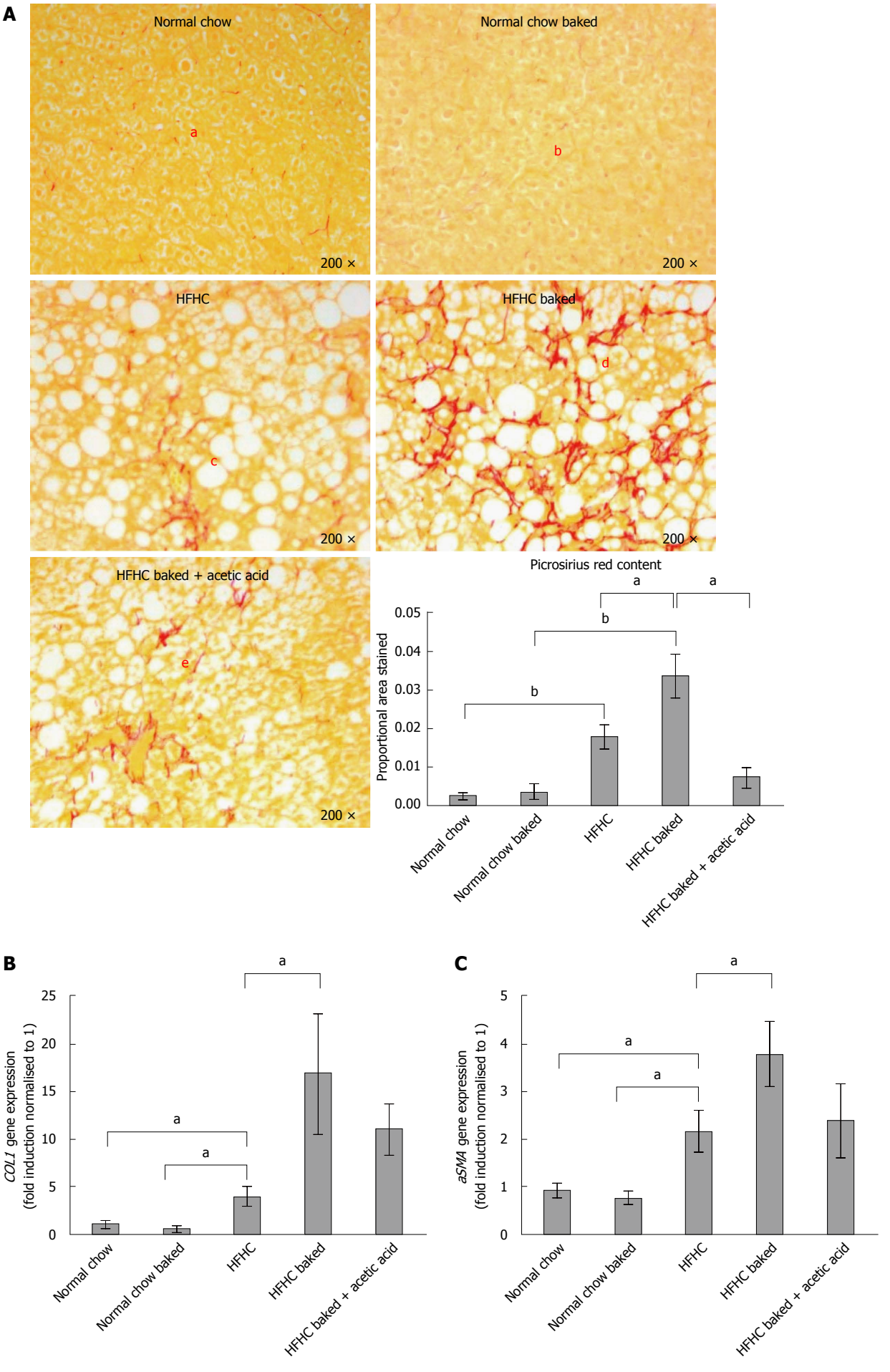


Figure 1 Novel 33 wk high fructose, high cholesterol dietary model induced changes of non-alcoholic steatohepatitis. A: Haematoxylin and eosin stained sections showing ballooning after high fructose, high cholesterol (HFHC) diets (examples of ballooning circled). At right, ballooning scores determined morphometrically; B: Oxidative stress revealed by the immunohistochemical localisation of 4-hydroxynonenal; C: Steatosis grade (grade 0, no fat; grade 1, steatosis occupying less than 33% of the hepatic parenchyma; grade 2, 34%-66% of the hepatic parenchyma; grade 3, more than 66% of the hepatic parenchyma); D: Plasma levels of alanine transaminase (ALT) in the different treatment groups; and E: Lobular inflammation (grade 0:none; grade 1, 1-2 foci/field; grade 2, 3-4 foci/field; grade 3, more than 4 foci/field). Ballooning and oxidative stress were significantly increased by raising the AGE content of the HFHC diet. These changes were attenuated when dietary AGE content was reduced by acetic acid premarination. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$.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Picrosirius red content
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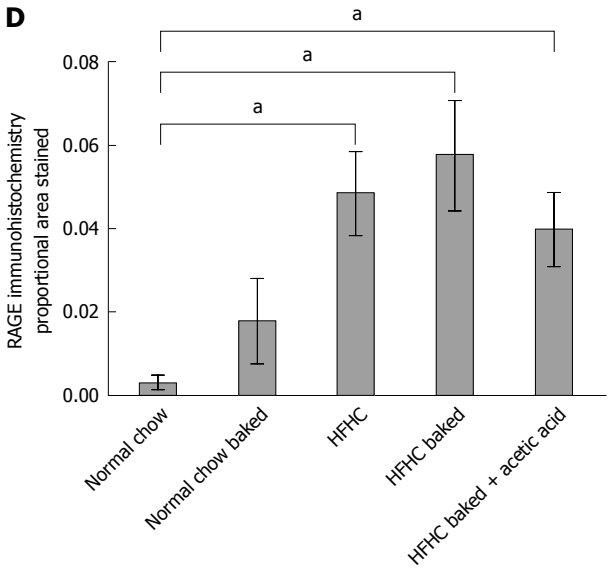


Figure 2 Elevation in profibrotic and proinflammatory cytokine expression in the liver. A: Picrosirius red staining of collagen showing centrilobular sinusoidal fibrosis induced by the 33 wk high fructose, high cholesterol (HFHC) dietary model (c). In animals receiving the HFHC diet, fibrosis was further increased by raising the advanced glycation end products (AGEs) content of the diet (d) and attenuated when dietary AGE content was reduced by acetic acid prearination prior to baking (e). However increasing the AGE content of normal chow did not cause increased fibrosis (compare a, b). Hepatic gene expression of COL1A (B) and αSMA (C) were also significantly increased by raising the AGE content of the diet. Expression of RAGE, a key receptor for AGEs, was elevated in the HFHC model but this was not affected by varying the AGE content of the diet, $^{a}P < 0.05$, $^{b}P < 0.01$ (D).

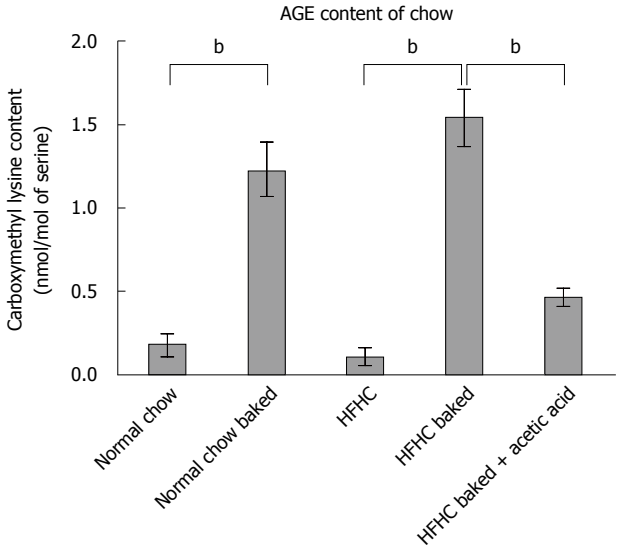


Figure 3 Levels of carboxymethyl lysine in the different diets. Baking the high fructose, high cholesterol (HFHC) diet and normal chow increased CML content of feed by > 6 fold. Levels of CML were markedly reduced by premarinating with vinegar prior to baking, $^{b}P < 0.01$.

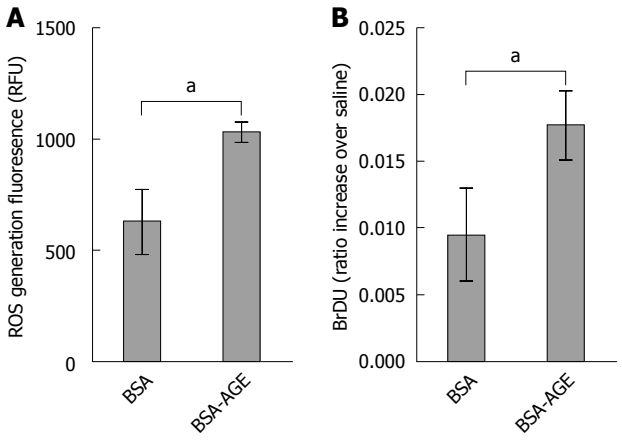


Figure 4 Effects of advanced glycation end products on reactive oxygen species and proliferation in isolated Kupffer cells exposed to physiological amounts of dietary advanced glycation end products compared with bovine serum albumin vehicle. A: Advanced glycation end products (AGEs) increased ROS generation as measured by 2',7'-dichlorodihydrofluorescein diacetate. B: AGEs increased cell proliferation as assessed by BrDU incorporation, $^{a}P \leqslant 0.05$.

increased ($P < 0.05$) with high oral intake of AGEs (Figure 2C).

The role of AGEs in mediating increased liver fibrosis was further supported by the finding that picrosirius staining, COL 1A gene expression and hepatic α-SMA gene expression were reduced to those observed in animals fed the HFHC diet alone, with vinegar pre-marination to reduce AGE content during baking (Figure 2B and C).

Pathological effects of dietary AGEs are RAGE dependent

In diabetes, many of the harmful effects of AGEs are thought to be mediated *via* AGE/RAGE signalling[35]. RAGE expression, as measured by immunohistochemistry[19], was minimal in healthy liver from animals fed normal chow however RAGE expression was increased in all HFHC groups compared to animals fed standard chow (Figure 2D).

Given the putative role of RAGE in mediating the

harmful effects of AGEs and the finding that its expression was increased in our dietary model, we performed a further study in which we examined whether RAGE deletion abrogated liver injury and the harmful effect of AGEs in the HFHC model. As in the initial study, in this experiment, HFHC diet induced liver fibrosis and 4HNE staining were increased by raising dietary AGE content and the area of fibrosis was expanded to include the periportal region (Figure 5A-C).

RAGE KO animals fed the HFHC diet had similar levels of fibrosis to wild type animals (5A). However RAGE KO animals appeared to be protected from the increased liver fibrosis induced by AGEs. Compared to wild type animals fed the high AGE/HFHC diet, there was less picrosirius staining and COL 1A gene expression in the RAGE deleted animals (Figure 5A-B). The increased oxidative stress in animals fed a HFHC baked diet was also abrogated significantly in corresponding RAGE KO animals (Figure 5C). However, HNE levels did not return back to normal chow levels. These findings suggest that AGEs act *via* RAGE to increase liver fibrosis in this model of experimental NASH.

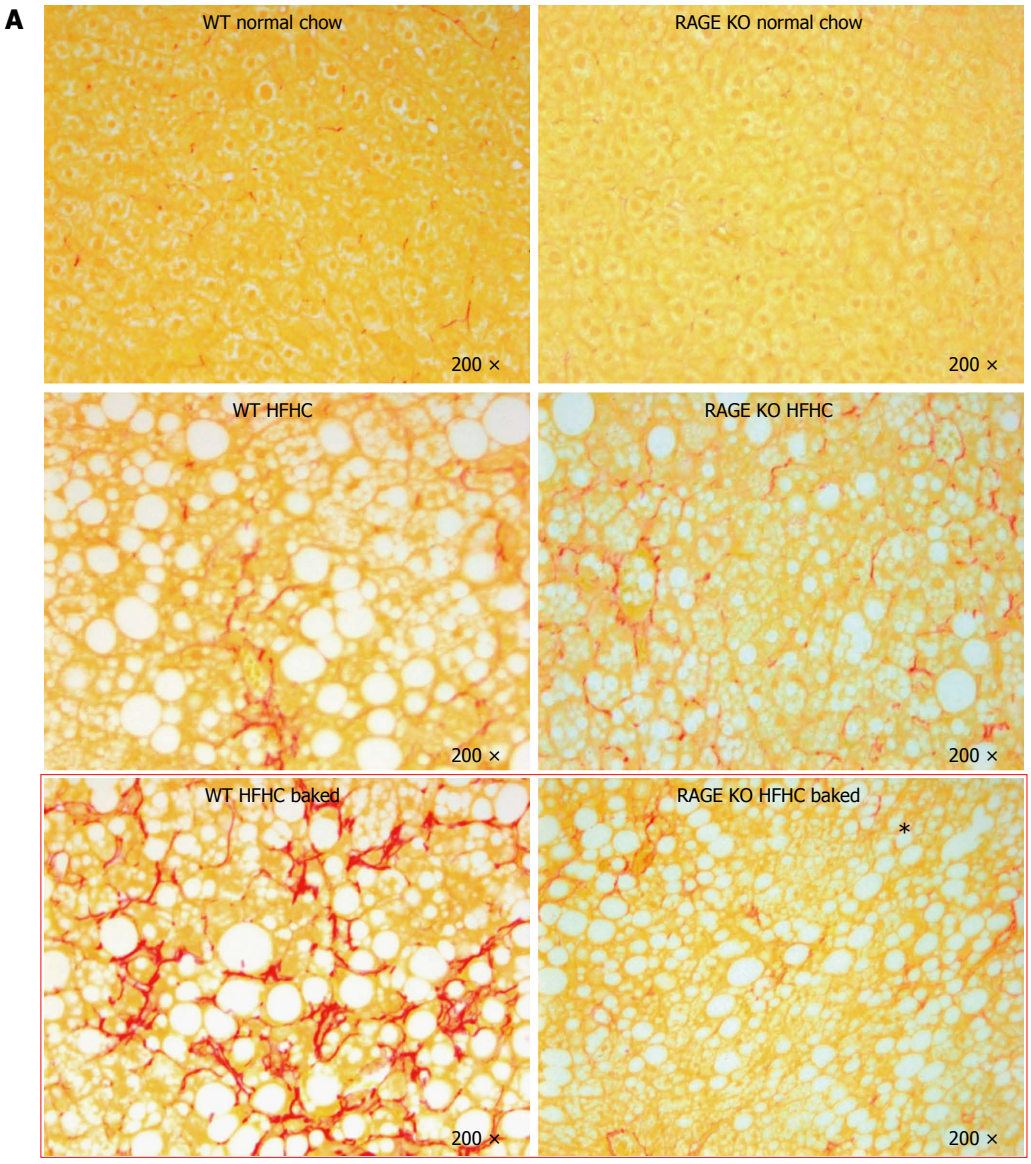
DISCUSSION

Our experiments show that AGEs may be important modifiable dietary cofactors which contribute to the development of liver fibrosis in NAFLD. The current findings are in keeping with previous studies which showed that AGEs administered intraperitoneally or in the diet exacerbate liver injury and fibrosis[12,19]. However these previous experiments were conducted in short term models of liver disease of limited relevance to human NAFLD[36]. For the present studies, therefore, a novel model of NAFLD was developed using physiological amounts of fat, cholesterol and fructose that occur in human diets and it reliably produced slowly progressive steatosis, ballooning, oxidative stress and predominantly pericentral sinusoidal fibrosis with a tempo of disease consistent with the long progressive natural history of human NASH. In this study, all of these key features of high fat diet induced NASH were exacerbated by increasing dietary AGE content

The two-hit hypothesis of NASH pathogenesis suggests that a second injury or cofactor is required for progression from simple benign steatosis to harmful steatohepatitis or fibrosis, cirrhosis and hepatocellular carcinoma[37]. There has been considerable interest in factors which could serve as this "second hit". In line with our previous study in MCD animals[19], we found that a high AGE containing diet has no effect on liver biochemistry or histology in animals without hepatic steatosis. However, we showed they act as a co-factor to increase injury in diseased livers.

AGEs have been shown to exert their effects through several receptors, the best studied of which is RAGE. Activation of RAGE stimulates multiple signal transduction pathways[38], ultimately leading to the generation of ROS[39]. This culminates in the activation of NF-κB, a redox sensitive transcription factor, which in turn translocates into the nucleus[40]. The promoter region of the RAGE gene contains an NF-κB binding site and therefore, one of the important consequences of NF-κB activation and translocation is upregulation of RAGE itself. This sets up a positive feedback loop and ensures maintenance of RAGE signalling. Furthermore, the generation of ROS triggered by RAGE activation causes increased AGE formation and contributes to a vicious cycle of AGE formation, generation of oxidative stress and further RAGE activation[7]. Our study showed that RAGE expression was minimal in healthy livers but upregulated in a non-diabetic model of fatty liver disease. These differences in RAGE expression may explain why the high AGE diet had no harmful effects in healthy controls but exacerbated liver injury in animals with NAFLD. However, since the dietary AGE content of normal mouse chow is so high it is also feasible that the dietary AGE content of normal mouse chow is so high that mice become preconditioned against the effects of further increases. In keeping with these findings, although RAGE KO animals developed NASH in response to the HFHC diet they were protected from the additional harmful effects of the high AGE diet.

Oxidative stress and the accumulation of superoxide is a key mediator of ballooning, cellular dysfunction and fibrosis in non-alcoholic steatohepatitis[7,41]. HNE, an end-product of peroxidation of membrane N-6-polyunsaturated fatty acids, is a particularly good marker of lipid oxidation during liver injury and is related to the intensity of necroinflammation[42]. Given the long- term nature of our model, assessment of oxidative stress in the liver was therefore performed by measuring HNE adducts which reflect accumulation of oxidative stress over time. This showed that HNE content was significantly elevated in all the HFHC groups (Figure 1C). Consistent with the known effects of AGEs, we found that a diet high in AGEs significantly increased oxidative stress in HFHC induced NASH. This was associated with increased ballooning, stellate cell activation as assessed by aSMA expression and increased fibrosis. These findings are consistent with our previous work which showed that in activated primary murine hepatic stellate cells which express RAGE, ROS production, cell activation and proliferation were markedly increased in the presence of AGEs. These effects were inhibited by RAGE blockade or NADPH oxidase inhibition. However, Kupffer cells (KCS) express RAGE[43] and uptake of AGEs in the liver occurs primarily *via* these cells[11]. It is known that KCs play a key role in promoting hepatic steatosis, steatohepatitis and ballooning in NASH[44]; and generation of ROS in KCs rather than by HSCs has been associated with NAFLD disease progression[45]. Given the important role of KCs in NAFLD progression and their key role in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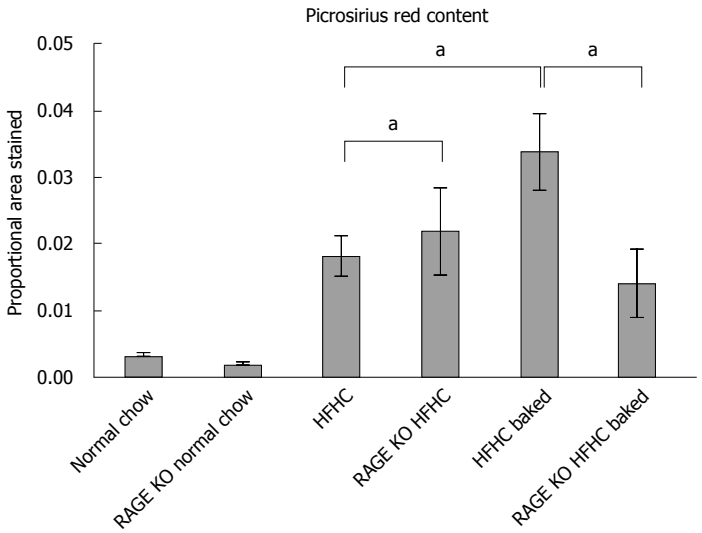
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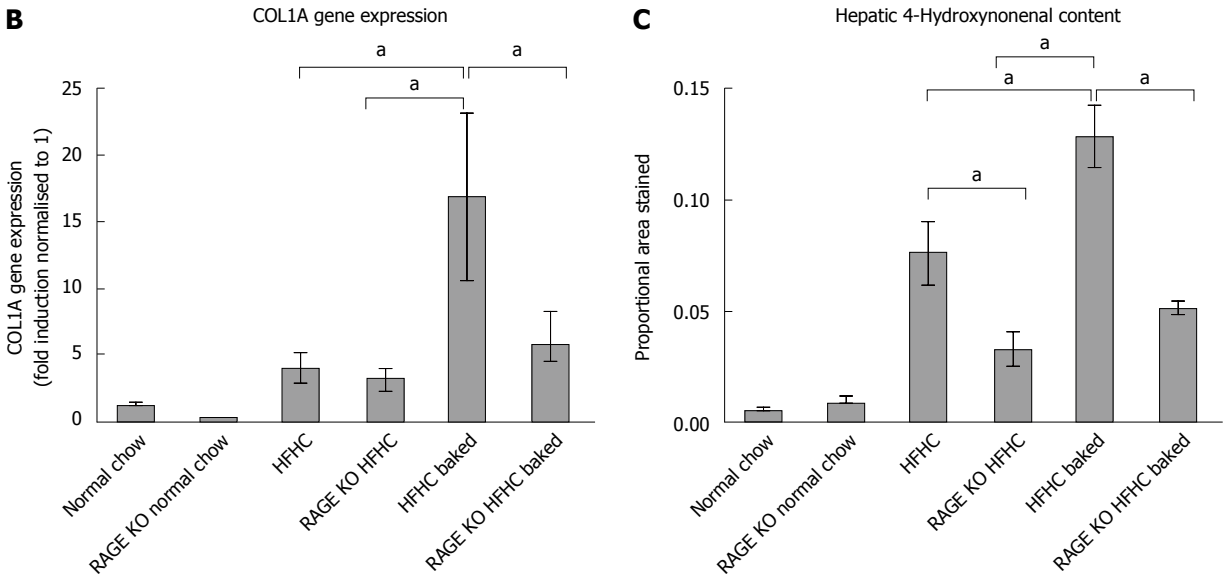

Figure 5 Effects of the high fructose, high cholesterol diet and dietary advanced glycation end products manipulation on fibrosis in the receptor for advanced glycation end products KO animals. A: The absence of receptor for advanced glycation end (RAGE) attenuated the effects of increasing the advanced glycation end products (AGEs) content of the high fructose, high cholesterol (HFHC) diet as evidenced by a reduction in picrosirius red staining to levels observed in animals on the HFHC diet alone; B: There was a commensurate reduction in COL1A gene expression; C: A reduction in hepatic 4-hydroxynonenal content ($^{a}P < 0.05$).

scavenging AGEs[46], we explored the effect of AGEs on primary KCs and showed that AGEs increase cell proliferation and the generation of ROS by these cells.

As expected, in our HFHC model there was marked upregulation of a number of key proinflammatory and fibrogenic cytokines. It is unclear why, despite showing that AGEs increase oxidative stress, hepatocyte ballooning and fibrosis, we did not find they increased inflammatory infiltration of the liver or in inflammatory cytokines expression. In keeping with this finding, in a very similar dietary model of NASH, Lo *et al*[47] found that although diabetes worsened steatosis induced liver fibrosis this was not associated with measurable increases in liver inflammatory infiltration or proinflammatory cytokine levels. These finding suggest that profibrotic effects of AGEs and diabetes may not be mediated primarily through increasing hepatic inflammation.

The formation of AGEs in foods involves the condensation of an amino group with the carbonyl group of a reducing carbohydrate (glucose, fructose, maltose, lactose or ribulose) to form intermediate Amadori products. Oxidation of Amadori products leads to a more stable compound, CML, used in studies as an indicator of the AGE content of foods[4]. A common method of high dietary AGE formation is *via* heating at high temperatures in non-aqueous environments (*e.g.,* baking or broiling)[4]. High levels of AGEs are thus found in many common foods such as baked breads and biscuits/cookies, toasted breakfast cereals, grilled steak, brewed beer, and roasted coffee beans. For example, toasting white bread increases its AGE content by more than 3 times[48]. Interestingly, acidifying such foods prior to cooking by marinating them in acetic acid (vinegar) or lemon juice, reduces their CML content substantially without compromising palatability[49]. In this study, we have found reducing AGEs production by vinegar marination prior to baking can abrogate the harmful effects of a high AGE diet in an animal model of NAFLD. This may be one mechanism by which the Mediterranean diet which includes the use of vinegar and lemon juice marination is beneficial for metabolic health and in NAFLD[50].

It has been shown that patients with NASH have higher levels of circulating AGEs than those with simple steatosis[46]. However whether this reflects an increased dietary exposure to AGEs or greater endogenous AGE production in patients who have both fatty liver and glucose intolerance is unclear. Studies examining the relationship between dietary exposure to AGEs and the histological severity of liver injury in non-diabetic patients will help clarify this issue.

In conclusion, we show that high dietary AGE exposure worsens liver pathology in experimental NASH, implicating AGE/RAGE signalling in fatty liver disease progression. Decreasing dietary AGEs by altering food selection and cooking methods may thus offset the possible harmful effects afforded by a high AGE diet. If confirmed in human studies, our findings have broad implications for the way we process foods and the dietary advice given to patients with NAFLD. They also suggest a possible role for therapies targeting the AGE/RAGE pathway in the treatment and prevention of NASH.

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COMMENTS

Background

There is considerable interest in identifying the factors which drive progression of uncomplicated non-alcoholic fatty liver disease (NAFLD) to non-alcoholic steatohepatitis (NASH), cirrhosis and liver cancer. Advanced glycation end products (AGEs) are a complex group of compounds formed in foods that are highly processed or dry heated at high temperatures which have been implicated in the pathogenesis of diabetic complications. Our previous work in a methionine choline deficient model of NASH has shown that AGEs may be an important environmental risk factor that serves as a second hit driving simple steatosis to steatohepatitis and cirrhosis. The aims of the current study were to determine whether AGEs drive NAFLD progression in a long term high fat, high cholesterol, high fructose dietary model of NASH that mimics many of the features of human NASH. We also aimed to examine the role of the receptor for AGEs (RAGE) and Kupffer cells in this process.

Research frontiers

There is major interest in identifying so-called second hits which drive liver inflammation and fibrosis in NAFLD. AGEs have been implicated in progression of a number of disease processes, including diabetic nephropathy, retinopathy, vasculopathy and neuropathy. This study investigates whether AGEs could represent an important potential dietary and pharmacological target in the management of the burgeoning problem of fatty liver disease.

Innovations and breakthroughs

This is the first study which examines the impact of altering dietary AGE content in a long term physiological model of NASH that utilises proportions of fats and fructose found typically in Western diets. We have found that changes in AGE content influence liver inflammation and fibrosis. This is also the first study to show that in RAGE knock out animals, these deleterious effects of AGEs are abrogated, suggesting a RAGE dependent pathway. Studies by Leung *et al* in isolated primary Kupffer cells also show AGEs increase cell proliferation and oxidative stress, suggesting a likely mechanism by which these compounds contribute to liver injury.

Applications

Food sourcing and preparation methods that reduce AGE content could influence the progression of NAFLD. This study also suggests that pharmacological therapies which target the AGE/RAGE pathway may have a role in treatment of NAFLD.

Terminology

AGEs, also known as glycotoxins, are a complex group of compounds that are formed when active sugar moieties become bound to proteins causing browning and other irreversible modifications. Foods that are highly processed or dry heated at high temperatures, such as broiled foods, have particularly high AGE content. However, they are also formed endogenously and this occurs at an increased rate in diabetes, most likely because of hyperglycaemia. There is now considerable evidence that accumulation of AGEs are implicated in the pathogenesis of diabetic renal, neurological, retinal and vascular complications. AGEs act on several receptors but the receptor for advanced glycation end products (RAGE), a member of the immunoglobulin superfamily of cell-surface molecules, is the best characterised of these receptors. Engagement with RAGE, which in turn increases inflammation and oxidative stress, is thought to be the main way in which AGEs impart these pathogenic effects. This receptor is expressed in a number of cell types, including endothelial cells, vascular smooth muscle cells, peripheral blood mononuclear cells, macrophages (including Kupffer cells) and HSCs.

Peer-review

A well designed and organized study.

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ORIGINAL ARTICLE

Basic Study

Different pre-S deletion patterns and their association with hepatitis B virus genotypes

Bing-Fang Chen

Bing-Fang Chen, School of Medicine, Fu Jen Catholic University, New Taipei City 24205, Taiwan

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Correspondence to: Bing-Fang Chen, PhD, Professor of School of Medicine, Fu Jen Catholic University, 510 Chung-Cheng Road, Hsin-Chuang Dist, New Taipei City 24205, Taiwan. nurs1018@mail.fju.edu.tw
Telephone: + 886-2-29053428
Fax: +886-2-29052096

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Abstract

AIM

To investigate the associations of different types of pre-S deletions with hepatitis B virus (HBV) genotypes.

METHODS

The sequences of the pre-S region, basal core promoter (BCP) mutation, and precore (PC) mutation were examined through direct DNA sequencing or clonal analysis and sequencing in 273 HBV carriers, namely 55 asymptomatic carriers, 55 carriers with chronic hepatitis (CH), 55 with liver cirrhosis (LC), 53 with liver cirrhotic hepatocellular carcinoma (LC-HCC), and 55 with noncirrhotic HCC. A total of 126 HBV carriers (46.2%) harbored pre-S deletions. The DNA sequences of pre-S deletion mutants from 43 age-matched genotype B (HBV/B)-infected carriers and 43 age-matched genotype C (HBV/C)-infected carriers were further examined, aligned, and compared.

RESULTS

No significant difference was observed in the mean age distribution (P = 0.464), male sex (P = 0.805), viral load (P = 0.635), or BCP mutation (P = 0.117) between the HBV/B and HBV/C groups. However, the rate of PC mutation was significantly higher in the HBV/B-infected carriers than in the HBV/C-infected carriers (P = 0.003). Both genotypes exhibited a high rate of deletion in the C-terminal half of the pre-S1 region and N-terminus of the pre-S2 region (86.0% and 79.1% in the HBV/B group; 69.8% and 72.1% in the HBV/C group, respectively). Epitope mapping showed that deletion in several epitope sites was frequent in both genotypes, particularly pS1-BT and pS2-B2. Conversely, the rate of pS2-B1 deletion was significantly higher

in the HBV/B group (72.1% *vs* 37.2%, *P* = 0.002), and the rate of pS2-T deletion was significantly higher in the HBV/C group (48.8% *vs* 25.6%, *P* = 0.044). Functional mapping showed that the rate of deletion in three functional sites (the nucleocapsid binding site, start codon of M, and site for viral secretion) located in the N-terminus of the pre-S2 region was significantly higher in the HBV/B group (*P* < 0.05). One type of N-terminus pre-S1 deletion mutant with deletion of the start codon of the L protein was frequently observed in the HBV/C group (20.9% *vs* 9.3%, *P* = 0.228), particularly in the LC patients (42.9% *vs* 12.5%). Different patterns of pre-S deletions were also found between the HBV/B and HBV/C groups according to different clinical outcomes. In CH patients, deletion in the site for polymerized human serum albumin was more frequent in the HBV/B group (88.9% *vs* 36.4%, *P* = 0.028). In the LC-HCC patients, the rate of deletion in the pre-S2 region was significantly higher in the HBV/B group than in the HBV/C group (*P* < 0.05).

CONCLUSION

HBV/B- and HBV/C-infected carriers exhibit different patterns of pre-S deletion, which may be associated with the progression of liver diseases.

Key words: Hepatitis B virus; Pre-S deletion; Chronic hepatitis; Hepatocellular carcinoma; Genotype; Liver cirrhosis

Core tip: This is a comprehensive study of the influence of HBV genotypes B (HBV/B) and C (HBV/C) on the emergence of different types of pre-S deletions. Different patterns of pre-S deletion were found in HBV/B and HBV/C. Deletion in the pS2-B1 epitope, nucleocapsid binding site, start codon of M, and site for viral secretion was frequently found in HBV/B. Conversely, deletion in pS2-T and the start codon of L was frequently found in HBV/C. The prevalence of different pre-S deletions differed between HBV/B and HBV/C among patients with liver disease.

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INTRODUCTION

Hepatitis B virus (HBV) is a small, enveloped DNA virus that causes acute and chronic diseases. Most acute infections are self-limiting, whereas chronic infection may lead to the development of chronic hepatitis (CH), liver cirrhosis (LC), and hepatocellular carcinoma (HCC)[1,2]. To date, research has identified 10 HBV genotypes, designated A to J on the basis of a divergence of > 8% over the entire genomic sequence. These 10 HBV genotypes are distributed in specific geographical locations[3,4]. All genotypes can lead to progressive liver disease, but the clinical implications of each genotype differ. For example, patients infected by the genotype C (HBV/C) or D (HBV/D) strain have a higher frequency of basal core promoter (BCP) mutations, a lower response rate to interferon therapy, and more rapid progression to liver fibrosis and HCC than those infected by the genotype B (HBV/B) or A (HBV/A) strain[3,4]. In addition, carriers infected by HBV/C have a higher rate of pre-S deletions than those infected by HBV/B[5,6]. Collectively, these data suggest pathogenic and therapeutic differences among the HBV genotypes[3,4].

Three different yet structurally related HBV viral surface proteins are translated from a single open reading frame, as follows: large (L), middle (M), and small (S) proteins. The S protein consists of 226 amino acids (aa). The M protein is an extension of the S protein, with an additional 55 aa (*i.e.*, pre-S2 region). The L protein is an extension of the M protein, with an additional 108-119 aa depending on the genotype (*i.e.*, pre-S1 region). The pre-S (pre-S1 and pre-S2) region has several functional domains and plays an essential role in the viral life cycle[7-13]. The pre-S1 region can be divided into two parts: N-terminal half (aa 1-57) and C-terminal half (aa 58-119). The N-terminal half of the pre-S1 region contains the hepatocyte binding site essential for the attachment of HBV to liver cells. The C-terminal half of the pre-S1 region contains several functional sites: (1) the S promoter and the CCAAT binding factor binding site necessary for S RNA transcription; (2) the heat shock protein 70 (Hsc70) binding site and the cytosolic anchorage determinant (CAD) essential for the dual topology of L proteins; and (3) the nucleocapsid binding site (NBS) required for virion morphogenesis. The pre-S2 region contains sites for nucleocapsid binding, polymerized human serum albumin (pHSA) binding, and viral secretion (VS). The HBV pre-S region also plays an essential role in immune responses, because pre-S region carries both B-cell and T-cell epitopes[13-18]. Many studies have suggested that HBV pre-S deletions are associated with the development of progressive liver diseases[5,6,19-23]. Some in vitro studies have shown that pre-S deletion mutants can cause the accumulation of L surface proteins in the endoplasmic reticulum (ER), resulting in ER stress[22-26]. Other related studies have suggested that ER stress results in the generation of large amounts of reactive oxygen species, which can cause oxidative DNA damage, inducing mutagenesis in the genome and ultimately resulting in HCC[27-29].

Current knowledge concerning these pre-S deletion mutants is focused on the frequencies of pre-S deletion and deletion patterns in the pre-S region according to

the clinical status[5,6,19-21]. Because the pre-S region has the highest genetic variability in the whole genome, HBV genotypes may influence deletions in the pre-S region. Knowledge concerning the prevalence of different types of pre-S deletions in different HBV genotypes is limited. Therefore, this study elucidated the prevalence of different types of pre-S deletions and their associations with HBV genotypes and examined the correlation of different types of pre-S deletion with HBV genotypes according to different clinical outcomes.

MATERIALS AND METHODS

Patients

This study included 273 patients with chronic HBV infection receiving long-term follow-up at the Gastroenterology Clinic of National Taiwan University Hospital. The study population comprised 55 asymptomatic HBV carriers with a normal serum alanine aminotransferase level for at least 3 years according to periodic biochemical examinations (every 3 or 6 mo) and 218 HBsAg-positive patients with histologically verified chronic liver disease. Among the HBsAg-positive patients, 55 had CH with active viral replication, 55 had LC without HCC, 53 had liver cirrhotic HCC (LC-HCC), and 55 had noncirrhotic HCC (NC-HCC). None of them were coinfected with hepatitis C virus or hepatitis D virus. Other causes of hepatitis, including autoimmune hepatitis and alcoholic liver diseases, were excluded clinically and serologically. The serum samples of each patient were stored at -70 ℃ until use.

Hepatitis virus serologic markers

Serum HBsAg was tested using a commercial assay (Ausria-Ⅱ, Abbott Laboratories, North Chicago, IL, United States).

Extraction of serum HBV DNA and quantification of HBV DNA

Serum viral DNA was extracted using a commercial kit (QIAamp DNA Blood Mini Kit, Qiagen Inc., Valencia, CA, United States). The extracted DNA was amplified for quantifying and genotyping HBV DNA and for direct sequencing of pre-S, BCP, and precore (PC) regions, as previously described[30].

HBV DNA was quantified and genotyped as previously described[31]. The sensitivity of this real-time polymerase chain reaction (PCR) method was 10^2 copies/mL.

Determination of PC nucleotide 1896 and BCP dinucleotide 1762/1764

The segments of PC/BCP DNAs (263 bp, nucleotide positions: 1704-1966) were amplified through nested PCR and sequenced as previously described[30].

Amplification, sequencing, and cloning of HBV pre-S gene

We performed direct sequencing and clonal analysis of the pre-S region as previously described[29]. Briefly, pre-S DNA was amplified using nested PCR with two sets of HBV genotype B and genotype C-copositive primers. To avoid false-positive PCR results, precautions were strictly followed. The PCR products were electrophoresed on 2.5% agarose gel to investigate the presence of pre-S deletions. All PCR products were also directly sequenced to identify any sequence diversity or deletion.

The pre-S segments that could not be sequenced directly or those that had various small amplicons in the presence or absence of full-size amplicons (564 bp) were further investigated by clonal analysis and sequencing, as previously described[30].

Alignment

Sequence alignment was performed using the Biology WorkBench 3.2-CLUSTALW software program (http://workbench.sdsc.edu).

Ethical considerations

This study was performed in accordance with the principles of the 1975 Declaration of Helsinki and was approved by the Ethics Committees of the National Taiwan University Hospital and Fu Jen Catholic University. The sera were sampled after obtaining their written informed consent from each patient.

Statistical analysis

Data were analyzed using the Fisher exact test, χ^2 test, or contingency table with Yates' correction when appropriate. A two-sided P value of < 0.05 was considered statistically significant. All statistical analyses were performed using SPSS 12.0 for Windows (SPSS, Chicago, IL, United States).

RESULTS

Baseline characteristics of the study population

A total of 126 HBV carriers (46.2%) harbored pre-S deletions. Table 1 compares the demographic, clinical, and virological characteristics between carriers with and without pre-S deletion. No significant difference was observed in the mean age distribution ($P = 0.54$), male sex ($P = 0.054$), or PC mutation ($P = 0.132$) between them. Carriers with pre-S deletion were more frequently infected with the HBV/C strain (47.6% *vs* 28.6%, $P = 0.003$), had a higher viral load (73.0% *vs* 45.7%, $P < 0.001$), had a higher occurrence of the BCP mutation (77.8% *vs* 54.4%, $P < 0.001$), and had more progressive liver disease (CH, LC, and HCC) (96.8% *vs* 65.3 %, $P < 0.001$), particularly LC patients (28.6% *vs* 12.9%, $P = 0.001$), than those without

Table 1 Characteristics of the study population classified according to the presence of pre-S deletion *n* (%)

Features	wild type pre-S (*n* = 147)	Pre-S deletion (*n* = 126)	*P* value
Age (mean ± STD)	45.4 ± 6.6	45.9 ± 7.0	0.540
Male	101 (68.7)	100 (79.4)	0.054
Genotype C	42 (28.6)	60 (47.6)	0.003
Genotype B	94 (63.9)	62 (49.2)	
Mixed genotypes	11 (7.5)	4 (3.2)	
HBV DNA ≥ 10^5 copies/mL	67 (45.7)	92 (73.0)	< 0.001
BCP mutation (A1762T, G1764A)	80 (54.4)	98 (77.8)	< 0.001
PC mutation (G1896A)	87 (59.2)	86 (68.3)	0.132
Clinical categories			
ASC	51 (34.7)	4 (3.2)	
Progressive liver diseases	96 (65.3)	122 (96.8)	< 0.001
CH	24 (16.3)	31 (24.6)	0.098
LC	19 (12.9)	36 (28.6)	0.001
NC-HCC	29 (19.7)	24 (19.0)	1.000
LC-HCC	24 (16.3)	31 (24.6)	0.098

ASC: Asymptomatic carriers; CH: Chronic hepatitis; HBV: Hepatitis B virus; LC: Liver cirrhosis; LC-HCC: Liver cirrhotic hepatocellular carcinoma; NC-HCC: Noncirrhotic hepatocellular carcinoma; BCP: Basal core promoter; PC: Precore.

Table 2 Clinical and virological characteristics of hepatitis B virus carriers with pre-S deletion *n* (%)

Features	HBV/B (*n* = 43)	HBV/C (*n* = 43)	*P* value
Age (mean ± STD)	44.5 ± 8.8	45.8 ± 7.6	0.464
Male	33 (76.7)	31 (72.1)	0.805
HBV DNA ≥ 10^5 copies/mL	29 (67.4)	32 (74.4)	0.635
BCP mutation (A1762T, G1764A)	30 (69.8)	37 (86.0)	0.117
PC mutation (G1896A)	38 (88.4)	25 (58.1)	0.003
Clinical categories			
ASC	2 (4.7)	2 (4.7)	1.000
Progressive liver diseases	41 (95.3)	41 (95.3)	
CH	9 (20.9)	11 (25.6)	0.799
LC	8 (18.6)	14 (32.6)	0.216
NC-HCC	14 (32.6)	4 (9.3)	0.015
LC-HCC	10 (23.3)	12 (27.9)	0.805

ASC: Asymptomatic carriers; CH: Chronic hepatitis; HBV: Hepatitis B virus; LC: Liver cirrhosis; LC-HCC: Liver cirrhotic hepatocellular carcinoma; NC-HCC: Noncirrhotic hepatocellular carcinoma; BCP: Basal core promoter; PC: Precore.

pre-S deletion.

To examine the role of viral factors and to exclude the influence of HBV infection duration on the occurrence of pre-S deletion, 43 age-matched HBV/B and 43 age-matched HBV/C infected carriers were selected to examine the associations of different types of pre-S deletion with HBV genotypes. Table 2 presents the clinical and virological characteristics of 86 HBV carriers with pre-S deletion classified according to genotype. No significant difference was observed in the mean age distribution (P = 0.464), male sex (P = 0.805), viral load (P = 0.635), or BCP mutation (P = 0.117) between the HBV/B and HBV/C groups. However, the HBV/B group had a higher rate of PC mutation (88.4% *vs* 58.1%, P = 0.003) and NC-HCC (32.6% *vs* 9.3%, P = 0.003).

Table 3 Frequencies of different types of pre-S deletions in HBV/B- and HBV/C-infected carriers *n* (%)

Deletion	HBV/B (*n* = 43)	HBV/C (*n* = 43)	*P* value
N-terminal half of pre-S1 (aa 1-57)	17 (39.5)	17 (39.5)	1.000
C-terminal half of pre-S1 (aa 58-119)	37 (86.0)	30 (69.8)	0.117
N-terminus of pre-S2 (aa 1-31)	34 (79.1)	31 (72.1)	0.616
d183 M	10 (23.3)	9 (20.9)	1.000
Epitope Mapping			
Pre-S1 region			
pS1-T1 (aa 12-48 of pre-S1)	15 (34.9)	9 (20.9)	0.229
pS1-B1 (aa 12-53 of pre-S1)	15 (34.9)	9 (20.9)	0.229
pS1-B2 (aa 72-78 of pre-S1)	18 (41.9)	22 (51.2)	0.517
pS1-BT (aa 94-117 of pre-S1)	31 (72.1)	22 (51.2)	0.075
Pre-S2 region			
pS2-B1 (aa 1-6 of pre-S2)	31 (72.1)	16 (37.2)	0.002
pS2-B2 (aa 3-15 of pre-S2)	33 (76.7)	27 (62.8)	0.240
pS2-B3 (aa 13-24 of pre-S2)	19 (44.2)	24 (55.8)	0.388
pS2-T1 (aa 21-48 of pre-S2)	11 (25.6)	21 (48.8)	0.044
Functional Mapping			
Pre-S1 region (aa 1-119)			
The start codon of L	4 (9.3)	9 (20.9)	0.228
HBS (aa 2-48)	16 (37.2)	15 (34.9)	1.000
S promoter (nt 3045-3189)	31 (72.1)	30 (69.8)	1.000
CBF (nt 3137-3147)	23 (53.5)	15 (34.9)	0.128
HSC70 (aa 74-118)	31 (72.1)	29 (67.4)	0.815
CAD (aa 81-105)	30 (69.8)	23 (53.5)	0.379
Pre-S1/S2 region (aa1-174)			
NBS (aa 103-127)	39 (90.7)	27 (62.8)	0.004
Pre-S2 region (aa1-55)			
The start codon of M[1]	30 (69.8)	18 (41.9)	0.016
VS (aa 1-5 of pre-S2)	32 (74.4)	16 (37.2)	0.001
pHSA (aa 3-16 of pre-S2)	34 (79.1)	25 (58.1)	0.062

[1]Deletion or mutation of the start codon of the M protein. HBS: Hepatocyte binding site; CBF: CCAAT binding factor; HSC70: Heat shock protein 70; CAD: Cytosolic anchorage determinant; NBS: Nucleocapsid binding site; VS: Viral secretion; pHSA: Polymerized human serum albumin.

Association of different types of pre-S deletion with HBV genotypes

To investigate whether particular types of deletion in the pre-S region are associated with the HBV genotype, pre-S deletion subgenomes from 43 HBV/B and 43 HBV/C infected carriers were sequenced and aligned. The locations of these pre-S deletions are depicted in a supplementary file. All deletions were in-frame deletions with sizes ranging from 3 to 507 bp, and the deletions ended at aa 143 of the pre-S region, except for two deletions that ended at aa 150 and aa 171. Sequence alignments of pre-S deletion mutants indicated that deletion in the C-terminal half of the pre-S1 and N-terminus of pre-S2 region was more frequent in the HBV/B group (86.0% and 79.1%) than in the HBV/C group (69.8% and 72.1%), although the difference was nonsignificant (Table 3). One pre-S1

deletion mutant, d183M, which had a 183-nt in-frame deletion in the C-terminal half of the pre-S1 region, was observed in 10 of the 43 (23.3%) HBV/B-infected carriers and 9 of the 43 (20.9%) HBV/C-infected carriers (Table 3).

Epitope mapping revealed frequent deletion in two epitopes (pS1-BT and pS2-B2) in both genotypes. Conversely, deletion in pS2-B1 was significantly more frequent in the HBV/B group (72.1% *vs* 37.2%, P = 0.002), and deletion in pS2-T1 was significantly more frequent in the HBV/C group (48.8% *vs* 25.6%, P = 0.044) (Table 3).

Functional mapping revealed frequent deletion in five functional domains (S promoter, HSC70, NBS, VS, and pHSA) in the HBV/B group (rate > 70%). The rate of deletion in NBS (90.7% *vs* 62.8%, P = 0.004), the start codon of M (69.8.2% *vs* 41.9%, P = 0.016), and VS (74.4% *vs* 37.2%, P = 0.001) was significantly higher in the HBV/B group than in the HBV/C group (Table 3). Similar to HBV/B, deletion in the S-promoter, HSC70, and NBS domains was frequently observed in the HBV/C group (rate > 60%) (Table 3). Only one type of N-terminus pre-S1 deletion mutant with deletion in the start codon of the L protein was more frequent in the HBV/C group than in the HBV/B group (20.9% *vs* 9.3%, P = 0.228) (Table 3).

Correlation of different types of pre-S deletion mutant with HBV genotypes according to different clinical outcomes

The frequencies of different types of pre-S deletion mutant were compared between the HBV/B and HBV/C groups according to different clinical outcomes. The results indicated that the patterns of pre-S deletion were considerably different in the LC-HCC patients. HBV/B-infected patients had significantly higher rates of deletion in N-terminus of pre-S2 (100% *vs* 58.3%, P = 0.04), pS2-B1 (100% *vs* 25%, P < 0.001), pS2-B2 (100% *vs* 58.3%, P = 0.04), NBS (100% *vs* 50%, P = 0.015), the start codon of M (90% *vs* 25%, P = 0.004), VS (100% *vs* 25%, P < 0.001), and pHSA (100% *vs* 58.3%, P = 0.04) than did the HBV/C-infected patients (Table 4). In the asymptomatic carriers, different types of pre-S deletion seemed to correlate with the HBV genotype, but the sample size (n = 2) was too small for statistical analysis. In the CH patients, deletion in pS1-BT and four functional sites (S promoter, HSC70, CAD, and NBS), which are located in the C-terminal half of the pre-S1 region, was frequent in both genotypes. Conversely, deletion in pHSA was more frequent in the HBV/B group than in the HBV/C group (88.9% *vs* 36.4%, P = 0.028). In the LC patients, no significant differences were observed between the HBV/B and HBV/C groups, except that deletion in the start codon of L was more frequent in the HBV/C group (42.9% *vs* 12.5%, P = 0.193) (Table 4). In NC-HCC, deletion in the site for CCAAT binding factor was more frequent in the HBV/B group than in the HBV/C group (57.1% *vs* 0.0%, P = 0.092) (Table 4).

DISCUSSION

The HBV genotypes influence several aspects of HBV infection, including clinical outcomes, response to antiviral therapy, and host immune response. Pre-S deletion mutants are frequently found in patients with chronic HBV infection and are correlated with the progression of liver disease[5,6,19-23]. In the present study, the influence of the HBV genotype on the emergence of different types of pre-S deletion was examined. The results show that the frequencies of some types of pre-S deletion mutant differed between the HBV/B and HBV/C groups, whereas the frequencies of other types of deletion mutant were similar in both genotypes. Sequence alignment analysis indicated that frequent deletion in the C-terminal half of the pre-S1 and N-terminus of the pre-S2 regions was observed in both genotypes (86.0% and 79.1% in the HBV/B group; 69.8% and 72.1% in the HBV/C group, respectively). Epitope mapping of these pre-S deletion mutants showed frequent deletions in several epitope sites in both genotypes, particularly pS1-BT (72.7% in the HBV/B group and 55.8% in the HBV/C group) and pS2-B2 (75.0% in the HBV/B group and 65.1% in the HBV/C group). By contrast, the rate of pS2-B1 deletion was considerably high in the HBV/B group, and the rate of pS2-T deletion was considerably high in the HBV/C group (Table 3). Previous studies have shown that several genotype-specific antibodies are induced and react with the variable pre-S1 and pre-S2 sequences[13,14]. The host immune response to these genotype-specific epitopes may increase the selection pressure for pS2-B1 and pS2-T deletion mutants in HBV/B and HBV/C, respectively.

Functional mapping also demonstrated the similarities and differences between HBV/B and HBV/C. Deletion in the S promoter and the HSC70 site was frequently found in both genotypes. Conversely, deletion in three functional sites (NBS, the start codon of M, and VS) located in the N-terminus of the pre-S2 region was significantly more frequent in the HBV/B group (P < 0.05), and one type of N-terminus pre-S1 deletion mutant with deletion of the start codon of the L protein was frequently observed in the HBV/C group (20.9% *vs* 9.3%, P = 0.228), particularly in the LC patients (42.9% *vs* 12.5%). Previous studies in Korea have also demonstrated frequent deletion in the start codon of the L protein in HBV/C[32,33]. Unexpectedly, the tendency of deletion in functional sites is opposite between HBV/B and HBV/C. Biswas *et al*[34] similarly showed that HBV/A and HBV/C have a higher rate of pre-S2 mutation/deletion, whereas HBV/D has a higher rate of pre-S1 deletion.

The correlation of different types of pre-S deletion with the HBV genotype was further examined according to different clinical outcomes. In the CH and LC

Table 4 Frequencies of different types of pre-S deletions in HBV/B- and HBV/C-infected carriers according to their clinical outcome

Deletion	ASC		*P* value[1]	CH		*P* value[1]	LC		*P* value[1]	NC-HCC		*P* value[1]	LC-HCC		*P* value[1]
	B (*n* = 2)	C (*n* = 2)		B (*n* = 9)	C (*n* = 11)		B (*n* = 8)	C (*n* = 14)		B (*n* = 14)	C (*n* = 4)		B (*n* = 10)	C (*n* = 12)	
N-terminal half of pre-S1 (aa 1-57)	50.0%	50.0%		22.2%	27.3%		50.0%	50.0%		28.6%	50.0%		60.0%	33.3%	
C-terminal half of pre-S1 (aa 58-119)	50.0%	0		88.9%	81.8%		87.5%	71.4%		92.9%	75.0%		80.0%	66.7%	
N-terminus of pre-S2 (aa 1-31)	0	100.0%		88.9%	72.7%		62.5%	78.6%		78.6%	75.0%		100.0%	58.3%	0.040
d183 M	50.0%	0		33.3%	36.4%		25.0%	28.6%		28.6%	0		0	8.3%	
Epitope Mapping															
pS1-T1 (aa 12-48 of pre-S1)	50.0%	0		11.1%	18.2%		50.0%	28.6%		21.4%	25.0%		60.0%	16.7%	0.074
pS1-B1 (aa 12-53 of pre-S1)	50.0%	0		11.1%	18.2%		50.0%	28.6%		28.6%	25.0%		50.0%	16.7%	
pS1-B2 (aa 72-78 of pre-S1)	50.0%	0		44.4%	54.5%		37.5%	57.1%		50.0%	25.0%		30.0%	58.3%	
pS1-BT (aa 94-117 of pre-S1)	50.0%	0		88.9%	81.8%		62.5%	64.3%		71.4%	25.0%		70.0%	25.0%	0.084
pS2-B1 (aa 1-6 of pre-S2)	0	0		88.9%	45.5%	0.070	50.0%	42.9%		64.3%	50.0%		100.0%	25.0%	< 0.001
pS2-B2 (aa 3-15 of pre-S2)	0	100.0%		88.9%	54.5%		62.5%	64.3%		71.4%	75.0%		100.0%	58.3%	0.040
pS2-B3 (aa 13-24 of pre-S2)	0	100.0%		44.4%	45.5%		37.5%	64.3%		35.7%	50.0%		70.0%	50.0%	
pS2-T1 (aa 21-48 of pre-S2)	0	100.0%		33.3%	36.4%		25.0%	57.1%		14.3%	50.0%		40.0%	41.7%	
Functional mapping															
The start codon of L	0	50.0%		11.1%	9.1%		12.5%	42.9%		0	25.0%		20.0%	0	
HBS (aa 2-48)	50.0%	50.0%		22.2%	18.2%		50.0%	50.0%		21.4%	50.0%		60.0%	25.0%	
S promoter (nt 3045-3189)	50.0%	0		88.9%	81.8%		75.0%	71.4%		64.3%	75.0%		70.0%	66.7%	
CBF (nt 3137-3147)	50.0%	0		66.7%	54.5%		50.0%	42.9%		57.1%	0	0.092	40.0%	25.0%	
HSC70 (aa 74-118)	50.0%	0		88.9%	81.8%		62.5%	71.4%		71.4%	50.0%		70.0%	66.7%	
CAD (aa 81-105)	50.0%	0		88.9%	81.8%		62.5%	64.3%		64.3%	50.0%		70.0%	25.0%	0.084
NBS (aa 103-127)	50.0%	0		100.0%	90.9%		75.0%	64.3%		92.9%	50.0%		100.0%	50.0%	0.015
The start codon of M	0	0		77.8%	54.5%		50.0%	50.0%		71.4%	50.0%		90.0%	25.0%	0.004
VS (aa 1-5 of pre-S2)	0	0		88.9%	45.5%	0.070	50.0%	42.9%		71.4%	50.0%		100.0%	25.0%	< 0.001
pHSA (aa 3-16 of pre-S2)	0	100.0%		88.9%	36.4%	0.028	62.5%	64.3%		78.6%	75.0%		100.0%	58.3%	0.040

[1]$P < 0.1$. ASC: Asymptomatic carriers; CH: Chronic hepatitis; HBV: Hepatitis B virus; LC: Liver cirrhosis; LC-HCC: Liver cirrhotic hepatocellular carcinoma; NC-HCC: Noncirrhotic hepatocellular carcinoma; HBS: Hepatocyte binding site; CBF: CCAAT binding factor; HSC70: Heat shock protein 70; CAD: Cytosolic anchorage determinant; NBS: Nucleocapsid binding site; VS: Viral secretion; pHSA: Polymerized human serum albumin.

patients, frequent deletion in the C-terminal half of pre-S1 was observed in both genotypes. Significant differences were observed between the HBV/B- and HBV/C-infected patients with LC-HCC. Deletion in the N-terminus of pre-S2 region, including two epitope sites (pS2-B1 and pS2-B2) and three functional sites (the start codon of M, VS, and pHSA), was significantly more frequent in the HBV/B-infected LC-HCC patients ($P < 0.05$). In Asia, HBV/B and HBV/C commonly coexist. However, their distribution differs by country[3,4]. Previous studies have shown that pre-S2 deletion is associated with the development of HCC in Taiwan[23,35-37]. This finding may be due to HBV/B being more prevalent than HBV/C in Taiwan. HBV/C is predominant in South Korea, where studies have shown that N-terminus pre-S1 deletion mutant with deletion of the start codon of the L protein correlates with the development of HCC[32,33]. The results of the current study indicate that the tendency of different types of pre-S deletion varies across the HBV genotypes. Therefore, the difference in genotype prevalence in different countries may influence the pattern of pre-S deletion associated with progressive liver disease.

Notably, one type of N-terminus pre-S1 deletion mutant with deletion of the start codon of the L protein was frequently observed in the HBV/C group (20.9% *vs* 9.3%, $P = 0.228$), particularly in the LC patients (42.9% *vs* 12.5%). These N-terminus pre-S1 deletion mutants are similar to genotype D, which has a deletion of 11 aa in the N-terminus of the L surface protein, as reported in Korean HBV/C-infected patients[32,33]. Deletion in the start codon of the L protein may result in no synthesis of L surface proteins. Because the L protein is essential for binding to hepatocytes, formation of virion envelope, and retention of surface proteins, the absence of L

proteins not only inhibits infection and virion assembly, but also facilitates extracellular secretion of surface proteins. The intracellular retention of surface proteins of some genotype D strains has been reported in mixed infection with genotype A, which can induce hepatic carcinogenesis by activating the ER stress pathway[38]. A recent study showed that the L start codon deletion mutant resulted in the absence of L proteins and increased levels of intracellular viral mRNA and extracellular HBsAg[39]. This finding suggests that accumulated intracellular viral mRNA might activate the intracellular toll-like receptors, leading to the subsequent activation of nuclear factor kappa B pathways, chronic inflammation, and carcinogenesis[39]. The precise pathogenesis caused by these L start codon deletion mutants should be further researched in the future to determine whether it is similar to that caused by genotype D.

Previous studies have frequently detected the C-terminal half pre-S1 deletion mutant d183M in the sera of individuals with CH and cirrhosis in different countries[40-46]. This pre-S1 deletion mutant has also been found in a child with occult HBV infection[47]. In the present study, the frequency of this mutant was examined in HBV carriers with pre-S deletion. The results show that d183M was detected in 23.2% and 20.9% of the HBV/B- and HBV/C-infected carriers, respectively, and the frequency of d183M was higher in the CH patients with HBV/B (33.3%) or HBV/C (36.4%). The d183M mutant may be generated by a splicing event, because the boundary of the deletion contains consensus donor and acceptor splice sites that are conserved among all known HBV genotypes[48]. This phenomenon may explain why this unique C-terminal half pre-S1 deletion is frequently observed in patients with HBV infection. Such a deletion results in the removal of (1) the Hsc70 binding site and the CAD essential for the dual topology of L proteins; (2) the NBS required for virion morphogenesis; and (3) the S promoter necessary for S and M surface proteins. Functional characterization of this mutant revealed a defect in HBsAg secretion and viral packaging and subsequent virion secretion[26,43-46]. Western blotting and immunofluorescence analysis showed that the mutant L surface proteins are poorly secreted, heterogeneous, and accumulated within the ER[26]. Studies have demonstrated that mutant L surface proteins exhibit direct cytopathic activity when they retained in the cell[49,50]. Several clinical reports have shown that alanine aminotransferase flare-up and liver fibrosis occur following this mutation[43,44]. These clinical and functional studies strongly suggest that this mutant causes liver inflammation. Therefore, the prevalence and impact of d183M on the clinical course of infection should be investigated in a large population.

It is suggested that multiple risk factors may contribute to the pathogenesis of HBV infection. Chronic inflammation, the effect of cytokines, and the integration of HBV DNA into the host cellular genome are crucial factors in the development of HCC. In addition, HBV mutations in X, BCP, PC, and the pre-S/S region are associated with the severity of liver disease and the development of HCC[6,19-22,51,52]. The dinucleotide substitution (A1762T, G1764A) is the most common mutation in BCP. This BCP mutation is associated with the higher occurrence of HCC and LC[6,19,20,51]. Mutations in PC (G1896A) and X (C1653T and T1753V) are also associated with the development of HCC[6,51,52]. Moreover, pre-S/S mutations are associated with fulminant hepatitis, fibrosing cholestatic hepatitis, and the development of cirrhosis and HCC[6,19-22]. Recent researches suggested that microRNA is involved in HBV-related HCC[53], All of these studies indicated that both viral and host factors affect HBV pathogenesis. Additional studies should be conducted to define their role in the progression of liver disease.

To the best of our knowledge, the present study is the first comprehensive study of the influence of the HBV genotype on the emergence of different types of pre-S deletion mutant. In conclusion, some patterns of pre-S deletion differ between HBV/B and HBV/C. The prevalence of different pre-S deletion mutants differs between HBV/B and HBV/C among patients with liver disease. These differences are related to the different HBV genotypes and the different roles of pre-S1 and pre-S2 deletions in the progression of liver disease. The precise mechanisms are yet to be elucidated. Knowledge concerning HBV pre-S deletion may improve our understanding of HBV-associated hepatopathogenesis and enable establishing strategies to reduce the incidence of progressive liver diseases in patients with hepatitis infection.

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COMMENTS

Background

Chronic hepatitis B virus (HBV) infection may lead to the development of chronic hepatitis (CH), liver cirrhosis (LC), and hepatocellular carcinoma (HCC). To date, research has identified 10 HBV genotypes, designated A to J. All genotypes can lead to progressive liver disease, but the clinical impaction of each genotype differs. For example, patients infected by the genotype C (HBV/C) strain have a higher frequency of basal core promoter (BCP) mutations, a lower response rate to interferon therapy, and more rapid progression to liver fibrosis and HCC than those infected by the genotype B (HBV/B) strain.

Research frontiers

Naturally occurring HBV pre-S deletion mutants have been identified and are associated with the development of progressive liver disease in hepatitis B carriers. Because the pre-S region has the highest genetic variability in the whole genome, the HBV genotypes may influence the deletions in the pre-S region. Knowledge concerning the prevalence of different types of pre-S

deletions in different HBV genotypes is limited.

Innovations and breakthroughs

This study is the first comprehensive study to investigate the associations of different types of pre-S deletion with HBV genotypes. The results reveal different patterns of pre-S deletion between HBV/B- and HBV/C-infected carriers. This finding may be attributable to the progression of liver disease.

Applications

These findings show that the different patterns of pre-S deletion mutants are associated with the HBV genotype. Different types of pre-S deletion are also found between HBV/B- and HBV/C-infected patients with different types of liver disease. Knowledge concerning HBV pre-S deletion may improve our understanding of HBV-associated hepatopathogenesis and enable establishing strategies to reduce the incidence of progressive liver diseases in patients with hepatitis infection.

Peer-review

Author of this manuscript described the differential patterns of pre-S deletions in association with HBV genotypes. This is an interesting and innovative work. It is very useful information to predict the prognosis and clinical outcomes. It would be better to compare/describe the pre-S/S mutations including pre-S deletions, too.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Second-generation direct-acting-antiviral hepatitis C virus treatment: Efficacy, safety, and predictors of SVR12

Christoph R Werner, Julia M Schwarz, Daniel P Egetemeyr, Robert Beck, Nisar P Malek, Ulrich M Lauer, Christoph P Berg

Christoph R Werner, Julia M Schwarz, Daniel P Egetemeyr, Nisar P Malek, Ulrich M Lauer, Christoph P Berg, Department of Gastroenterology, Hepatology, and Infectiology, University Hospital Tübingen, 72076 Tübingen, Germany

Robert Beck, Institute of Medical Virology, University Hospital Tübingen, 72076 Tübingen, Germany

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Informed consent statement: The institutional review board of the Medical Faculty of the University of Tübingen approved this retrospective analysis and waived the need for written informed consent because of the anonymous evaluation of patient data from patient records.

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Correspondence to: Christoph P Berg, MD, Department of Gastroenterology, Hepatology, and Infectiology, University Hospital Tübingen, Otfried-Müller-Str. 10, 72076 Tübingen, Germany. christoph.berg@med.uni-tuebingen.de
Telephone: +49-7071-2982723
Fax: +49-7071-294423

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Abstract

AIM

To gather data on the antiviral efficacy and safety of second generation direct acting antiviral (DAA) treatment with respect to sustained virological response (SVR) 12 wk after conclusion of treatment, and to determine predictors of SVR12 in this setting.

METHODS

Two hundred and sixty patients treated with SOF combination partners PR (n = 51), R (n = 10), SMV (n = 30), DCV (n = 81), LDV (n = 73), or 3D (n = 15).

144/260 were pre-treated, 89/260 had liver cirrhosis, 56/260 had portal hypertension with platelets < 100/nL, 25/260 had a MELD score ⩾ 10 and 17/260 were post-liver transplantation patients. 194/260 had HCV GT1, 44/260 HCV GT3.

RESULTS

Two hundred and forty/256 (93.7%) patients achieved SVR12 (mITT); 4/260 were lost to follow-up. SVR12 rates for subgroups were: 92% for SOF/DCV, 93% for each SOF/SMV, SOF/PR, 94% for SOF/LDV, 100% for 3D, 94% for pretreated, 87% for liver cirrhosis, 82% for patients with platelets < 100/nL, 88% post-liver transplantation, 95% for GT1a, 93% for GT1b, 90% for GT3, 100% for GT2, 4, and 6. 12 patients suffered from relapse, 6 prematurely discontinued treatment, of which 4 died. Negative predictors of SVR12 were a platelet count < 100/nL, MELD score ⩾ 10 (P < 0.0001), liver cirrhosis (P = 0.005) at baseline. In Interferon-free treatment GT3 had significantly lower SVR rates than GT1 (P = 0.016). Side effects were mild.

CONCLUSION

Excellent SVR12 rates and the favorable side-effect profile of DAA-combination therapy can be well translated into "real-world". Patients with advanced liver disease, signs of portal hypertension, especially with platelets < 100/nL and patients with GT3 are in special need for further research efforts to overcome comparatively higher rates of virological failure.

Key words: Sofosbuvir; Simeprevir; Ledipasvir; Hepatitis C; Liver transplant; Sustained virological response; Liver cirrhosis; Side effects; Resistance; Daclatasvir

Core tip: From 2014 on the second wave of direct acting antiviral agents was available for treatment of chronic hepatitis C infection. Due to the more heterogeneous character of patients in the "real world", the therapeutic performance of these new drugs outside randomized clinical trials is of interest. Therefore, in this monocentric retrospective cohort study, we analyzed the efficacy, safety, and predictors of sustained virological response 12 for treatment with combinations of second generation direct acting antivirals in a "real-world" setting.

Werner CR, Schwarz JM, Egetemeyr DP, Beck R, Malek NP, Lauer UM, Berg CP. Second-generation direct-acting-antiviral hepatitis C virus treatment: Efficacy, safety, and predictors of SVR12. *World J Gastroenterol* 2016; 22(35): 8050-8059 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i35/8050.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i35.8050

INTRODUCTION

Chronic infection with Hepatitis C virus (HCV) is still one of the main causes for liver disease with a prevalence of 0.2%-2% in Western countries, while worldwide about 80 million people are threatened by HCV[1-4]. After many years of just moderate sustained viral response (SVR) rates of around 50%-60% through all genotypes (GT) under Interferon (IFN)-based treatment regimens[5-9], in 2011 the first direct acting antiviral compounds (DAA), the protease inhibitors (PI) Telaprevir and Boceprevir had been approved for treatment of HCV GT1[10-14]. Beyond that, promising SVR results obtained in the clinical trials were shown to be achieved also in "real-world" settings[15,16]. However, treatment with first generation DAA was only subject to GT1 patients, and antiviral potency was counteracted by aggravated side-effects. For the second wave of DAA, diverse drug classes were developed: (1) polymerase-; (2) NS5A-, as well as (3) new protease inhibitors (PI). From early 2014 on, consecutively the first-in-class polymerase inhibitor Sofosbuvir (SOF), a second wave PI Simeprevir (SMV), the first-in-class NS5A inhibitor Daclatasvir (DCV), and another NS5A inhibitor Ledipasvir (LDV), were approved. Accordingly, for the first time, IFN-free treatments consisting of combinations of these DAA, with or without Ribavirin (R), were possible, showing impressive SVR rates and a superior side effect profile. From 2014 until 2015, SOF constituted the "backbone" of most combination treatments: in combination with R alone (SOF/R), with pegylated Interferon and R (SOF/PR), or combined with SMV (SOF/SMV), DCV (SOF/DCV), or LDV (SOF/LDV) with or without R. In 2015, this "monopoly" was tackled by the fixed combination of Dasabuvir, a non-nucleosidic polymerase inhibitor, with Ombitasvir, and Paritaprevir/r (3D).

Now, after more than two years since the approval of SOF and its combination partners, and a year after approval of the 3D regimen, we here summarize our experiences with these combination treatments being obtained in the "real-world" setting of a tertiary center. This retrospective analysis was conducted to gather data on the antiviral efficacy and safety of second generation DAA treatment with respect to sustained virological response 12 wk after conclusion of treatment (SVR12), and to determine predictors of SVR12 in this setting.

MATERIALS AND METHODS

The clinical characteristics of our retrospective cohort are presented in Table 1. The study cohort includes all 260 consecutive patients, who were treated at our center with a DAA containing therapy between January 2014 and December 2015. Treatment decisions were

Table 1 Characteristics of study cohort patients

Demographics		
n		260
Age	(yr)[1]	53 (44-60)
Sex	Male/Female *n* (%)	157 (60)/103 (40)
Baseline viral characteristics		
Genotype 1a/1b/1x	*n* (%)/*n* (%)/*n* (%)	76 (29)/115 (44)/3(1)
Genotype 2/3	*n* (%)/*n* (%)	8 (3)/44 (17)
Genotype 4/5/6	*n* (%)/*n* (%)	11 (4)/1 (0.4)/2 (0.8)
Baseline viral load	(IU/mL)[1]	1.33 Mio. (414.750-3.4 Mio.)
Baseline viral load ⩾ 800.000 IU/mL	*n* (%)	169 (65)
Baseline viral load ⩾ 6 Mio IU/mL	*n* (%)	28 (11)
Special populations		
LCi[2]	*n* (%)	89 (34)
Low platelets (⩽ 100/nL)	*n* (%)	56 (22)
Post Liver Transplantation	*n* (%)	17 (7)
Patients ⩾ 60 yr	*n* (%)	72 (28)
Patients with MELD score ⩾ 10	*n* (%)	25 (10)
Treatment history		
Treatment experienced	*n* (%)	144 (55)
Treatment regimens [thereof liver cirrhosis *n* (%)]		
SOF PR	*n* (%)/LCi *n* (%)	51 (20)/6 (12)
SOF R	*n* (%)/LCi *n* (%)	10 (4)/4 (40)
SOF SMV	*n* (%)/LCi *n* (%)/R *n* (%)	30 (12)/16 (53)/15 (19)
SOF DCV	*n* (%)/LCi *n* (%)/R *n* (%)	81 (31)/42 (52)/12 (40)
SOF LDV	*n* (%)/LCi *n* (%)/R *n* (%)	73 (28)/17 (23)/16 (22)
3D	*n* (%)/LCi *n* (%)/R *n* (%)	15 (6)/4 (27)/6 (40)
Baseline clinical chemistry		
WBC	(/μL)[1]	5935 (4683-7670)
Hemoglobin	(g/dL)[1]	14.5 (13.1-15.6)
Platelets	(thousand/μL)[1]	174 (113-228)
Creatinine	(mg/dL)[1]	0.7 (0.6-0.8)
Total Bilirubin	(mg/dL)[1]	0.7 (0.5-1.0)
INR	INR[1]	1 (1-1)
ALT	IU/l[1]	67 (44-105)

[1]Data are presented as medians (interquartile ranges in parentheses); [2]By liver histology, Fibroscan (> 12.5 kPa), or by clinical diagnosis (*e.g.*, esophageal varices, ascites, distinct ultrasound signs of portal hypertension or liver cirrhosis). 1x: Genotype 1, no subtype differentiable; LCi: Liver cirrhosis; MELD: Model of end stage liver disease; P: Pegylated Interferon; R: Ribavirin; SOF: Sofosbuvir; SMV: Simeprevir; LDV: Ledipasvir; DCV: Daclatasvir; 3D: Dasabuvir, Ombitasvir, and Paritaprevir/r; WBC: White blood cell count; INR: International normalized ratio; ALT: Alanine-aminotransferase.

based on antiviral activity against GT of respective DAA according to approval, severity of liver disease, comorbidities, approval of DAA at time-point of treatment start, and economic reasons.

If possible, IFN-free treatments were favored. Treatment of patients presenting hepatic impairment was postponed, if possible, until IFN-free regimens were available. 17 patients were treated in the context of post-liver transplantation, two of these with cholestatic recurrence of HCV after liver transplantation who were treated with SOF/DCV in a compassionate use program. Following the dates of approval of the different DAA, patients were treated with SOF/R or SOF/PR respectively, when being started with treatment in early 2014, until later on additionally a SOF/SMV combination became available. From autumn 2014 on, patients were, if possible, treated with SOF/DCV, and again later on this year, patients could be treated also with SOF/LDV. From Early 2015 on, patients could also be treated with the 3D regimen. Proportions of different treatment regimens are shown in Table 1.

Baseline clinical chemistry is shown in Table 1. 13/260 patients had leukopenia (leukocyte count < 3000/μL), 56/260 patients presented with thrombopenia (platelet count < 100/nL). Transaminases were elevated in 223/260 patients (ALT > 35 IU/mL). Data of albumin levels were available only on an occasional basis.

Diagnosis of liver cirrhosis was based upon liver histology, Fibroscan (> 12.5 kPa), or clinical diagnosis (*e.g.*, ascites, esophageal varices, distinct sonographic signs of portal hypertension or liver cirrhosis). For assessment of severity of liver disease, we calculated the MELD score. In this retrospective analysis, Child Turcotte Pugh score or other assessment scores for severity of liver disease could not be calculated due to lack of data[17,18]. For retrospective identification of patients with possible portal hypertension, a threshold of 100 platelets/nL was assumed.

For virological analyses Roche CobasAmpliprep/Roche CobasTaqMan [Roche Diagnostics GmbH, Mannheim, Germany; lower limit of quantification (LLOQ) 15 IU/mL] was used.

The institutional review board of the Medical Faculty of the University of Tübingen approved this retrospective analysis and waived the need for written informed consent because of the anonymous evaluation of patient data from patient records.

Data were statistically analyzed using Microsoft Office Excel, SPSS, and Graph Pad Prism. 4/260 patients were lost to follow-up. These patients were excluded from analysis [modified intention to treat analysis (mITT)].

RESULTS

Analysis of SVR12 rates

The overall SVR12 rate is shown in Figure 1 (93.7%; 240/256 patients mITT; of those, 2/256 patients discontinued treatment prematurely, but achieved SVR 12; 4/260 patients were lost to follow-up and were excluded from analysis. SVR12 rates according to GT, and diverse treatment regimens are shown in Figure 2. Additionally, SVR12 rates according to several baseline characteristics (sex, age, cirrhosis status, platelet count, MELD score, viral load, and treatment experienced patients) and early viral kinetics are shown in Table 2. SVR12 rates in different GT (1a, 1b, 2, 3, 4, and 6) ranged from 90%-100%. One additional patient with HCV GT 5 achieved SVR 12.

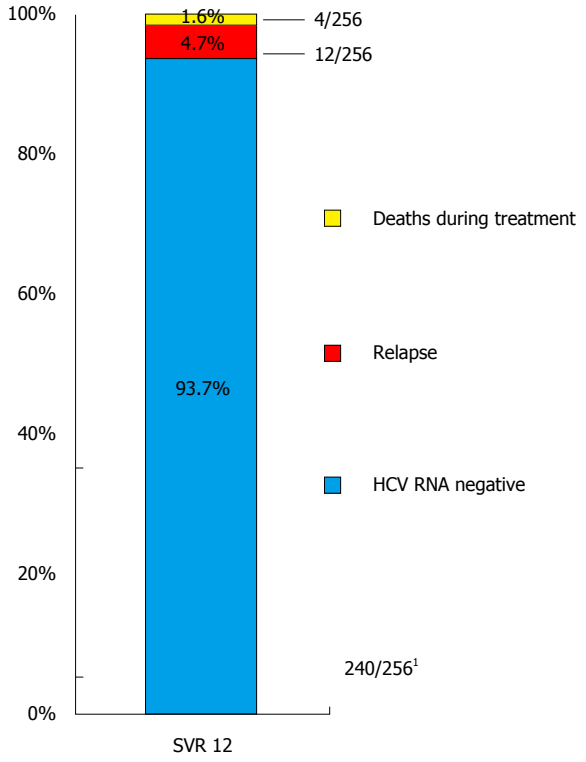

Figure 1 Overall treatment outcome. [1]Two hundred and sixty consecutive patients were treated with second generation direct acting antiviral (DAA) combinations; 14/260 patients lost to follow-up were not included in our analysis [modified intention-to-treat (mITT) analysis]. Additionally, 2 patients discontinued treatment prematurely, but achieved sustained virological response (SVR); accordingly these 2 patients also were counted as SVR12 patients.

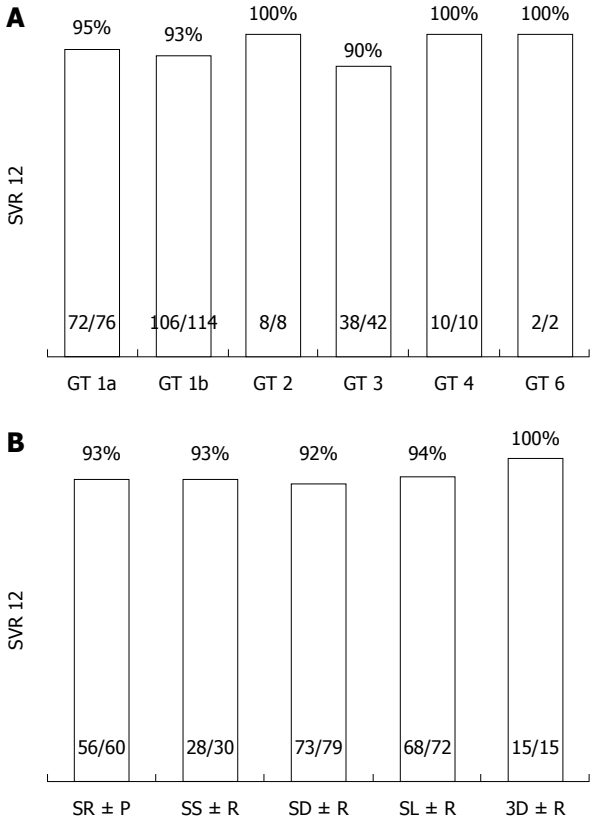

Figure 2 SVR12 rates with respect to GT (A), and diverse direct acting antiviracombinations (B, both modified intention-to-treat). This mITT analysis excluded patients, who were lost to follow-up (n = 4). Additionally, three GT1x patients and one GT 5 patient achieved SVR12. For further subgroup analyses according to baseline parameters see Table 2. 3D: Dasabuvir, Ombitasvir, and Paritaprevir/r; GT: Genotype; GT1x: Genotype 1, no subtype differentiable; P: Pegylated Interferon; R: Ribavirin; SR: Sofosbuvir, Ribavirin; SS: Sofosbuvir, Simeprevir; SL: Sofosbuvir, Ledipasvir; SD: Sofosbuvir, Daclatasvir; SVR: Sustained virological response.

Depending on different treatment regimens, SVR12 rates ranged from 92%-100%. Special subgroups of patients known to be "hard-to-treat" (patients post-liver transplantation (n = 17)], patients with liver cirrhosis (n = 89), patients of older age (≥ 60 years; n = 72), and treatment experienced patients (n = 144) achieved highly acceptable SVR12 rates in the range of 88%-95%. However, patients with low platelet count at baseline (< 100/nL; n = 56), and patients with a MELD score ≥ 10 at baseline (n = 25) achieved SVR12 rates of 82%, and 72%, respectively.

Predictors of SVR12

For evaluation of predictors of SVR12, see Table 2. Presence of liver cirrhosis (P = 0.005), a platelet count < 100/nL (P < 0.0001), and a MELD score ≥ 10 (P < 0.0001) at baseline were significant negative predictors of SVR12 in our study cohort (univariate analysis). Multivariate analysis identified a platelet count < 100/nL (P = 0.031), and a MELD score ≥ 10 (P < 0.0001) at baseline as independent predictors for achievement of a diminished SVR12 rate (see Table 2 for details).

With respect to GT, in a "per-protocol" subgroup analysis of GT1 and GT3 patients strictly treated with IFN-free protocols, GT3 patients had a significantly lower SVR12 rate than GT1 patients (P = 0.016, univariate analysis).

However, in our cohort, treatment experience, sex, age, baseline viral load, and early virological kinetics were found not to be significant as predictors of SVR12.

Virological failure

Virological failure was a rare event. No primary non-response or virological breakthrough occurred in our cohort. In addition, only 12 of the 256 patients (4.7%, mITT) suffered from virological relapse during follow-up (see Table 3 for baseline characteristics, treatment details, and resistance analysis of respective patients). One patient (No. 9 in Table 3) exhibiting a liver cirrhosis with Child-Turcotte-Pugh (CTP) stadium A suffered from relapse after SOF/PR. In this patient liver function deteriorated upon relapse to CTP stadium C, and therefore the patient had to be listed for liver transplantation (at a MELD score of 28). After having achieved an active listing status, treatment was initiated with SOF/DCV for 24 wk, leading to SVR12. During treatment, the liver function restored to CTP stadium A, and thus, the patient could be de-listed for liver transplantation again.

Table 2 Predictive model of sustained virological response analyzed for all patients treated with second generation direct acting antiviracombination therapy (n = 256)

	SVR 12 (n/N; %)[2]	Univariate analysis		Multivariate analysis	
		Odds ratio (95%CI)	Wald *P* value	Odds ratio (95%CI)	Wald *P* value
Viral kinetics					
RVR	143/152; 94%				
vs Non-RVR	83/89; 93%	1.149 (0.395, 3.341)	0.799		0.728
Baseline demographic parameters					
Fibrosis					
Liver Cirrhosis	76/87; 87%				
vs no Liver Cirrhosis	164/169; 97%	0.211 (0.071, 0.627)	0.005		0.290
Transplant Status					
LTx	15/17; 88%				
vs no LTx	225/239; 94%	0.467 (0.097, 2.246)	0.467		0.972
Sex					
Male	140/153; 92%				
vs female	100/103; 97%	0.329 (0.091, 1.184)	0.089		0.282
Age					
Patients ⩾ 60 yr	68/71; 96%				
vs < 60 yr	172/185; 93%	0.999 (0.959, 1.04)	0.948		0.078
Baseline viral load					
High viral load (> 6 Mio IU/mL)	28/28; 100%				
vs low viral load (⩽ 6 Mio IU/mL)	212/228; 93%	NA	0.998		0.251
Genotype					
1	180/192; 93%				
vs 3	38/42; 90%	1.606 (0.510, 5.060)	0.418		0.424
Genotype[1]					
1 IFN-free, per protocol	160/164/; 98%				
vs 3 IFN-free, per protocol	16/20/; 80%	5.000 (1.355, 18.45)	0.016		NA
Baseline platelet count					
Platelets ⩽ 100/nL	45/55; 82%				
vs > 100/nL	195/201; 97%	0.138 (0.048, 0.401)	< 0.0001	0.24 (0.072, 0.88)	0.031
Baseline MELD ⩾ 10					
MELD ⩾ 10	18/25; 72%				
vs MELD < 10	222/231; 96%	0.104 (0.035, 0.313)	< 0.0001	0.117 (0.037, 0.373)	< 0.0001
Previous treatment response					
Pre-treated	133/142; 94%				
vs treatment-naive	107/114; 94%	0.967 (0.349, 2.681)	0.948		0.457

[1]This analysis excluded patients co-treated with IFN, and patients with premature discontinuation ("per protocol analysis"); [2]Number of patients who achieved SVR12 in category/total number in category. Univariate and multivariate models for prediction of SVR [n = 256, modified intention-to-treat (mITT) analysis] were used. This mITT analysis excluded patients, who were lost to follow-up (n = 4). Significant calculations (P < 0.05). RVR: Rapid viral response (hepatitis C virus RNA negative at treatment week 4); LTx: Liver transplantation; SVR: Sustained virological response; NA: Not available.

For GT1, 8/194 patients (4%) suffered from virological relapse, for GT3 a rate of 3/41 (9%) was found. All GT3 patients with relapse had advanced liver disease (cirrhosis or post-liver transplantation), while the GT1 cohort of relapsers was more heterogeneous. This emphasizes the new paradigm of GT3 (with advanced liver disease) being one of the harder-to-treat GT in the era of DAA (possibly due to an unintended "misfit design" of DAA with respect to this distinct genotype). In the group of IFN-free treated patients, the difference in SVR12 rates between GT1 and 3 was significant (P = 0.016, univariate analysis; Table 2).

In all patients with virological relapse after IFN-free DAA treatment resistance analysis was performed: Patients receiving a NS5A inhibitor always revealed NS5A-specific resistance associated variants (RAV) after relapse. Only one patient was detected with a NS5B-polymerase RAV (see Table 3 for details).

Premature discontinuation of treatment

Altogether, 6 out of 256 (2.3%) patients discontinued treatment prematurely (see Table 3 for baseline characteristics, treatment details, and outcome). Four of those patients died during or shortly after discontinuation of treatment: two patients developed sepsis, one patient died from right-sided heart failure, and one patient died from cerebral hemorrhage. All these patients suffered from decompensated liver disease at baseline, two of those additionally were on immunosuppression due to prior organ transplantation (liver, and heart, respectively). Notably, these numbers are much too small to calculate significant associations between the respective causes of death and the DAA-class which had been used for HCV treatment, but the mere fact that 4 of our patients died emphasizes the need for treatment of those patients in an experienced tertiary center. However, these patients would have

Table 3 Characteristics of patients, who died during treatment, discontinued treatment prematurely, or suffered from virological

	Age (yr)	Sex	BL viral load, IU/mL	GT	LCi	BL MELD	Previous treatment	Current treatment	Treatment duration	Cause for premature discontinuation	Outcome	RAV NS3 protease	RAV NS5A	RAV NS5B polymerase
Patients, who died during treatment, or discontinued treatment prematurely														
No. 1	63	male	1.41 Mio	1b	Yes	12	PR	SOF/SMV	12	Sepsis	death			
No. 2	66	male	1.69 Mio	1b	Yes, HTx	14	-	SOF/LDV	5	Intracranial bleeding	death			
No. 3	55	male	56200	1b	Yes, LTx	24	TVR/PR	SOF/SMV/R	14	Re-LTx, sepsis	death			
No. 4	49	male	157000	1a	Yes,	14	PR	SOF/LDV/R	8	Right-heart failure	death			
No. 5	79	female	5.85 Mio	1b	Yes	6	TVR/PR	SOF/SMV	10	Back-pain	SVR 24			
No. 6	55	male	396000	1a	Yes, TIPSS	10	PR	SOF/DCV	16	Hepatic encephalopathy	SVR 24			
Patients with relapse after end of treatment														
No. 7	54	male	10300	1a	Yes	8	PR	SOF/PR	12			NA	NA	NA
No. 8	63	male	321000	1b	-	6	-	SOF/PR	12			NA	NA	NA
No. 9	43	female	408000	1a	Yes	8	-	SOF/PR	12			NA	NA	NA
No. 10	40	male	2.74 Mio	1b	-	6	PR	SOF/PR	12			NA	NA	NA
No. 11	48	male	4.89 Mio	3a	No, LTx	11	PR	SOF/DCV/R	24			F43F/L	A30K	-
No. 12	56	female	1.38 Mio	3a	Yes	7	PR	SOF/DCV	24			-	Y93H	S282S/T
No. 13	38	male	3.39 Mio	1b	-	6	-	SOF/DCV	12			Q80K	L31M, Y93H	-
No. 14	52	male	2.47 Mio	3a	Yes	10	-	SOF/DCV/R	24			-	Y93H	-
No. 15	55	female	3.03 Mio	1a	-	6	TVR/PR	SOF/DCV	12			T54S, R155K	L31M/L	-
No. 16	49	male	8340	3	Yes	6	-	SOF/LDV/R	24			-	A30S	-
No. 17	52	male	239000	1b	Yes	6	-	SOF/LDV/R	12			T54S, V55I, Q80K	Q30E	-
No. 18	57	male	137 (on SOF/R)	1a	Yes, HCC	11	SOF/R	SOF/DCV	12			-	L31M, P58S, Y93C	-

BL: Baseline; GT: Genotype; HCC: Hepatocellular carcinoma; LCi: Liver cirrhosis; MELD: Model of end stage liver disease; P: Pegylated Interferon; R: Ribavirin; SOF: Sofosbuvir; SMV: Simeprevir; LDV: Ledipasvir; DCV: Daclatasvir; TVR: Telaprevir; RAV: Resistance associated variant; HTx: Heart transplantation; LTx: Liver transplantation; TIPSS: Transjugular portosystemic stent shunt; NA: Not available.

never been treated at time of IFN-containing regimens.

One patient discontinued due to back-pain after 10 wk of treatment, without contacting our center; nevertheless, this patient was found later on of having achieved SVR 24. Another patient with HCV GT1a, liver cirrhosis, and a transjugular portosystemic shunt (TIPSS) was discontinued after 4 mo of treatment due to several episodes of hepatic encephalopathy. Nevertheless, this patient also achieved SVR 24.

Side effects

One hundred and forty-seven/237 (62%) patients with data available for side effects reported side effects during treatment (depicted in detail in Table 4). In general, side effects were mild in intensity, thus, just one patient discontinued treatment prematurely due to a self-reported intolerance (*i.e.,* back-pain; Table 3). Overall, fatigue, headache, bone pain or joint pain, and myalgia were the most frequently reported side effects. As expected, the rate of side effects was found to differ significantly between patients with IFN-containing regimens versus patients with IFN-free treatment.

Hospital admissions were required during treatment with DAA-based regimens in 22 (8.6%) patients. Some of those patients had to be hospitalized twice or more. Reasons for hospitalization were: infections (n = 8; 4%), hepatic decompensation (n = 3; 1.5%), cardiovascular disease (n = 3), cholestatic hepatitis after liver transplantation (n = 2), treatment of hepatocellular carcinoma (n = 2), lumbago, vomitus, and diabetes insipidus, right upper quadrant pain (n = 1, each). Of

Table 4 Patient self-reported side effects during treatment, and for comparison of self-reported side effects of PEG-interferon containing regimen and interferon-free treatment Fisher's exact test was used

Side effects	Overall[1]		PEG-IFN co-treatment[2]		IFN-free treatment[3]		*P* value
Fatigue	93	39.2%	26	54.2%	67	35.4%	0.020
Cephalgia	52	21.9%	9	18.8%	43	22.8%	0.697
Bone/joint pain/myalgia	38	16.0%	12	25.0%	26	13.8%	0.077
Nausea/vomiting	38	16.0%	14	29.2%	24	12.7%	0.008
							0.163
Insomnia	28	11.8%	16	33.3%	12	6.3%	< 0.0001
Vertigo	16	6.8%	6	12.5%	10	5.3%	0.102
Flu-like symptoms	14	5.9%	12	25.0%	2	1.1%	< 0.0001
Abdominal discomfort/pain	17	7.2%	3	6.3%	15	7.9%	1.000
Pruritus	18	7.6%	4	8.3%	13	6.9%	0.755
Diarrhea	13	5.5%	7	14.6%	6	3.2%	0.006
Any rash	14	5.9%	7	14.6%	7	3.7%	0.010
Anorexia	9	3.8%	4	8.3%	5	2.6%	0.085
Nervousness	7	3.0%	4	8.3%	3	1.6%	0.033
Depression/fear	5	2.1%	2	4.2%	3	1.6%	0.267
Dyspnoea	7	3.0%	4	8.3%	3	1.6%	0.030
Concentration weakness	4	1.7%	2	4.2%	2	1.1%	0.183
Visual changes	4	1.7%	1	2.1%	3	1.6%	1.000
Loss of hair	4	1.7%	1	2.1%	3	1.6%	1.000
Tachycardia/palpitations	2	0.8%	2	4.3%	0	0.0%	0.040
Meteorism	3	1.3%	0	0.0%	3	1.6%	1.000
Aggression	2	0.8%	2	4.2%	0	0.0%	0.040
Fever/chills	1	0.4%	1	2.1%	0	0.0%	0.200
Attacks of sweating	1	0.4%	1	2.1%	0	0.0%	0.200
Gingivitis	1	0.4%	0	0.0%	1	0.5%	1.000
Cough	1	0.4%	1	2.1%	0	0.0%	0.200
Neurological symptoms	1	0.4%	0	0.0%	1	0.7%	1.000

[1]Side effect reports were available in 237 of 260 patients; [2]In PEG-IFN co-treated patients (n = 51), side effect reports were available in 48 patients; [3]In IFN-free treated patients (n = 209), side effects reports were available in 189 patients. Significant calculations (P < 0.05) are printed in bold. IFN: Interferon.

those hospitalized, 4 patients deceased: Two patients died from infectious complications, and two patients died from cardiovascular disease (right-heart failure, intracerebral bleeding, respectively; see above, and Table 3 for details).

One hundred and ten patients received R as part of their combination treatment. Of those, 5 patients had to reduce R dosage due to anemia (4.5%), and 2 due to renal impairment. 2 patients had to completely withdraw R, one due to pruritus, and the other due to hemolysis, respectively.

DISCUSSION

This "real-world" monocentric retrospective cohort study analyzing safety, efficacy, and predictors of SVR12 of second generation DAA treatment shows impressive overall SVR rates (93.7%).

Due to the relatively small number of patients and the retrospective character of this study a comparison between subgroups according to DAA combination partners is only of limited significance.

Furthermore, due to the lesser tolerability and presumed lower activity of SOF/PR in patients with advanced liver disease, this treatment *a priori* was reserved in our hands to patients showing up with a well-compensated liver function only.

The results in our heterogeneous cohort, containing meaningful fractions of hard-to-treat patients (liver cirrhosis, portal hypertension, post-liver transplantation) are similar to SVR rates of so far published study trials: our cohort of PR or R co-treated patients achieved a SVR rate of 93%, while in the NEUTRINO trial the cohort treated with SOF/PR achieved a SVR rate in previously untreated patients of 90%[19], and the VA-real world cohort achieved SVR12 in 66.8%-79%[20].

In other "real-world" analyses with SMV ± R as combination partners of SOF, SVR rates of 74.1%-84.2% were achieved[20-22]. In the OPTIMIST study, a phase Ⅲ trial, a SVR12 rate of 97% in non-cirrhotic patients[23], while in the OPTIMIST-2 study treating cirrhotic patients with SOF, SMV ± R, SVR 12 in 83% of patients was achieved[24]. However, in our cohort, with more than half of patients being patients with liver cirrhosis, we achieved a SVR rate of 93% with this combination of drugs.

For patients with SOF, DCV ± R as combination partners, SVR rates of 86%-93% have been reported[25]. In our cohort, we were in line with those results and could achieve a SVR rate of 92%, including two patients with recurrent cholestatic Hepatitis C post-liver

transplantation, one of those being a non-responder to a prior Telaprevir triple therapy being undertaken post-liver transplantation, and both decompensated at the beginning of treatment.

In previously treated and untreated patients with HCV, a combination of LDV ± R, and SOF led to SVR rates of 94%-99%[26-28], which is in line with results of our cohort, in which we could register a SVR12 rate of 94%, while in another real-world analysis, SVR rates of 91.3%-92% were achieved[29].

For our small group of 3D regimen-receiving patients, we could achieve SVR in 100%, while in the Phase Ⅲ trials SVR rates of 91.8%-99.5% were observed[30,31].

Thus, altogether, favorable SVR rates achieved in the randomized controlled clinical trials could be translated into the "real-world", and importantly, in our cohort; even extra hard-to-treat groups of patients exhibited favorable treatment outcomes (SVR12 post-liver transplantation: 88%, SVR12 in cirrhotic patients with platelets < 100/nL: 82%), which exceed results with former treatment options by far[15,32].

Nevertheless, patients with liver cirrhosis show significantly lower response rates (87% with liver cirrhosis, 97% without; $P = 0.005$), and especially those with advanced portal hypertension (platelets < 100/nL), or high MELD score (≥ 10) show significantly lower SVR12 rates than patients without ($P < 0.0001$, uni- and multivariate analysis). These findings were also observed in other real world studies with low platelets, low albumin and liver cirrhosis as negative predictors of SVR in larger cohorts[21,33,34]. Thus, this subgroup of patients still resembles a group of patients in the "catch 22"-situation of being in the greatest need of treatment while showing the lowest response rates. Moreover, the whole drug class of HCV protease inhibitors (represented by SMV, and Paritaprevir/r) is not recommendeded for those patients (CTP B "plus") due to hepatotoxicity. Therefore, new strategies are needed to tackle this problem, *e.g.,* by implementation of screening programs to identify patients infected with HCV at an early stage of their liver disease, and more importantly by development of more potent agents in the near future. However, 4 patients were lost in our cohort. Even if not associated with anti-HCV medication, this emphasizes, that treatment of patients with advanced liver disease should remain in the hands of experienced tertiary centers even in times of "easy" treatment with second generation DAA.

In our cohort, results of early viral kinetics had no impact on prediction of SVR12, thus costly "in-between" measurements of HCV viral load possibly are expendable.

Previous treatment with PR may not play a role any more in treatment decisions, as in our cohort previous treatment was not identified as a negative predictor of SVR, as it was in the HCV-TARGET cohort[21].

Most probably due to the favorable tolerability and low-toxicity profile of second generation DAA, and the omission of pegylated Interferon, now also senior patients benefit from SOF-based DAA treatment on the same scale as younger patients do[21].

Another subgroup of patients in need of further research efforts seems to be the one with GT3: Even though we could achieve a favorable overall SVR rate of 90%, at least in IFN-free treatment regimens, the SVR rates in a "per-protocol" analysis are significantly lower in GT3 patients than in GT1 (80% in GT3, 98% in GT1, respectively, $P = 0.016$; univariate analysis), again with a special negative focus on patients with GT3 and concomitant advanced liver disease. Moreover, means are limited with respect to treatment of GT3 due to the insufficient antiviral activity of protease- and NS5A-inhibitors (except DCV) in this GT. However, new pangenotypic NS5A inhibitors like Velpatasvir or upcoming new pangenotypic protease inhibitors hopefully close that gap in the near future.

However, since DAA treatment forms RAVs in the viriom of patients, pretreatment with DAA of any generation has to be considered more and more in future treatment attempts after any prior exposure to DAA. All patients in our cohort, who suffered from virological relapse showed RAVs at time-point of relapse. Especially NS5A-RAVs are frequent due to the low resistance barrier of NS5A-inhibitors, as exemplified also in our "real-word" cohort. Since NS5A-RAVs lead to just minimal impairment of viral "fitness", unfortunately they are detectable for a long time in exposed patients[35]. While for some RAVs (like NS5A L31M, Y93H) clear associations between existence of RAV and virological failure exist, for others (like NS5A A30S) this association is not well established[35]. This may lead to confusion in case of a future re-treatment, if minor RAVs have been detected, and even more in case of a baseline test in treatment naïve patients. However, as the population of patients with relapse after DAA treatment grows, the need for controlled trials with new DAA-combinations (*e.g.,* pangenotypic protease inhibitor) for those patients to address this problem is obvious. Therefore, after virological relapse we recommend resistance testing for individualized adjustment of future DAA therapies.

In our retrospective analysis excellent SVR12 rates of second generation DAA could be translated from the large study trials into "real-world" scenarios. Patients with advanced liver disease, signs of portal hypertension, especially with platelets < 100/nL, or MELD ≥ 10, and patients with GT3 are at relatively higher risk to suffer from virological failure and development of resistance associated variants after exposure to DAA. To overcome this unsolved problem, further research efforts are needed.

COMMENTS

Background

From 2014 on, successively the second wave of direct acting antiviral agents (Sofosbuvir, Daclatasvir, Simeprevir, Ledipasvir, Dasabuvir, Ombitasvir,

Paritaprevir/r) was available for treatment of chronic hepatitis C infection. In randomized clinical trials, superb rates of sustained virological response (SVR) 12 could be achieved.

Research frontiers

Due to the more heterogeneous character of patients in the "real world", the therapeutic performance of these new drugs outside randomized clinical trials is of interest. Therefore, in this monocentric retrospective cohort study, we analyzed the efficacy, safety, and predictors of SVR 12 for treatment with combinations of second generation direct acting antivirals in a "real-world" setting.

Innovations and breakthroughs

In this retrospective study, similar SVR rates could be achieved compared to randomized clinical trials. However, certain subgroups of patients have significantly lower viral response rates: Significant negative predictors of SVR12 were a platelet count < 100/nL, a MELD score ≥ 10 (both $P < 0.0001$), liver cirrhosis at baseline ($P = 0.005$). Moreover, in Interferon-free treatment patients with HCV genotype 3 had significantly lower SVR rates than patients with HCV genotype 1 ($P = 0.016$). In the future, these subgroups of patients should be more in the focus of research efforts to overcome lower rates of SVR.

Applications

Current retrospective analysis shows that excellent SVR12 rates and the favorable side-effect profile of direct acting antiviral-combination therapy can be well translated into "real-world".

Peer-review

Good level-study to be ameliorated in the presentation of characteristics of cirrhotic patients that are an important part of the studied population.

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ORIGINAL ARTICLE

Retrospective Study

Trajectories of endoscopic Barrett esophagus: Chronological changes in a community-based cohort

Shouji Shimoyama, Toshihisa Ogawa, Toshiyuki Toma

Shouji Shimoyama, Toshihisa Ogawa, Toshiyuki Toma, Gastrointestinal Unit, Settlement Clinic, Adachi-ku, Tokyo 120-0003, Japan

Author contributions: Shimoyama S designed and performed the research, analyzed the data, and wrote the paper; Ogawa T and Toma T performed the research.

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Correspondence to: Dr. Shouji Shimoyama, Gastrointestinal Unit, Settlement Clinic, 4-20-7, Towa, Adachi-ku, Tokyo 120-0003, Japan. shimoyama@apost.plala.or.jp
Telephone: +81-3-36057747
Fax: +81-3-36050244

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Abstract

AIM

To elucidate longitudinal changes of an endoscopic Barrett esophagus (BE), especially of short segment endoscopic BE (SSBE).

METHODS

This study comprised 779 patients who underwent two or more endoscopies between January 2009 and December 2015. The intervals between the first and the last endoscopy were at least 6 mo. The diagnosis of endoscopic BE was based on the criteria proposed by the Japan Esophageal Society and was classified as long segment (LSBE) and SSBE, the latter being further divided into partial and circumferential types. The potential background factors that were deemed to affect BE change included age, gender, antacid therapy use, gastroesophageal reflux disease-suggested symptoms, esophagitis, and hiatus hernia. Time trends of a new appearance and complete regression were investigated by Kaplan-Meier curves. The factors that may affect appearance and complete regression were investigated by χ^2 and Student-*t* tests, and multivariable Cox regression analysis.

RESULTS

Incidences of SSBE and LSBE were respectively 21.7% and 0%, with a mean age of 68 years. Complete regression of SSBE was observed in 61.5% of initial SSBE patients, while 12.1% of initially disease free patients experienced an appearance of SSBE. Complete regressions and appearances of BE occurred constantly over time, accounting for 80% and 17% of 5-year cumulative rates. No LSBE development from SSBE was observed. A hiatus hernia was the only significant factor that facilitated BE development (P = 0.03) or hampered (P = 0.007) BE regression.

CONCLUSION

Both appearances and complete regressions of SSBE occurred over time. A hiatus hernia was the only significant factor affecting the BE story.

Key words: Barrett esophagus; Longitudinal vessels; Progression; Regression; Hiatus hernia

Core tip: The authors demonstrated that the appearance or complete regression of Barrett esophagus (BE) occurs constantly over time. Both phenomena are associated with a hiatus hernia but not gastroesophageal reflux disease (GERD)-suggested symptoms, suggesting that the appearance of BE occurs silently. These findings imply that a lack of GERD-suggested symptoms is not sufficient to exclude patients from screening an upper gastrointestinal endoscopy for identifying BE. The endoscopists should bear in mind that, along with the silent BE story, they should not miss the chance for the detection of BE and subsequent esophageal adenocarcinoma at an early, presymptomatic stage.

Shimoyama S, Ogawa T, Toma T. Trajectories of endoscopic Barrett esophagus: Chronological changes in a community-based cohort. *World J Gastroenterol* 2016; 22(35): 8060-8066 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i35/8060.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i35.8060

INTRODUCTION

Barrett esophagus (BE), a disease in which a squamous epithelium at the distal esophagus is replaced by a specialized intestinal metaplastic epithelium, has received increasing public health concern because of its potential for lesions that develop into esophageal adenocarcinoma. It is one of the histological consequences of long lasting gastroesophageal reflux disease (GERD), and is classified into two types according to its length. Long segment BE (LSBE) denotes the length of specialized intestinal metaplasia of 3 cm or greater, while short segment BE (SSBE) is less than 3 cm. In contrast to the Western criteria[1,2], the Japan Esophageal Society proposed the concept of endoscopically diagnosed BE by the detection of longitudinal vessels in the distal side of the squamocolumnar junction (SCJ) without any histological evidence[3,4]. No requirement of biopsy specimens for the diagnosis of BE has lead general practitioners to promote a deeper awareness of the disease among the general population. We have previously published proof of the substantial incidence (22.1%) of SSBE[5], a rate comparable to or even higher than those assumed with great variation reported from Western[6] and Asian[7] countries. Surprisingly, we have also found that two thirds of SSBE patients were asymptomatic[5].

Whether the longer length of BE correlates to higher malignant potential of esophageal adenocarcinoma is controversial. Several studies have elucidated that SSBE and LSBE carry equivalent risks of esophageal adenocarcinoma[8,9], while a longer Barrett length possesses a higher risk of dysplasia[10,11] or subsequent esophageal adenocarcinoma than a shorter one[11,12]. Accordingly, every 1cm increase in BE length resulted in a 28% increase in the risk of high-grade dysplasia or esophageal adenocarcinoma[13]. These results, although inconsistent, give credence to the notion that SSBE should not be overlooked, and that the development of SSBE as well as its progression from SSBE to LSBE may at least increase risks of esophageal adenocarcinoma. Inversely, a regression of BE, if it occurs, may be expected to lessen cancer risks.

In this regard, it is highly important to investigate changes in BE length and to explore factors associated with its change. Although many studies focusing on comparisons between longer and shorter BE at a given point consistently proved that a longer BE length was associated with obesity[14], the Caucasian race[15], older age[16], a longer hiatus hernia[16-19], a longer duration of acid exposure and subsequent GERD symptoms[16,18], while proton pump inhibitor (PPI) use correlated with shorter BE[16,17], the specific factors that could affect the elongation or regression of BE in an individual patient are still debated. With regard to LSBE, several investigators found that a regression of LSBE was accomplished by PPI use[20,21], no hiatal hernia[22], or length of columnar epithelium and less severe GERD[23], while others did not[24-26]. In addition to such inconsistencies in LSBE, research investigating chronological changes for SSBE are scanty in the literature[16,27,28]. Motivated by the limited knowledge on this matter, we have conducted a community-based longitudinal prospective study and demonstrated that both the appearance and complete regression of SSBE could occur constantly over time as well as that a hiatus hernia contributes to both phenomena.

MATERIALS AND METHODS

Between January 2009 and December 2015, 1883 patients underwent a referral or screening endoscopy. The various clinical indications of referral endoscopy have been listed previously[5]. In brief, indications of referral endoscopy included GERD suggested symptoms as listed later or other gastrointestinal symptoms such as abdominal pain or loss of appetite with or without a clinically important weight loss. Other indications unrelated to gastrointestinal symptoms included abnormalities of laboratory findings or positive fecal occult blood test. Patients were excluded from this study if they underwent: (1) therapeutic or urgent endoscopies; (2) previous gastric or esophageal surgery including antireflux surgery; or (3) only a single endoscopy during the study period. Patients with a total follow up period of less than 6 mo were also excluded. Patients undergoing previous endoscopic mucosal resection outside the areas of SCJ were permitted. Consequently, 779 patients were eligible for inclusion in this study, each required to have two

Table 1 Background patient demographics

Mean age, yr (mean ± SD, range)	68.1 ± 10.8 (27-95)
Gender (male/female)	326/453 (41.8/58.2)
Antiacid therapy (present/absent)	292/487 (37.5/62.5)
GERD-suggested symptoms (present/absent)	414/365 (53.1/46.9)
Esophagitis (present/absent)	81/698 (10.4/89.6)
Hiatus hernia (present/absent)	704/75 (90.4/9.6)

Number (%) of patients otherwise stated (n = 779). GERD: Gastroesophageal reflux disease.

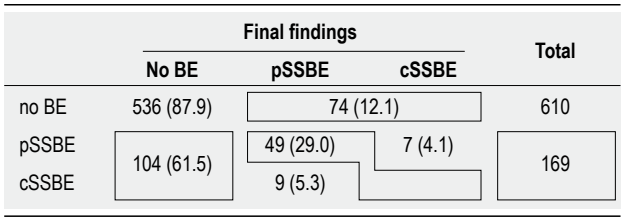

Figure 1 Patient distribution of Barrett esophagus change during the study period. Data are expressed as absolute numbers (percentage). Disease free: n = 536; Persistence: n = 49; Appearance: n = 74; Progression: n = 7; Complete regression: n = 104; Partial regression: n = 9. BE: Barrett esophagus; pSSBE: Partial type short segment Barrett esophagus; cSSBE: Circumferential type Barrett esophagus.

or more endoscopies during the study period; the presence or absence of, as well as the degrees of BE, were recorded at each endoscopy. The last date of follow up was 31st December 2015.

The diagnostic procedures and definition of BE have described previously[5]. In brief, we have noticed three important landmarks during endoscopic procedures with only minimal air inflation: a SCJ, diaphragmatic hiatus, and esophagogastric junction oriented by longitudinal vessels - if present, before the fiberscope was inserted into the stomach to avoid any push and pull effect of the endoscope. SCJ was defined as the distinct color difference between a whitish-gray smooth epithelium and reddish-orange velvety gastric mucosa. The diaphragmatic hiatus was defined at the point where the tubular esophagus flared to become the sac-like stomach. The distal limit of the longitudinal vessels emanating from the SCJ was defined as the esophagogastric junction[29,30]. Therefore, the area of longitudinal vessels located distal to the SCJ can be considered BE without any requirement of histological evidence[3,4], according to the Japan Esophageal Society (JES). Therefore, the area between the diaphragmatic hiatus and distal end of the longitudinal vessels, or between the diaphragmatic hiatus and SCJ in the case where no longitudinal vessels are observed, is recognized as a hiatus hernia. The BE was divided into two types according to its length: long segment BE (LSBE), when circumferentially recognized with a minimal length of 3 cm or more, or short segment BE (SSBE), for length of less than 3 cm. SSBE was further categorized as circumferential (cSSBE) and partial (pSSBE) types.

The chronological changes for BE during the follow up period were categorized into 6 groups using the following nomenclature: (1) disease free, no BE recognized; (2) persistence, pSSBE or cSSBE at the first endoscopy and no change thereafter; (3) appearance, no SSBE at the first endoscopy with a subsequent appearance of pSSBE or cSSBE at a follow-up endoscopy; (4) progression, progression from pSSBE to cSSBE; (5) complete regression, disappearance of pSSBE or cSSBE; and (6) partial regression, a regression from cSSBE to pSSBE. The background factors that potentially affect BE change included age, gender, antacid therapy use, GERD-suggested symptoms, esophagitis, and a hiatus hernia. GERD-suggested symptoms included heartburn, regurgitation, dysphagia, odynophagia, epigastralgia, belching, nausea and vomiting, and non-cardiac chest pain, as proposed in the published questionnaires. Esophagitis was diagnosed according to the Los Angeles Classification[31], and a grade A or higher was considered evidence of its presence. Use of a histamine-2 receptor antagonist or PPI was considered antacid therapy. These factors, except age, were dichotomized by categorizing either a presence or absence.

Statistical analysis

The relationship between these factors and changes in BE were investigated by univariate and multivariable[32] analyses. Fisher's exact test and Student's t test were respectively used for comparison of categorical and two mean values. Kaplan-Meier curves were used for the chronological changes for BE, especially for appearance and complete regression. For patients classified as disease-free at the first endoscopy, disease-free probability was calculated by using a time-length between the date of the first endoscopy and the date when the first appearance of pSSBE or cSSBE was noticed. For patients with SSBE at their first endoscopy, complete regression probability was calculated by using a time-length between the date of the first endoscopy and the date when the complete regression of pSSBE or cSSBE was first noticed.

RESULTS

The 779 patients were followed prospectively by a total of 2712 endoscopies for an average of 40.7 ± 21.3 mo (range, 6-81 mo) comprising a total of 31720 patient-months. Patient baseline characteristics are presented in Table 1. Overall, 292 (37.5%) patients took PPI or histamine-2 receptor antagonists.

The patient distributions of the 6 categories of BE change are given in Figure 1. The incidence of SSBE at the first endoscopy was 21.7% (169 patients). Among these, complete regression and progression from pSSBE to cSSBE was respectively observed in 104 (61.5%) and 7 (4.1%) patients at their first endoscopy, while 49 (29.0%) SSBE remained stable

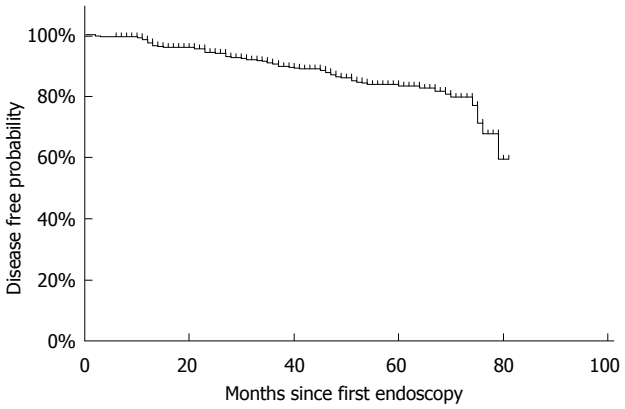

Figure 2 Kaplan-Meier curve illustrating disease free probability in patients with no short segment endoscopic Barrett esophagus at the first endoscopy.

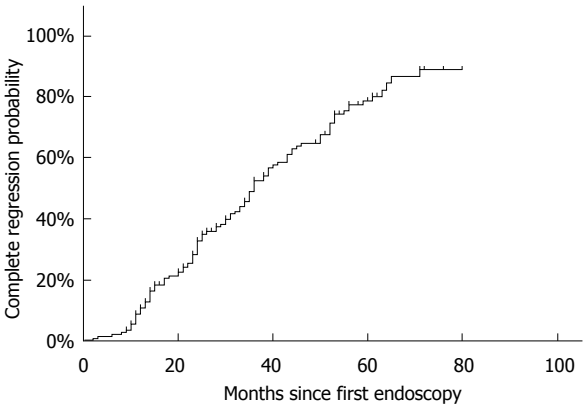

Figure 3 Kaplan-Meier curve illustrating complete regression probability in patients with short segment endoscopic Barrett esophagus at the first endoscopy.

during the study period. Among the 610 disease-free patients at the first endoscopy, SSBE developed in 74 patients, accounting for 12.1% of the appearance rate. None of the SSBE progressed to LSBE.

Kaplan-Meier analyses revealed that the appearance and complete regression occurred constantly over time (Figures 2 and 3). Five-year cumulative disease-free and complete regression probabilities were 83% and 80%. This meant that 5-year and annual appearance probabilities of SSBE were respectively 17% and 3.4%. The median persistent period of SSBE who experienced complete regression was 36 mo.

Multivariable Cox regression analysis revealed that a hiatus hernia was the only significant factor which both facilitates BE appearance and hampers BE regression (Table 2). Esophagitis appeared to be a marginally significant factor that hampers BE regression.

DISCUSSION

The strength of our study is the simultaneous, multivariate, and longitudinal analyses investigating time trends of appearances or regressions of SSBE. Our main findings are that both the appearance and complete regression of SSBE occurred steadily over time, and that a hiatus hernia was the strongest and the only significant factor related to both phenomena.

In the West, the diagnosis of BE requires multiple, systematic targeted biopsies confirming specialized intestinal metaplasia[1] or columnar lined epithelium[2], as well as an endoscopic diagnosis of BE following Prague C and M criteria[33]. The proximal margin of the gastric fold is considered the gastroesophageal junction. On the other hand, it is widely accepted in Japan that longitudinal vessels emanating from, and located distally to the SCJ, can be considered BE, and no histological evidence of a goblet cell is mandatory[3,4]. We have previously discussed the merits of the Japanese criteria with regard to easy adoption of these criteria which enables endoscopists to facilitate BE description even for patients with conditions liable to bleeding[5]. Especially, the upper limit of the gastric folds is not located at a fixed position because it moves upward and downward according to breathing and the distending volume of air in the esophagus[34]. Noteworthily, the Western experts also recognized the distal margin of the palisade vessels and value of the Japanese criteria[35,36].

We have found in this study that a new appearance of SSBE occurred constantly over time, the 5-year cumulative appearance rate being approximately 17%. To the best of our knowledge, this is the first report elucidating longitudinal time-trend profiles of new appearances of BE. Furthermore, a progression from pSSBE to cSSBE was seen in only a small fraction of patients (4.1%), findings which are indirectly supported by the observation of the occurrence of BE in a fairly rapid fashion, quickly reaching its maximal length with little increase thereafter[37].

In the present study, the complete regression rate was 61.5% at a mean follow-up length of 41 mo, and the 5-year cumulative complete regression rate was 80%. Controversies appear to exist in the literature concerning the rates and time-trend of BE regression. The complete regression rate in the present study is higher than those in the literature, ranging from 7%-44%[16,22,27,28]. In addition, antireflux surgery achieved steady SSBE regression[38], while a lack of further regression even after PPI therapy was noted[20]. However, direct comparisons of regression rates between previous studies and ours must be viewed with caution. In the previous studies, most patients were LSBE or the initial length of BE was at least 5 mm[16,22,27], while only SSBE patients even with partial types, regardless of length, were included in the present study. Although different criteria of SSBE at study entry undoubtedly make investigations difficult to compare, our results suggest that the cohort including pSSBE exhibits a substantial rate of regression. Indeed, the probability of BE regression may be length dependent[23,27] - a longer length of BE

Table 2 Factors associated with appearance or regression of e short segment Barrett esophagus

		Appearance		Complete regression	
		Hazard ratio (95%CI)	*P* value	Hazard ratio (95%CI)	*P* value
Age		0.98 (0.96-1.00)	0.11	1.00 (0.98-1.02)	0.750
Gender	Male	1		1	
	Female	1.01 (0.63-1.62)	0.98	1.32 (0.88-1.97)	0.180
Antiacid therapy	Absent	1		1	
	Present	1.19 (0.74-1.93)	0.47	0.73 (0.48-1.14)	0.170
GERD-suggested symptoms	Absent	1		1	
	Present	0.77 (0.46-1.19)	0.22	0.98 (0.64-1.50)	0.920
Esophagitis	Absent	1		1	
	Present	1.09 (0.55-2.15)	0.80	0.51 (0.26-1.01)	0.052
Hiatus hernia	Absent	1		1	
	Present	8.66 (1.20-62.6)	0.03	0.13 (0.03-0.58)	0.007

Multivariable Cox regression analysis. GERD: Gastroesophageal reflux disease.

is less likely to regress completely.

Our multivariable analysis revealed that presence of a hiatus hernia was the only significant factor that both facilitates BE appearance and hampers BE regression. In addition, esophagitis was a marginally significant factor that hampers BE regression. These findings were supported by previous findings of extremely higher rates of a hiatus hernia among BE patients[19]. In addition, management of reflux resulted in a higher likelihood of BE regression, since antacid medication[20,21], antireflux surgery[38,39], and the presence or absence of hiatal hernia[22] were noted to be significant factors associated with BE regression. Given the role of antacid therapies on BE regression, it is conceivable that reduced lower esophageal sphincter pressure and subsequently developed hiatus hernia may promote BE. These considerations imply the importance of acid in the pathogenesis of BE.

Hiatus hernia is placed at the upstream condition that plays a causal role on lower esophageal disorders such as GERD, esophagitis, and eventual prescription of antacid medication. This provides one explanation why other background factors than hiatus hernia were not selected as significant factors associated with BE change. In this regard, the mode of categorizing hiatus hernia may be important, and a mere dichotomized category of hiatus hernia in the present study is a potential limitation. However, as discussed earlier, the length of hiatus hernia may not be fixed and therefore, the presence of hiatus hernia - a proof of reduced lower esophageal sphincter pressure - is applied in the present study rather than its length. Another potential limitation in this study is that body mass index or degree of obese - another potential factor that associated with hiatus hernia - was not included as a background factor. However, it should be noted that an association between obesity and BE has been controversial[40,41].

Surprisingly, our multivariable analysis elucidated that GERD-related symptoms were not a significant factor in the appearance or regression of BE. Although reflux symptoms correlated with longer BE, GERD alone is not sufficient to recommend screening an upper gastrointestinal endoscopy for identifying BE[42]. We have previously demonstrated that two-thirds of SSBE patients had silent symptoms, suggesting that asymptomatic patients do carry SSBE and their appearance cannot be predicted by symptoms. These previous findings from ours[5] and others[42-45] could raise awareness that changes in findings around the region of SCJ, either appearance or complete regression, could occur regardless of GERD-related symptoms. This could further imply that excluding patients from screening based on a lack of GERD-related symptoms could surpress chances of detection of esophageal adenocarcinoma at an early, presymptomatic stage.

In the present study, only 7 patients (4.1%) experienced progression from pSSBE to cSSBE. The small number of patients experiencing the progression of SSBE in the present study precludes drawing a firm conclusion with regard to factors affecting BE progression. The progression rate of 4.1% in the present study was comparable to a previous study[46], in which the majority of SSBE patients remained stable and its elongation was observed in only 6% of patients.

Our clinic is one of the institutions which cover over 130000 residents. Under these circumstances, our study was conducted at a single community unit with patients undergoing an endoscopy regardless of GERD symptoms. Our clinic is a primary institution where the mean number of endoscopies performed is approximately 600 per year. Our study holds up as a representative population sample, being aged 27 to 95 years of residents easily accessible to our clinic, and thus may differ from all the potentially biased cohorts reported earlier. This could further support the idea that the regression rate could be extrapolated to the general Japanese population. Although our study did not include Caucasian patients, endoscopists should continuously be aware of a BE story - both the regression and appearance of SSBE steadily occurring - under the circumstances of a substantial incidence of

hiatus hernias and with the knowledge that substantial numbers of BE patients are asymptomatic[5,37,42].

In conclusion, both appearances and complete regressions of SSBE occurred over time. A hiatus hernia was the only significant factor affecting the BE story, while substantial numbers of BE patients are asymptomatic.

COMMENTS

Background

Despite a growing awareness of Barrett esophagus (BE) in the context of the risk of esophageal adenocarcinoma, knowledge on time-trends of appearances and changes of BE, especially short segment endoscopic BE (SSBE), is scarce in the literature.

Research frontiers

Against the background of BE as a susceptible lesion of esophageal adenocarcinoma, it is important to elucidate longitudinal changes for BE and factors associated with it. With easy adoption by general practitioners, the authors applied endoscopic diagnoses of BE by detecting longitudinal vessels proposed by the Japanese criteria. No requirement of histological findings enables the facilitation of an endoscopic diagnosis of BE among the general population and for general practitioners.

Innovations and breakthroughs

The appearance or complete regression of BE occurs constantly over time. Both phenomena are associated with a hiatus hernia but not gastroesophageal reflux disease-suggested symptoms, suggesting that the appearance of BE occurs silently among a non-biased study population that resembles that seen by the general practitioner.

Applications

Despite the incidence of a hiatus hernia being dependent on its diagnostic criteria, a higher incidence of hiatus hernias and the existence of silent SSBE motivates endoscopists to assess the distal esophagus in all patients undergoing an upper endoscopy for any indication. This stance allows the detection of SSBE at an early stage to enable patients to enter a follow-up program as well as the early detection of eventual esophageal adenocarcinoma.

Terminology

Endoscopic BE is defined according to the Japan Esophageal Society. The existence of longitudinal vessels emanating from the squamocolumnar junction is defined as endoscopic BE. BE is classified into two categories according to its length, long segment BE being 3 cm or more, and short segment BE being less than 3 cm. The length is further classified as partial and circumferential types. Changes in BE was classified as disease free, or showing persistence, appearance, progression, complete regression, and partial regression.

Peer-review

Although this study is based on the endoscopic diagnosis of BE, the authors' unbiased population could be considered more suitable than surrogate unrepresentative clinical samples for quantifying the magnitude of the BE story. This study contributes important evidence and reliable connections between the BE story and a silent hiatus hernia, and thus provides guidance for not overlooking the chance for the detection of BE as well as subsequent esophageal adenocarcinoma at an early, presymptomatic stage.

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35>