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REVIEW

Antiviral therapy of hepatitis C as curative treatment of indolent B-cell lymphoma

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Abstract

The association of hepatitis C virus (HCV) and B-cell non-Hodgkin lymphomas (NHL) has been highlighted by several epidemiological and biological insights; however the most convincing evidence is represented by interventional studies demonstrating the capability of antiviral treatment (AT) with interferon (IFN) with or without ribavirin to induce the regression of indolent lymphomas, especially of marginal-zone origin. In the largest published retrospective study (100 patients) the overall response rate (ORR) after first-line IFN-based AT was 77% (44% complete responses) and responses were sustainable (median duration of response 33 mo). These results were confirmed by a recent meta-analysis on 254 patients, demonstrating an ORR of 73%. Moreover this analysis confirmed the highly significant correlation between the achievement of viral eradication sustained virological response (SVR) and hematological responses. Two large prospective studies demonstrated that AT is associated with improved survival and argue in favor of current guidelines' recommendation of AT as preferential first-line option in asymptomatic patients with HCV-associated indolent NHL. The recently approved direct-acting antiviral agents (DAAs) revolutionized the treatment of HCV infection, leading to SVR approaching 100% in all genotypes. Very preliminary data of IFN-free DAAs therapy in indolent HCV-positive NHL seem to confirm their activity in inducing lymphoma regression.

Key words: Non-hodgkin lymphomas; Hepatitis C virus; Antiviral therapy; Interferon; Ribavirin; Sofosbuvir; Direct-acting antiviral agents; Marginal zone lymphomas

Core tip: In the last decade many clinical studies demonstrated that front line antiviral therapy (AT)

with interferon (IFN) and ribavirin, is able to induce a 70%-75% response rate in patients with hepatitis C virus (HCV)-associated indolent non-Hodgkin lymphoma who do not need immediate conventional treatment. Hematological response was durable, and invariably related to the viral eradication. International guidelines indicate that AT should be the treatment of choice in such patients. Very preliminary data about the use of the new direct-acting antiviral agents (DAAs) suggest a similar activity in inducing lymphoma response. We discuss available literature about IFN-based AT and preliminary experiences with DAAs in the treatment of HCV-associated indolent lymphomas.

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INTRODUCTION

The association between hepatitis C virus (HCV) and B-cell non-Hodgkin lymphomas (NHL) is now widely accepted, as a result of the large body of evidences from epidemiological, biological and especially therapeutic studies carried out in the last 15 years. Among B-cell NHL indolent subtypes, HCV has been consistently associated with marginal-zone lymphomas (MZL), while within aggressive histotypes the highest correlation has been found with diffuse large B-cell lymphomas (DLBCL)[1-3].

The most convincing argument in favor of an etiological link between HCV and specific histotypes of B-cell NHL is indeed represented by the highly frequent success of HCV-directed antiviral therapy (AT) in inducing lymphoma regression in patients with HCV-positive indolent lymphomas. Across all the published studies, lymphoma response has been significantly related to the obtainment of viral eradication. As a result, current international guidelines support the use of front-line AT in asymptomatic patients with HCV-associated indolent lymphomas who do not require immediate conventional immune-chemotherapy approach[2].

Many pathogenetic models have been proposed to elucidate possible mechanisms of HCV-related lymphomagenesis and consequential lymphoma regression induced by AT-induced viral eradication: The most accepted and supported by experimental evidences rely on chronic antigenic stimulation of lymphocyte receptors by viral antigens resulting in B cells proliferation, similarly to the *Helicobacter pylori*-induced gastric MALT lymphoma ("antigen-driven proliferation")[1] and, alternatively, on HCV replication inside B-cells with oncogenic effects mediated by intracellular viral proteins HCV ("intracellular viral replication"). Also from the biological point of view a great expectation is represented by the introduction in HCV-related B-cell non-Hodgkin lymphomas (B-NHL) of the new direct-acting antiviral agents (DAAs) that, after nearly 25 years of interferon (IFN)-based therapy, are revolutionizing HCV treatment by inducing nearly 100% sustained virological responses (SVRs). As the cytostatic effect of IFN could not be completely ruled out, the evaluation of new "IFN-free" AT as curative-intent primary treatment of HCV-associated indolent lymphomas may ultimately clarify the real grade HCV-dependency of different lymphoma subtypes. Recent case reports seem to confirm the efficacy of different new schedules of IFN-free AT in inducing lymphoma regression, however more large and possibly prospective series are needed to clarify this issue. If this would be proven by prospective studies, IFN-free AT may be regarded as a real "chemo-free" targeted therapy for HCV-related indolent lymphomas.

The aim of this paper is to summarize the up-to now reported experiences with both IFN-based and new DAA-based AT delivered as curative treatment in patients with HCV-associated indolent B-NHL.

HCV INFECTION AND INDOLENT LYMPHOMAS

Epidemiological studies

It is estimated that 3% of the world population (170 million people) is chronically infected by HCV. HCV prevalence varies consistently in different geographic areas, with lowest rates being reported in Northern Europe and Scandinavia (0.1%-0.4%) while in Italy, Egypt, Japan and southern parts of United States, prevalence estimates exceeds 2%. HCV has been linked to a spectrum of lymphoproliferative disorders with or without cryoglobulinemia[2]. The first evidences about the link between HCV and B-NHL were established by epidemiologic studies. A recent updated meta-analysis including 19 case-control studies (9038 cases and 12224 controls) and 4 cohort studies confirmed the previous reported data of 2.0-2.5-fold increased risk of developing B-cell NHL in HCV-infected patients (2.4 relative risk in case-control studies and 2.0 in cohort studies), with higher risk in countries with higher HCV prevalence[3]. For instance, in countries with high HCV prevalence like Italy (4.4%; 1.6% in the North and 7.3% in the South)[4], the fraction of B-cell NHL attributable to HCV may be estimated close to 10%, while in countries with low prevalence as in Northern Europe only < 1% of B-NHL may be considered to be linked to HCV[5]. Concerning specific NHL subtypes, the large international "*Interlymph*" study reported a significant association of HCV infection and MZL (OR = 2.47), DLBCL (OR = 2.24), and lymphoplasmacytic lymphoma (LPL, OR = 2.57)[6]. Moreover, retrospective studies reported a high HCV seroprevalence among patients features suggesting

the transformation from a previously unrecognized low with MZL and DLBCL[11-13]. Notably, many studies focusing on HCV-positive DLBCL showed histologic as well as molecular -grade lymphoma, mainly MZL[12-15].

An interesting confirmation of the association of HCV and B-cell NHL may be argued by a Japanese large cohort prospective study evaluating the differential rate of NHL development in two cohorts of HCV-infected patients treated (n = 2701) or not (n = 501) with IFN-based AT. While in patients who received IFN without obtaining SVR the rate of new diagnosis of NHL resulted similar to those who did not receive IFN (0.4%, 1.5% and 2.6% at 5, 10 and 15 years, respectively), no patient obtaining SVR after AT developed NHL. In other words, the eradication of HCV infection by AT was able to prevent the development of lymphoma[16]. A confirmation of these findings was recently furnished by a Taiwanese population-based cohort study (11.679 HCV and 46716 non-HCV patients followed for 8 years). The incidence rates of NHL were significantly greater in the HCV cohort than the non-HCV cohort (37.0 *vs* 17.5 per 100000 person-years) and multivariate analysis showed that HCV infection was associated with an increased rate of NHL (HR = 2). Noteworthy, similarly to the Japanese study, the incidence rate of NHL development was 0 per 100000 person-years in HCV-patients who received IFN-based AT (n = 958) compared with 41 per 100000 person-years in the untreated group[17].

Clinical and pathological studies

In the WHO classification (2008) three MZL entities are listed[18]: SMZL, NMZL and extranodal MZL of MALT-type (MALT lymphoma). Marginal zone B-cells play an important role in various infectious and autoimmune conditions and marginal zone-related neoplasms often retain features of these cells. In addition to epidemiological studies, the association between HCV and MZL is supported by several large clinical-pathological MZL series, especially in SMZL. In a large multicentre series of SMZL from Italy HCV serology was positive in 49 out of 255 available cases (19%), who presented with more frequent presence of nodal disease, cryoglobulinemia and serum monoclonal component[7]. In 2005, French authors described a clinical trial of SMZL with villous lymphocytes, type Ⅱ mixed cryoglobulinemia and HCV infection in 18 patients[24]. NMZL is a rare entity accounting for less than 2% of NHL; in the largest published series[10], HCV serology was positive in 9 out of 38 patients (24%). Considering MALT lymphomas, a multicenter Italian study on 172 patients, pointed out the high rate of positivity of HCV serology (35%), with 92% of tested cases being positive for HCV-RNA. Interestingly, a prominent prevalence of HCV infection was reported in three peculiar MALT lymphoma sites: Salivary glands (47%), skin (43%) and orbit (36%)[10]. At this regard a new peculiar clinical and pathological entity of extranodal MALT MZL of the skin has been characterized in 12 HCV-positive patients, who presented single or multiple subcutaneous "lipoma-like" nodules and displayed indolent clinical course and responsiveness to AT[25]. LPL and Waldenström macroglobulinemia have been associated with HCV infection and mixed cryoglobulinemia in some but not all series, perhaps related to geographic differences[26]. Follicular lymphoma (FL) and small lymphocytic lymphoma (SLL), on the contrary, have been rarely associated with HCV infection. Other residual cases of low-grade miscellaneous lymphoproliferative disorders frequently reported as low-grade "B-NHL not otherwise specified" (B-NHL NOS), characterized by exclusive bone marrow and leukemic involvement with flow cytometry features different from those of chronic lymphocytic leukemia (CLL), have been sometimes associated with HCV infection[27].

Besides reports focusing specifically on single HCV-associated histotypes of indolent NHL, two different large prospective observational studies provided a comprehensive overview of subtypes distribution and clinical picture of HCV-positive NHL. The French "ANRS Lympho-C" observational study enrolled between 2006 and 2012 116 consecutive patients with HCV infection and a new diagnosis of NHL, including 39%MZL and 39% DLBCL. Median age was 62 years, 52% had genotype 1 and 25% genotype 2. Patients with MZL had more frequently serum rheumatoid factor positivity (68% *vs* 35%) and monoclonal component (74% *vs* 44%) with respect to patients with DLBCL[28].

ANTIVIRAL TREATMENT OF HCV-POSITIVE INDOLENT LYMPHOMAS

The seminal work by Hermine *et al*[30] in SMZL with villous lymphocytes in 2002 has opened the way to several following consistent data from the literature demonstrating that AT should be considered as a reliable frontline approach in HCV-associated indolent lymphomas when there is no immediate need of conventional treatment. Such behaviour has been recommended by recently updated haematological (ESMO[31] and NCCN[32]) and hepatological (EASL[33]) international guidelines. Among specific NHL subtypes, this treatment modality has been more frequently adopted in MZL although many studies in IFN era extended the validity of this approach for all indolent histologies when associated to HCV infection. Table 1 summarizes the results of anti-lymphoma IFN-based AT experiences [with or without ribavirin (RBV)] in low-grade NHL.

On the contrary, front-line AT it is clearly insufficient in HCV-positive aggressive lymphomas and could not be considered as a curative treatment modality in this setting, in which an immediately active therapy is needed. However AT may be a logical recommendation in HCV-positive DLBCL after completion of standard

Table 1 Interferon-based antiviral treatment in hepatitis C virus-infected patients with low-grade non-Hodgkin lymphomas

	Year	No. of patients	Diagnosis	Genotypes	Cryoglobulinemia	Antiviral treatment	Virologic response	NHL response
Bauduer[35]	1996	1	MZL of MALT (oral cavity)	NA	-	α-IFN	1	1 PR
Caramaschi *et al*[36]	1999	1	MZL of MALT (salivary glands)	NA	-	α-IFN	NA	1 CR
Moccia *et al*[37]	1999	3	SMZL	NA	-	α-IFN	NA	2 CR
Patriarca *et al*[38]	2001	1	LPL	2a/2c	-	α-IFN	1	1 CR
Hermine *et al*[30]	2002	9	SLVL	NA	6	α-IFN	7	7 CR
Casato *et al*[39]	2002	1	Leukemic MZL	NA	1	α-IFN	Decreased HCV-RNA	1 CR
Pitini *et al*[63]	2004	2	SMZL	NA	-	α-IFN	2	2 CR
Tursi *et al*[64]	2004	16	MZL of MALT (stomach)	NA	-	α-IFN-2b + RBV	11/16	16 CR
Kelaidi *et al*[41]	2004	8	SMZL (*n* = 4)	3a (*n* = 1), 5a (*n* = 1)	8	α-IFN-2b + RBV	5 SVR, 2 PR	5 CR
			Disseminated MZL (*n* = 1)	-				
			Leukemic MZL (*n* = 1)	1b				
			MZL of MALT (*n* = 2) (1 duodenus; 1 ileus)	4c/4d				
Vallisa *et al*[42]	2005	13	SMZL (*n* = 4)	1b (*n* = 2), 2b (*n* = 1)	5	Peg-IFN + RBV	7 SVR, 1 PR	7 CR, 2 PR
			NMZL (*n* = 2)	2a/2c (*n* = 1), 1b (*n* = 1)				
			MZL of MALT (*n* = 2)	1b (*n* = 2)				
			FL (*n* = 1)	2a				
			LPL (*n* = 4)	2a/2c (*n* = 1), 1b (*n* = 1), na (*n* = 2)				
Svoboda *et al*[65]	2005	1	MZL of MALT (salivary gland, liver)	2b	-	Peg-IFN + RBV	1	CR
Saadoun *et al*[24]	2005	18	SLVL	1 (*n* = 7) 2 (*n* = 4) 3 (*n* = 1) 4 (*n* = 1)	18	α-IFN (+ RBV in 10)	14 CR, 4 PR	14 CR, 4 PR
Paulli *et al*[25]	2009	2	Subcutaneous MZL of MALT	2a/2c, 2b	2	Peg-IFN + RBV	2 CR	1 CR, 1 PR
Mazzaro *et al*[43]	2009	18	1 SLVL	1b (*n* = 11)	13	α -IFN + RBV (*n* = 8)	9 SVR	9 CR, 4 PR
			1 FL	2a/2c (*n* = 7)		Peg-IFN + RBV (*n* = 10)		
			16 LPL					
Oda *et al*[66]	2010	1	B-NHL (liver)	2a	-	Peg-IFN + RBV	SVR	CR
Pellicelli *et al*[67]	2011	9	3 SMZL	1b (*n* = 2)	4	Peg-IFN + RBV	7 SVR	5 CR, 2 PR
			3 MZL of MALT	2 (*n* = 2)				
			1 NMZL	2a (*n* = 2)				
			2 FL	2a/2c (*n* = 3)				
Mauro *et al*[68]	2012	1	LPL	1b	1	Peg-IFN + RBV (2nd line)	SVR	CR
Arcaini *et al*[45]	2014	100	23 SMZL	1 (*n* = 37)	34	α-IFN (*n* = 33) (+ RBV in 26)	80% SVR	44% CR, 33% PR
		(1st-line)	12 NMZL	2 (*n* = 52)		Peg-IFN (*n* = 67) (+ RBV in 57)		
			25 MZL of MALT	3 (*n* = 5)				
			7 LPL	5 (*n* = 1)				
			5 FL	NA (*n* = 5)				
			1 SLL					
			27 Low-grade NHL NOS					
		34	12 SMZL	1 (*n* = 15)	10	α-IFN (*n* = 14) (+ RBV in 10)	67% SVR	56% CR, 29% PR
		(2nd-line)	2 NMZL	2 (*n* = 13)		Peg-IFN (*n* = 20) (+ RBV in 15)		
			6 MZL of MALT	3 (*n* = 2)				
			2 LPL	5 (*n* = 1)				
			7 FL	NA (*n* = 4)				
			3 SLL					
			2 Low-grade NHL NOS					

Michot *et al*[28]	2015	14 (AT alone)	14 MZL	NA	NA	Peg-IFN + RBV (n = 12)	79% SVR (AT alone)	57% CR, 21% PR
		8 (At + Rituximab)	8 MZL	NA	NA	PegIFN + RBV + 4 Rituximab (n = 8)	NA (AT + Rituximab)	38% CR, 62% PR

SMZL: Splenic marginal zone lymphoma; NMZL: Nodal marginal zone lymphoma; SLVL: Splenic lymphoma with villous lymphocytes; MZL: Marginal zone lymphoma; FL: Follicular lymphoma; LPL: Lymphoplasmacytic lymphoma; MCL: Mantle cell lymphoma; SLL: Small lymphocytic lymphoma; NHL: Non-Hodgkin lymphoma; NOS: Not otherwise specified; IFN: Interferon; RBV: Ribavirine; CR: Complete response; PR: Partial response; SVR: Sustained virologic response.

R-CHOP immune-chemotherapy with the aim to eliminate a potential lymphoma trigger and reduce the risk of relapse[14,34].

First experiences (2002-2011) in the interferon era

The majority of the initial studies reporting the use of IFN as primary lymphoma treatment in HCV-infected patients diagnosed with a lymphoproliferative disorder relied on single case reports or small case series[35-39], and included also patients with type-2 mixed cryoglobulinemia with evidence of B-cell monoclonality (IgH and/or Bcl-2 rearrangement)[40].

In 2002 Hermine *et al*[30] reported data on AT in 9 patients with splenic lymphoma with villous lymphocytes and HCV infection treated with IFN-2αb. Six patients presented also symptomatic cryoglobulinemia. Complete hematological response and SVR were observed in 7 out of 9 patients. The remaining 2 patients who did not respond were subsequently treated with IFN plus RBV and obtained HCV-RNA clearance as well as lymphoma response [one complete response (CR) and one partial response (PR)]. In contrast, none of 6 matched patients with SLVL without HCV infection treated with IFN experienced any grade of lymphoma regression.

In 2005 the same French group expanded these results in 18 patients with chronic HCV infection, mixed cryoglobulinemia and SLVL treated with IFN (+ RBV in 10). Fourteen patients (78%) obtained a CR after clearance of HCV-RNA. Two patients who obtained only a virologic PR and 2 non-responders achieved nevertheless a PR of lymphoma, with an overall response rate (ORR) of 100%[24].

Another study reported the use AT with IFN and RBV as first-line treatment in 8 HCV-positive patients with different MZL subtypes (4 SMZL with or without villous lymphocytes; 1 disseminated MZL, 1 leukemic MZL and 2 intestinal MALT-lymphomas): Overall, 5 out of 8 patients (60%) obtained a CR of lymphoma, which was related to SVR in the majority of cases[41].

Among most significant initial experiences, an Italian multicenter study reported results of AT in 13 HCV-infected patients with various low-grade B-NHL subtypes[42]. All patients received peg-IFN and RBV, 10 as first-line and 3 as second or third-line of therapy. Among 12 assessable patients, 7 achieved CR, 2 PR (ORR = 75%), 2 had stable disease (SD) and one progressed during therapy. Similarly to previous reports, hematologic responses resulted significantly associated to clearance of HCV viral load, as 7 out of 9 responders achieved prior SVR. Although number of cases was small, this study suggested for the first time that AT with peg-IFN and RBV may be equally effective also in a wide range of HCV-positive low-grade NHL subtypes other than MZL, as CRs were actually observed without significant differences in all indolent NHL histologies (2 out of 4 non-MZL and 5 out of 8 MZL).

In 2009 Mazzaro *et al*[43], reported a series of 18 patients with HCV-positive low grade B-cell NHL (16 LPL, 1 FL, 1 SLVL) treated frontline with PEG-IFN or standard IFN (plus RBV). SVR and CRs resulted higher in the group treated with PEG-IFN (6 out of 10 patients, 60%) with respect to the group treated with standard IFN (3 out of 8 patients, 37%). All the 9 patients who obtained SVR experienced also CR of lymphoma, thus confirming the previously found strict relationship between the achievement of virological and hematological CR.

Most recent studies (2014-2015)

In 2014, the Fondazione Italiana Linfomi (FIL) reported data[45] on 100 patients with HCV-positive indolent NHL (23 SMZL, 12 NMZL, 25 MALT-lymphomas, 7 LPL, 5 FL, 1 SLL, 27 indolent B-NHL NOS), all characterized by an indolent course of disease without the need to receive immediate conventional anti-lymphoma therapy, were treated with first-line AT. Thirty-three patients received IFN and 67 received PEG-IFN-based AT (with or without RBV). Six patients discontinued AT due toxicity, while 7 patients interrupted early AT due to lymphoma progression and lack of virological response. Forty-four (44%) patients achieved a CR and 33 (33%) a PR, with an ORR of 77%; 14 patients had SD. Median duration of response (DOR) was 33 months. A SVR was achieved in 80 patients (80%). Lymphoma response resulted significantly associated to the achievement of a SVR (P = 0.003) while it was not recorded a significant difference in ORR between patients with MZL or non-MZL histology (82% *vs* 70%, P = 0.3). At a median follow-up of 3.6 years, 9 patients progressed and 13 experienced lymphoma relapse after initial response to AT, with a resulting 5-year PFS of 63%. Five-year OS was 92%; only 2 patients died due to lymphoma progression.

Thirty-four patients were treated with second-line AT for relapse after a conventional first-line therapy: Among them, 19 (56%) achieved a CR and 10 (29%)

a PR (ORR 85%); a SVR was achieved in 22 patients (67%). The median DOR was 26 months and 5-year PFS was 63%.

These recent data unequivocally confirm the high rates of lymphoma regression in patients with HCV-positive indolent NHL treated with AT without significant differences between various histologies, although MZL represent the more frequent subtype. Moreover, the established association of hematological response with virological response confirms previous findings and is in accordance with the proposed pathogenetic model of chronic antigenic stimulation in HCV-positive indolent lymphoma, thus underlying the importance of virus eradication.

Another large cohort of patients with HCV-NHL (n = 116), including 45 patients with MZL, was recently published. However, of 38 patients with MZL treated with IFN-based AT, only 14 received AT alone, while 8 patients with high tumor burden were treated with AT with the addition of 4 weekly dose of rituximab, a schedule adopted from therapy of type 2 cryoglobulinemia. Among the 14 patients receiving with AT alone, 8 obtained a CR and 3 a PR (ORR 79%), while all patients treated with the combination of AT and rituximab responded (3 CR, 5 PR; ORR 100%).

A highly relevant finding confirmed independently by the two last cited studies is represented by the positive impact of AT on the prognosis of the patients with HCV-associated indolent lymphoma. The FIL "*HCV-LNH outcome survey*" included 704 consecutive HIV-negative HCV-positive patients with indolent NHL diagnosed and treated from 1993 to 2009 in 39 centres of the FIL; 134 patients received AT as first or second-line therapy, as previously described. In the whole cohort, 5-year OS was 78% and 5-year PFS was 48%. In multivariate analysis, use of AT during the patients' life (*i.e.*, as first-line or as subsequent line of therapy) had positive impact on OS. In details, in patients who performed AT the overall risk of death was significantly reduced (HR = 0.21, P = 0.014)[45]. Similarly, in the French *"ANRS Lympho-C Study"* the use of AT resulted significantly associated with improved OS in MZL at multivariate analysis (HR = 0.11)[28]. This obviously means that, at least in these unselected retrospective series, AT is able to prolong the survival of patients with HCV-positive indolent NHL and further emphasizes the validity of this treatment strategy as the cornerstone treatment of HCV-related in indolent NHL in a long-term perspective.

A recently published meta-analysis specifically evaluated this issue. The primary endpoint was the correlation between SVR and lymphoma response, while secondary endpoints were overall lymphoma response rate and differential efficacy within various histotypes. Overall, 254 patients from 20 studies were included. Overall lymphoma response rate following AT was 73% (95%CI: 67%-78%) and a strong statistical association between SVR and lymphoma response was confirmed: In particular patients obtaining SVR displayed 83% response rate compared to 53% response rate of those failing to achieve SVR (P = 0.0002). A trend towards a better response to AT in HCV-associated MZL (ORR 81%) compared to non-MZL histotypes (ORR 71%, P = 0.07) was observed. In summary, the results of this updated meta-analysis further justifies the current recommendation for AT as first-line treatment in patients who do not need immediate conventional treatment and support the hypothesis of a causal relationship of HCV and lymphomagenesis[46].

MOLECULAR FEATURES OF HCV-ASSOCIATED INDOLENT LYMPHOMAS TREATED WITH INTERFERON-BASED ANTIVIRAL TREATMENT

Only scanty data concerning molecular features of HCV-related indolent NHL, especially in the setting of AT, have been presented, mainly due to the retrospective nature and to the low number of patients analyzed in these studies.

Concerning mutational profile, no difference between HCV-positive and HCV-negative cases was found in the genomic landscape of SMZL by integrating whole-exome sequencing and copy-number analysis. In particular, rate of mutation in NOTCH pathway genes (*NOTCH2* in about 20%-40%, *NOTCH1* in about 5%, *SPEN*, *DTX1* or *MAML2*), NF-αB signaling pathway genes (*IKBKB* in about 10%, *TNAIF3* in about 5%), *KLF2* (about 20%-40%) or TP53 (about 15%) did not differ according HCV-status[47]. Interestingly the only molecular difference between HCV-positive and HCV-negative SMZL was detected by miRNA expression analysis: In particular, HCV-positive SMZL patients revealed a downregulation of the tumor suppressive miR26b[49].

From the immune-genetic point of view, many biologic studies focusing on HCV-related lymphomagenesis, demonstrated that HCV-positive NHL frequently carry signs of somatic hypermutation and preferential usage of restricted repertoire of VH (*e.g.,* V_H1-69) and VL genes (*e.g.,* V_k3-20/15)[50], suggesting a possible role of antigen selection in expansion of the B-cell clone, that would be therefore still antigen-dependent, at least until a certain postulated critical turning-point. According to this hypothesis, stereotyped B-cell receptors were found in 12% SMZL cases, including HCV-positive cases, pointing out the role of antigen selection (both HCV and non-HCV restricted) in SMZL development[51].

On the basis of the previous findings in patients with HCV-related type-Ⅱ mixed cryoglobulinemia, the first interventional studies evaluating AT in patients with NHL looked out to the achievement of molecular response. In the seminal study of Hermine *et al*[30], as well as in the subsequent report by Saadoun *et al*[24],

and in the Italian multicenter study[42], despite the achievement of clinical CR in the majority of patients, none of the patients evaluated by investigation of IgH-specific rearrangement or Bcl2/IgH translocation obtained a molecular response after AT. This finding differed from what reported in patients affected only by type Ⅱ mixed cryoglobulinemia, a benign lymphoproliferative disorder that may evolve in overt in NHL in 5%-10% of cases, where the disappearance of B-cell clones from the blood of HCV-infected patients after AT has been reported[40]. One can speculate that this differential pattern of molecular responses may be related to the more advanced stage of neoplastic transformation at molecular level of B-cells in low-grade NHL, which imply the achievement of a higher grade of antigen-independency with respected to type Ⅱ mixed cryoglobulinemia.

NEW DAAS ERA IN THE TREATMENT OF HCV INFECTION

HCV therapy is undergoing a revolution. After nearly 25 years of improvements of IFN-based therapies, enormous research efforts led ultimately to the license of a large number of new DAAs. DAAs include different classes of antiviral agents that inhibit HCV viral-specific non-structural (NS) proteins: Protease inhibitors (NS3), NS5A replication-complex inhibitors, nucleoside and non-nucleoside NS5B (viral RNA-polymerase) inhibitors[52].

More than 90% of infections were reported to be cured in phase Ⅱ and Ⅲ trials, with or without peg-IFN and/or RBV. A plethora of new DAA with differential activity across different HCV genotypes is undergoing clinical investigation, with the aim to develop IFN- and possibly RBV-free oral regimen with efficacy approaching 100% and without significant toxicity.

Sofosbuvir (SOF) is a nucleotide analog inhibitor of viral NS5B polymerase, the key enzyme mediating HCV-RNA replication. The triphosphate form of SOF mimics the natural cellular uridine nucleotide and is incorporated by the HCV RNA-polymerase into the elongating RNA primer strand, resulting in chain termination. In the "Valence" study the combination of SOF and RBV (12 wk) demonstrated 93% SVR in genotype 2 patients, while in genotype 3 patients the same combination administered for 24 wk obtained 85% SVR[53]. Ledipasvir (LDV) is a potent inhibitor of HCV NS5A, a viral phosphoprotein that plays an important role in viral replication, assembly, and secretion. The combination of LDV (90 mg) and SOF (400 mg) at fixed-dose combination (FDC) has been primarily studied as an all-oral IFN-free combination regimen in treatment-naïve and treatment-experienced patients as the first once-daily single tablet regimen to treat the majority of chronic HCV genotype 1 and 4 infection. LDV-SOF FDC obtained 99% SVR in genotype 1 treatment-naïve patients in "ION-1 study"[54], and 95% in genotype 4 patients in "NIH Synergy study"[55]. Moreover, preliminary data of *Electron-2 study* demonstrated that the combination of LDV-SOF and RBV administered for 12 wk induced 100% SVR in genotype 3 patients[52]. Finally, the recently reported "ASTRAL studies" pointed out the impressive results of the combination of SOF with the new pangenotypic NS5A inhibitor Velpatasvir, which demonstrated SVR of 97%-100% across all 6 genotypes and resulted superior to SOF-RBV in genotype 2 (SVR 99% *vs* 94%) and 3 (SVR 95% *vs* 80%) in a randomized comparison.

Preliminary data about interferon-free antiviral treatment in HCV-associated indolent lymphomas

As previously discussed, the lymphoma regression observed in nearly 75% of patients with IFN-based AT, which has been closely associated with HCV eradication, is strongly in favor of a causative role of HCV in a subset of patients with indolent NHL, although the direct anti-lymphoma properties of IFN cannot be ruled out. For this reason, the introduction in HCV-associated NHL therapy of the highly active DAA-based IFN-free regimens, that demonstrated SVR rates ⩾ 90% also in genotype 1, is expected to definitely clarify this point. To date, six cases of HCV-positive NHL treatment with DAA IFN-free regimen have been reported, three in SMZL, two in MALT-type MZL and one in leukemic MZL[58-61]. In all these cases the SVR obtained with various DAA IFN-free regimens (manly SOF-based) has been followed by partial or complete hematologic response (Table 2).

In details, in the first one case report by Italy, a 42-year old patient carrying genotype 1 HCV infection and SMZL with lymphocytosis has been treated with a 16 wk regimen of Faldaprevir (NS3/NS4 protease inhibitor), Deleobuvir (non-nucleoside NS5B inhibitor) and RBV. This DAA combination led to a RVR (HCV-RNA undetectable after 4 wk) and to a concurrent resolution of splenomegaly and lymphocytosis[58]. In the second case report from France a 57-year old female with genotype 3a HCV infection and stage Ⅳ disseminated MALT-type MZL (breast, humeral shaft and cervical lymph node involvement) has been treated for 4 wk with SOF and RBV and then, after obtainment of HCV-RNA clearance, with a 12-wk regimen with SOF and Daclatasvir (NS5A inhibitor). After 12 wk a SVR was confirmed (SVR12) and a CT scan showed a complete regression of lymphoma localizations (CR), which was still ongoing at the time of publication after 6 mo of follow-up[59]. Three other cases were described by another French paper focusing on the use of DAA in patients with lymphomas. The first patient was a 54-years old female with a history of previous intravenous drug abuse chronically infected with genotype 4 HCV. After diagnosis of a leukemic MZL (6000/mmc clonal B-cells in peripheral blood) with mixed cryoglobulinemia (cryocrit 5%), she underwent to AT with 12-wk of SOF and Simeprevir (NS3/NS4

Table 2 Direct-acting antiviral agents-based antiviral treatment in hepatitis C virus-infected patients with low-grade non-Hodgkin lymphomas

	Year	No. of patients	Diagnosis	Genotypes	Cryoglobulinemia	Antiviral treatment	Virologic response	NHL response
Rossotti *et al*[58]	2015	1	SMZL	1b	Yes (type Ⅱ MC)	FDV + DLV + RBV (16 w)	SVR	PR
Sultanik *et al*[59]	2015	1	MALT MZL (breast, humeral shaft, cervical lymph node)	3a	Yes (type Ⅱ MC)	SOF + RBV (4 w), then SOF + DCV (12 w)	SVR	CR (ongoing at 6 m)
Carrier *et al*[60]	2015	3	1 Leukemic MZL	4	3 (type Ⅱ MC)	SOF - SIM	3 SVR	PR
			1 MALT MZL (kidney)	1b		SOF - SIM + 4 Rtx		CR
			1 SMZL	1b		SOF - DCV		CR
Lim *et al*[61]	2015	1	SMZL	2	No	SOF + RBV (12 w)	SVR	CR (ongoing at 17 m)
Arcaini *et al*[62]	2015	20	9 SMZL	1 (n = 13)	10 (50%)	various	19 SVR	4 CR, 2 PR, 2 SD 1 PD
			1 NMZL (Nodal)	2 (n = 3)		SOF-based regimens		1 CR
			5 MALT MZL	3 (n = 3)				2 CR, 1 PR, 2 PD
			2 Leukemic MZL	4 (n = 1)		(+ 4 Rtx in 1 pts)		1 CR, 1 PR
			2 CLL					2 SD
			1 LPL					1 SD

DAA: Direct-acting antiviral agents; SMZL: Splenic marginal zone lymphoma; NMZL: Nodal marginal zone lymphoma; phocytes; MZL: Marginal zone lymphoma; LPL: Lymphoplasmacytic lymphoma; MC: Mixed cryoglobulinemia; FDV: Faldaprevir; DLV: Deleobuvir; RBV: Ribavirine; SOF: Sofosbuvir; DCV: Daclatasvir; SVR: Sustained virologic response; CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease; Rtx: Rituximab.

protease inhibitor), obtaining SVR12 together with partial hematologic response (1000/mmc clonal B-cells in peripheral blood) and clearance of cryoglobulins. The second patient (66-year old male) carrying genotype 1b and presenting with compensated cirrhosis (Child score 5) was diagnosed with stage Ⅳ SMZL with associated type Ⅱ mixed cryoglobulinemia (cryocrit 79%). He was treated with 24 wk combination of SOF and Daclatasvir, obtaining SVR12, haematological CR and reduction of circulating cryoglobulins (cryocrit 9%). Unfortunately he developed thereafter a metastatic hepatocellular carcinoma. The third patient (66 years-old female, genotype 1b) presented with rapidly progressive chronic renal insufficiency and 7% circulating cryoglobulins. A renal biopsy showed monomorphous small B-cell infiltrate and diagnosis of renal MALT-MZL was made. Due to the pre-dialisis life-threatening renal failure she was treated with 4 weekly doses of rituximab plus AT with SOF and Simeprevir for 12 wk, that enabled to obtain together with SVR12 a significant improvement of glomerular filtrate and the disappearance of MZL infiltrate at the control kidney biopsy (haematological CR), although a residual weak B-cell clonality (by study of immunoglobulin genes rearrangement) was still detectable[60]. The last published case report from Canada described a 70-year-old woman presenting with severe thrombocytopenia. Routine serology discovered a previously unrecognized genotype 2 HCV infection with high titer HCV-RNA. Peripheral blood and bone marrow testing revealed an aberrant monoclonal B-cell population (CD20-positive), although no splenomegaly or adenopaties were evident. After failure to a variety of immune-suppressive therapies for thrombocytopenia she underwent splenectomy and SMZL diagnosis was made. She was then treated with SOF and RBV for 12 wk resulting in RVR, resolution of thrombocytopenia and flow-cytometry negativity for residual clonal B-cells (CR), remaining ongoing at more than 18 mo of follow-up[61].

At the last ASH meeting (2015), Arcaini *et al*[62] presented preliminary data of a large international survey on 26 patients with indolent lymphoproliferative disorders (12 SMZL, 2 NMZL, 6 MALT-MZL, 2 leukemic MZL, 2 SLL/CLL, 1 LPL and 1 low grade B-NHL NOS) and HCV infection treated with IFN-free AT. Three patients previously received chemotherapy and 4 a course of IFN-based AT. HCV genotype was 1 in 63%, 2 in 17% and 3 in 12% of patients. The majority of patients (n = 24) received a SOF-based regimen and 2 other regimens. A RVR was obtained in 20 out of 21 evaluable patients (95%). Considering 20 patients evaluable for lymphoma response, 8 achieved a CR (40%), 4 a PR (20%), with an ORR of 60%, while 5 had SD and 3 progressed during AT. According to histological subtypes, ORR was 70% in MZL (12 out of 17 patients), including 6 out of 9 SMZL (66%), while none of the 3 patients with other subtypes responded (2 CLL and 1 LPL, all SD). In conclusion this preliminary study showed that a significant rate of hematological response can be achieved in HCV-associated MZL also with DAAs, thus suggesting that the eradication of HCV may be able *per se* to induce the regression of indolent NHLs. Although these data have to be confirmed by larger series with longer follow-up, MZL seem to be more sensible to IFN-free AT, while subtypes less clearly associated with HCV such as CLL probably are not responsible to it.

On the basis of these preliminary data, the FIL initiated the phase Ⅱ BArT study (B-cell Anti-lymphoma Treatment), the first prospective clinical trial aimed to evaluate the efficacy, both virological and

haematological, of an IFN-free antiviral regimen in patient with indolent NHL associated with HCV infection. In this multicenter study, 50 patients with genotype 1-4 chronically active (HCV-RNA positive) non-cirrhotic HCV infection and untreated indolent asymptomatic NHL (with low tumor burden) will receive appropriate IFN-free AT according to genotype.

CONCLUSION

A large body of studies carried out in the last decade demonstrated that front-line IFN-based anti-HCV AT is able to induce nearly 75% response rate in all HCV-associated indolent B-cell lymphomas that do not need immediate conventional immuno-chemotherapy. Moreover, AT delivered at any time during patients' life has been associated with significantly improved OS. As in IFN era lymphoma response has been durable and related to the obtainment of viral eradication. Very preliminary data about the use of DAAs as primary treatment of indolent NHL seem to confirm that together with high rate of SVR in all genotypes they are able to induce a proportion of tumor responses, despite the absence of IFN therapy. More mature data of retrospective experiences as well as ongoing prospective studies are needed to precisely clarify their impact in HCV-positive B-NHL. Moreover, a promising area of future investigation may deal with the combination of DAA and rituximab, especially in cases with high tumor-burden. Because of its safety, rapidity and efficacy, AT should be recommended front line to patients with HCV-positive indolent NHL, both for the expected curative activity on the tumor itself, and because eradicating HCV is *per se* beneficial to the patient, avoiding late complications of the infection and allowing better tolerability to eventual future conventional anti-lymphoma treatments.

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REVIEW

Towards a new paradigm of microscopic colitis: Incomplete and variant forms

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Abstract

Microscopic colitis (MC) is a chronic inflammatory bowel disease that has emerged in the last three decades as a leading cause of chronic watery diarrhoea. MC classically includes two main subtypes: lymphocytic colitis (LC) and collagenous colitis (CC). Other types of histopathological changes in the colonic mucosa have been described in patients with chronic diarrhoea, without fulfilling the conventional histopathological criteria for MC diagnosis. Whereas those unclassified alterations remained orphan for a long time, the use of the term incomplete MC (MCi) is nowadays universally accepted. However, it is still unresolved whether CC, LC and MCi should be considered as one clinical entity or if they represent three related conditions. In contrast to classical MC, the real epidemiological impact of MCi remains unknown, because only few epidemiological studies and case reports have been described. MCi presents clinical characteristics indistinguishable from complete MC with a good response to budesonide and cholestiramine. Although a number of medical treatments have been assayed in MC patients, currently, there is no causal treatment approach for MC and MCi, and only empirical strategies have been performed. Further studies are needed in order to identify their etiopathogenic mechanisms, and to better classify and treat MC.

Key words: Microscopic colitis; Incomplete microscopic

colitis; Collagenous colitis; Lymphocytic colitis

Core tip: Microscopic colitis (MC) includes two well-defined entities: collagenous colitis and lymphocytic colitis. Similar clinical manifestations, but variable histopathologic features have also been identified and recognized as additional forms of MC, as not all patients suffering from colitis fulfill the criteria for MC diagnosis. Introducing the histological diagnosis for incomplete MC subtypes could reduce the risk of missing patients with a treatable cause of chronic diarrhoea. The importance of developing research studies addressed at describing etiopathogenic mechanisms of MC subtypes is highlighted in this review.

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INTRODUCTION

Microscopic colitis (MC) is a chronic inflammatory bowel disease (IBD) that has emerged in the last three decades as a leading cause of chronic watery diarrhoea significantly affecting the patient health-related quality of life (HRQoL)[1]. The term MC was employed in the early 80's to describe a group of patients with watery diarrhoea and weight loss, exhibiting normal endoscopic findings, but with microscopic inflammation in the colonic mucosa, as identified in biopsy specimens[2]. Later, in 1993, two independent research groups suggested the use of MC as a generic term to cover any type of colitis in which there were specific histological changes without any macroscopic alteration, as evaluated by endoscopic or radiological analysis, including the two main entities known as collagenous colitis (CC) and lymphocytic colitis (LC). Since then, the clinical-histological definition of MC and its classical subtypes have been a matter of debate and nowadays several consensuses classify these patients[1,3,4]. Moreover, other types of histopathological changes in the colonic mucosa have also been described over time in patients with chronic diarrhoea and normal or close to normal endoscopic findings, without fulfilling the classical histopathological criteria for MC diagnosis. Whereas those unclassified alterations remained orphan for a long time, it is nowadays universally accepted the use of the term and concept of incomplete MC (MCi), which proposes those abnormalities as new entities. Furthermore, different variant forms of MC have also been reported under separate names to describe peculiar histopathological infiltrate in the colonic mucosa in patients with clinical manifestations of MC but without the key histological features for LC, CC or MCi[5]. Since it is not appropriate to consider these entities as classical MC, it is crucial to establish a defined and reliable clinical-pathological criterion to confirm the diagnosis of these emerging entities.

This review summarizes current evidence on the definition, epidemiological and clinical significance of MCi and variant forms of MC. We searched the PubMed, Cochrane, MEDLINE, and Scopus libraries using the following individual and combined key words: "borderline lymphocytic colitis", "minimal collagenous colitis", "microscopic colitis undesignated", "microscopic colitis incomplete", "microscopic colitis not otherwise specified", "minimal change colitis", "paucicellular lymphocytic colitis", "cryptal lymphocytic colitis", "lymphocytic colitis with giant cells", "collagenous colitis with giant cells", "pseudomembranous collagenous colitis", "atypical lymphocytic colitis", "atypical microscopic colitis" or "focal active colitis". References cited in the articles obtained were also searched in order to identify other potential sources of information. The results were limited to human studies available in English including all articles published before April 2016.

DEFINITION: EVOLUTION OF A NEW CONCEPT

MC is an umbrella term that includes two main presentations of chronic and relapsing inflammatory disease of the gastrointestinal tract with characteristic histopathological features that allow us distinguish CC from LC[4]. In both entities, there are some common histopathologic features, not pathognomonic of these conditions, such as: surface epithelial injury (mild in LC and marked in CC), elevated and homogeneously distributed mononuclear inflammation in the lamina propria (mainly of lymphocytes and plasma cells), little or no presence of crypt architectural distortion and possible focal IBD-like changes (cryptitis and Paneth cell metaplasia)[5]. LC is particularly defined by large number of surface intraepithelial lymphocytes (IELs: > 20 IELs per 100 surface epithelial cells) with little or no crypt architectural distortion (Figure 1), whereas CC is characterized by irregular thickened collagen band (> 10 μm) under the surface epithelium, independently of IELs infiltration[1,5] (Figure 2). This feature is most evident between the crypts immediately beneath the surface epithelial cells containing entrapped capillaries, red blood cells and mononuclear cells[1,5]. Whereas the histopathological criteria for MC diagnosis seems to be established in its classical forms, many doubts remain in those cases in which the histological aspect of the colonic mucosa is not completely normal but specific findings do not reach the cut-off values considered diagnostic for classical MC.

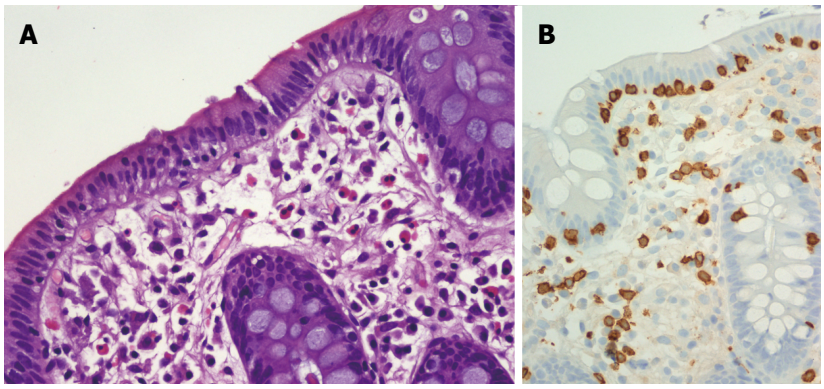

Figure 1 Photomicrographs of a colonic specimen from a lymphocytic colitis patient showing inflammatory hypercellularity in the lamina propria of colonic mucosa and clear presence of a greater number of intraepithelial lymphocyte cells. A: Hematoxylin-eosin staining, magnification × 200; B: More evident with the CD3 staining, magnification × 200.

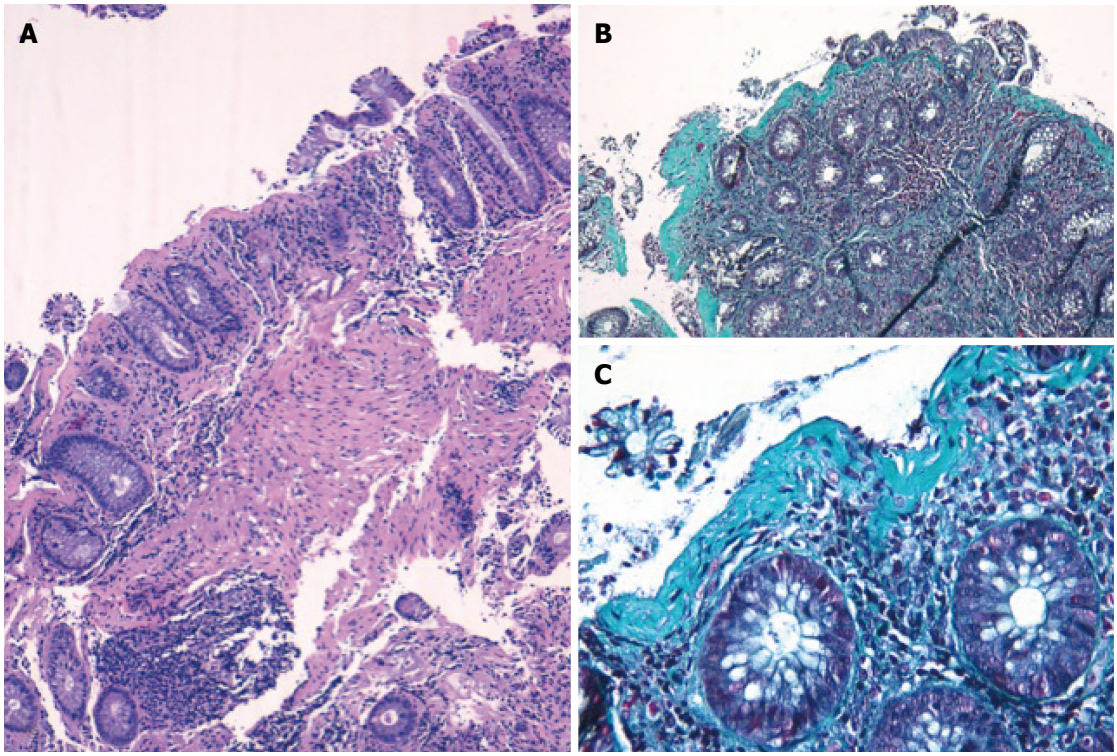

Figure 2 Photomicrographs of a colonic specimen from a collagenous colitis patient showing detachment of superficial epithelium. Hematoxylin-eosin staining, magnification × 100 (A) and thick subepithelial collagen band, Gomori's Trichrome staining (B and C, magnification × 100, × 400, respectively).

Incomplete microscopic colitis

In 2002, the terms "MC not otherwise specified" (MCNOS) as well as "undefined MC" (uMC) and "paucicellular colitis" were equally used to describe a subgroup of patients with chronic diarrhoea and an increased cellular infiltrate in the lamina propria with or without abnormal collagenous layer and/or elevated number of IELs, without completely fulfilling the criteria for MC diagnosis[6-10]. From 2007, some authors proposed classifying MC forms into five subtypes: CC, LC, "minimal change colitis" (crypt architectural abnormality in the form of cryptitis and crypt dilation in the absence of an increase in IELs and larger sub-epithelial collagenous band), MCNOS (increased inflammatory cell infiltrates in the lamina propria in the absence of other abnormalities) and MC with giant cells[11]. Despite the different phenotypes described, not all cases having clinical features of MC fulfill the histological diagnostic criteria for MC diagnosis. Consequently, the term and concept of "incomplete microscopic colitis" (MCi) emerged to recognize this MC subtype, to avoid overdiagnosis as well as underdiagnosis in order to guide therapy decision in the clinical practice[9,10,12] (Table 1).

While the minimum criteria required for MCi diagnosis is still under discussion, > 10 and < 20 IELs per 100 surface epithelial cells and > 5 μm and < 10 μm thickness of the collagen band in colonic biopsies

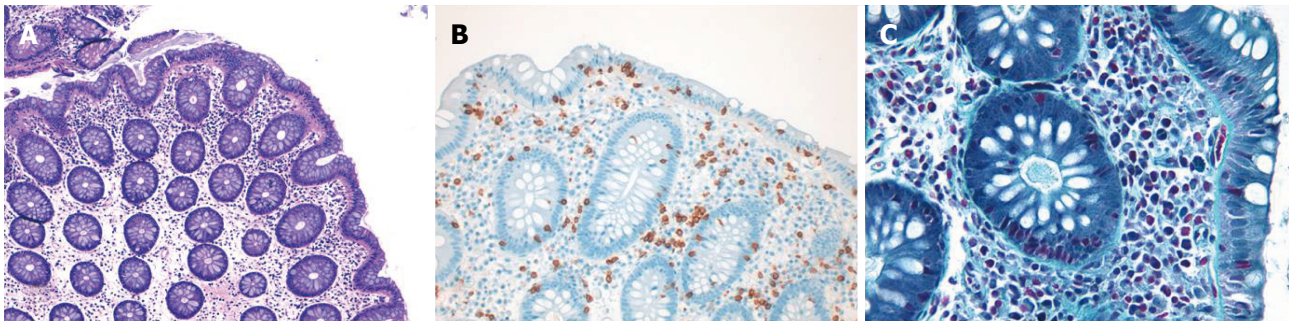

Figure 3 Photomicrographs of a colonic specimen from an incomplete lymphocytic colitis patient. Hematoxylin-eosin staining, magnification × 100 (A) with a mild increase in intraepithelial lymphocytes cells (B, CD3, magnification × 200) and a regular collagen band (C, Gomori's Trichrome staining, magnification × 400).

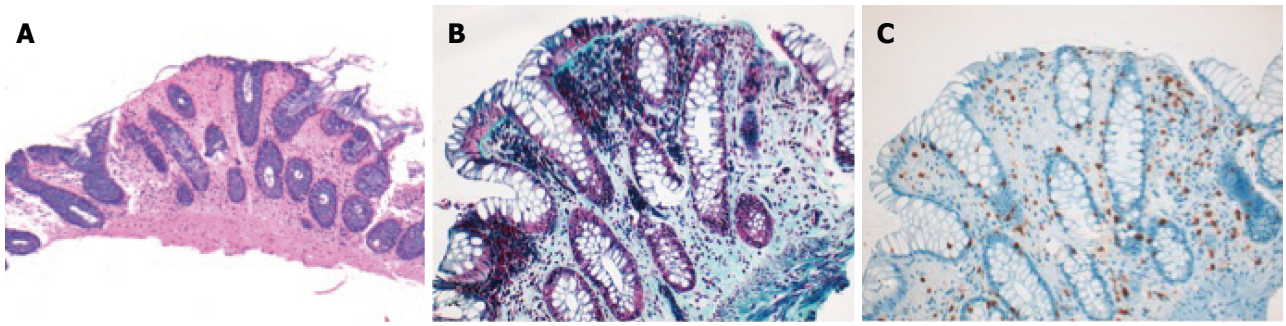

Figure 4 Photomicrographs of a colonic specimen from an incomplete collagenous colitis patient. Hematoxylin-eosin staining, magnification × 100 (A) with slight enhancement of subepithelial collagen band (B, Gomori's Trichrome staining, magnification × 200) without increased intraepithelial lymphocyte cells infiltration (C, CD3 inmunostaining, magnification × 200).

Table 1 Key histological features of classical and incomplete subtypes of microscopic colitis[4,5,25]

Subtype	IELs/100 epithelial cells	Thickness of collagen band
LC	> 20 IELs	< 10 μm
LCi	> 10 and < 20 IELs	< 5 μm
CC	< 20 IELs (sometimes > 20 IELs)	> 10 μm
CCi	< 20 IELs	> 5 and < 10 μm

LC: Lymphocytic colitis; CC: Collagenous colitis; IELs: Intraepithelial lymphocytes cells; LCi: Incomplete lymphocytic colitis; CCi: Incomplete collagenous colitis.

have recently been proposed as cut-off values for the diagnosis of incomplete LC (LCi) and incomplete CC (CCi) subtypes, respectively[5] (Figures 3 and 4). Previously, other terms were used to describe these CMi subtypes, although we can observe a slight difference in the cut-off values employed to define it over time, especially considering the LCi subtype. In fact, the heterogeneous minimal cut-off value for IELs infiltration used in different studies to define LCi reflects the difficulty in establishing an approved value, and reducing the risk of overdiagnosing these entities.

In any case, the differential diagnosis between complete and incomplete forms of MC is not an easy task. Morphological abnormalities in MC may be patchy[13-15], and MCi and fully established MC may coexist at different colonic segments of the same patient, making mandatory the collection of multiple and stepped biopsy samples along the large bowel to establish a correct histopathological diagnosis[4]. In fact, in a retrospective cohort of MC patients, among whom a large proportion of them had a repeated endoscopy after diagnosis, less than 2% had a primary endoscopy without histopathological abnormalities while 76% had chronic inflammation or MCi in their initial biopsies without a specific definition of MC or its subtypes[16]. Furthermore, in the 30% of 115 patients with MC subjected to a second endoscopy after a median of 21 mo no longer fulfilled the histological criteria of MC, and 9% switched MC subtype[16]. In this study, incomplete histological findings in MC were present in a significant number of patients later diagnosed with MC, suggesting that they could represent a different stage of the same disease, however more studies are needed to better determine the clinical-histopathological correlation over time[16,17]. Furthermore, chronic ileal inflammation was described in 15% of patients with MCi compared to 37%-57% with complete MC, observing that generally the colonic inflammation is most pronounced in the right colon adjacent to the terminal ileum. The significance of this nonspecific inflammation defined as the increase in the number of IELs is unknown, but its presence demonstrates that inflammation in MCi and in complete MC could affect the small intestine and is not

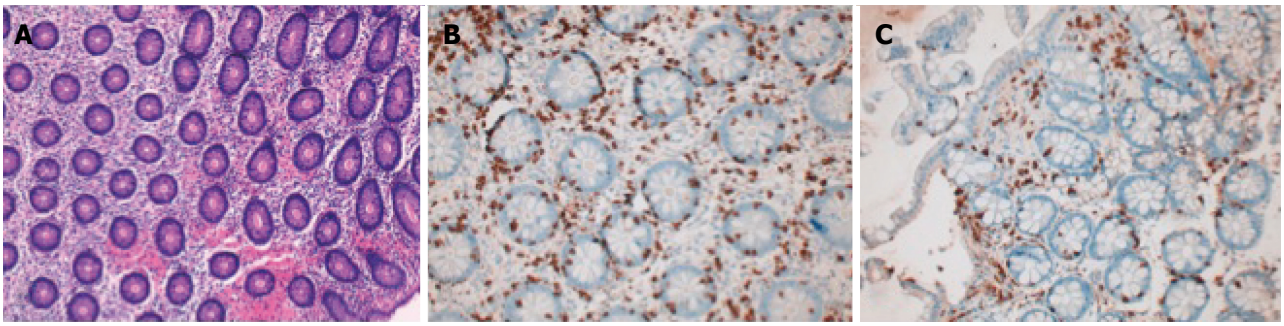

Figure 5 Photomicrographs of a colonic specimen from a cryptal lymphocytic colitis patient. Hematoxylin-eosin staining, magnification × 100 (A) showing only the presence of cryptal lymphocytosis (B, CD3, magnification × 200) and lack of intraepithelial lymphocytosis (C, CD3, magnification × 200).

confined only to the colonic mucosa, as documented in previous reports[18-20].

The difficulty in defining the histopathological diagnosis of all forms of MC is reflected by the intra and inter-observer variability even when applying the currently accepted diagnostic criteria for classical subtypes[21]. However, some authors investigated the ability to discriminate MCi from healthy mucosa and IBD/nonspecific reactive changes and concluded that there was very good intra-observer and inter-observer agreement (κ value varying from 0.88 to 0.96 and from 0.81 to 0.89, respectively) in separating MC/MCi group from non-MC. However, the ability to discriminate MCi from CC and LC was still low with the lowest number of cases agreed in MCi group (κ value ranging from 0.59 to 0.69)[22]. In fact, in a retrospective cohort of 93 patients, 15% of colonic biopsies primarily diagnosed as MCi changed to classical MC diagnosis through the study by two gastrointestinal pathologists[16]. Recently, the use of the CD3 immunohistochemical staining has demonstrated an improvement in the diagnostic agreement among pathologists, with a change in the diagnosis in 34% of cases, especially towards LCi[23]. Moreover, the lack of methodological agreement among pathologists also adds difficulty to MC and MCi diagnosis. For example, the assessment of the thickness of the collagen band by histologic analysis could be measured using an "eyeballed" histologic evaluation, a conventional calibrated micrometer scale or by performing semiautomatic micrometer measurements. Recently, automated image analysis software has been developed to measure the thickness of the subepithelial collagenous band in colonic biopsies of patients with CC and CCi stained with Van Gieson, providing a promising supplementary tool for the diagnosis of CC and CCi[24]. The authors showed that the overall agreement between all pathologists was κ value of 0.69 compared to κ value of 0.71 using the above-described software[24]. Despite the variety of methods, a gold standard for collagen band thickness quantification is still lacking[25]. To identify collagen, the trichrome stain is widely used, but immunohistochemistry with antibodies directed against Tenascin (extracellular matrix glycoprotein) represents a more sensitive method and may be a good alternative, as demonstrated in complete CC. In fact, in normal colon, the basement membrane is composed predominantly of type Ⅳ collagen while in complete CC it mainly consists of type Ⅵ collagen and Tenascin with lower amount of collagen types Ⅰ and Ⅲ[26-29]. Notably, it has been demonstrated that Tenascin immunostaining is also useful to detect minimal deposits of sub-epithelial collagen compared to hematoxylin-eosin, van Gieson's elastin and collagen-Ⅵ stainings (P < 0.001), being useful to discriminate between minimal collagenous colitis and normal mucosa[30,31]. However, more studies are needed in order to determine collagen composition and the pathogenetic mechanisms implicated in its formation, as well as to validate the Tenascin immunostaining use in a larger series of cases of CCi. On the basis of these observations, it would be desirable to establish a consensus on a more stringent panel selection of the best methods to evaluate the histological criterion abnormalities, in order to establish the diagnosis of MCi.

Variant forms of microscopic colitis

Other variant forms of MC have been reported in the literature in patients with clinical history of watery chronic diarrhoea. One study described two patients with a peculiar form of LC called cryptal lymphocytic colitis (Figure 5). It was defined as an increased number of IELs localized within the cryptal epithelium, with a mean number ranging from 39 (range, 33-43) to 46 (range, 32-55) per 100 crypt epithelial cells, while no changes in surface IELs were detected[32]. Immunohistochemistry analysis with anti-$CD3^+$, $CD8^+$ and TIA-1 antibodies showed that the cryptal IELs phenotype was of cytotoxic/suppressor T cells. Moreover, no signs of surface epithelial injury (mucin depletion, epithelial cells with cuboidal configuration, or mucin-depleted flattened epithelial cells) were reported[32]. It is feasible that IELs are attracted by signals derived from antigens present in the lumen of the crypts, as a consequence of the differences between non-adherent mucins in the lumen of the crypts and in the surface epithelium that could influence the selection of antigens in susceptible individuals[32]. However, the causes of selective cryptal

colorectal infiltration by lymphocytes remain unclear.

Other authors described some case reports of pseudomembranous CC as an unusual cause of chronic diarrhoea with a good response to budesonide treatment[33-38]. This variant form was histologically defined as the increased thickening of the sub-epithelial collagen band and pseudomembranes formation characterized by eruptive exudate composed of neuthophil leukocytes, necrotic debris and fibrin at the luminal surface of the colonic mucosa excluding ischemic, toxin-induced or infective aetiologies (*Clostridium difficile* infection)[36,38]. The colonoscopy showed normal colonic appearance in some patients, but inflammation and colonic ulcerations in others. Recently, a case of pseudomembranous CC with superimposed drug damage was reported, describing the presence of cholestyramine crystals on the mucosal surface[39]. However, it is still under debate whether the pseudomembranous formation constitutes part of the spectrum of CC itself or is associated with unknown superimposed infection.

Another variant described is focal active colitis (FAC), although considerable controversy exists regarding the clinical implication of its diagnosis. FAC is characterized by focal crypt damage caused by neutrophils that may be associated with infections, ischemia, Crohn's disease (CD), partially-treated ulcerative colitis or irritable bowel syndrome (IBS)[40]. Some reports showed an association between FAC and oral sodium phosphate ingestion, as it has commonly been used as an oral laxative agent, causing aphtoid ulcers and/or FAC in the colon and rectum[41-44]. Two previous studies described the prevalence of FAC ranging from 3.5% (11/316) to 6.6% (15/226) in IBS patients with normal endoscopic evaluation[41,45]. A follow-up study in 90 patients, showed a positive association of drugs in 24% of them [especially non-steroidal anti-inflammatory drugs (NSAIDs)] and a basal subtype of FAC and infection in 19%[46]. Moreover, in 16% of patients (predominantly women) a diagnosis of IBD was ultimately made (CD in the majority of the patients and ulcerative colitis only in two patients)[46]. The disease duration ranged from 4 mo to 7 years with a mean of 4.2 years and 8 patients (28%) developed CD, especially in pediatric group compared to adult population[47]. In conclusion, it is not currently clear whether FAC should be considered as a variant form of MC or an initial form of IBD.

An additional variant form of MC described by some authors is MC with giant cells, defined as the presence of multinucleated giant cells in an otherwise classic LC and CC, reporting until now 6 cases of LC with giant cells and 12 cases of CC with giant cells[48-50]. The sub-epithelial multinucleated giant cells that are present in the lamina propria are positive for the CD68 marker and seem to arise from merged sub-epithelial macrophages. The presence of giant cells does not appear to confer any additional adverse clinical outcome with doubtful clinical significance.

PATHOPHYSIOLOGICAL RELEVANCE OF LOW-GRADE INTESTINAL MUCOSAL INFLAMMATION

The colonic mucosa daily faces dietary antigens and microbial products present in the intestinal lumen. It is well established that the interactions between the intestinal immune cells and the luminal microorganisms directly regulate the physiological inflammatory state of the intestinal mucosa and that both, overreaction and underreaction have been implicated in the pathogenesis of several chronic gastrointestinal disorders.

Definition of healthy intestinal mucosa

The histological analysis of colonic specimens from healthy donors and non-affected areas from section margins of surgical specimens has allowed a better knowledge of tissue morphology and cell distribution in a non-disease condition. However, factors such as diet, antibiotic consumption, or physiological stress influence mucosal immune cell infiltrate, making it difficult to define reference values, which are especially helpful when diagnosing diseases with mild changes, such as MC and MCi. Despite the lack of histological criterion defining all features of normality, the colon from healthy donors has been described as harbosing about < 5 IELs (mostly $CD8^+$) per 100 surface epithelial cells, being slightly higher in the proximal than in the distal colon[25] (Figure 6). Sometimes a higher number of IELs is present over lymphoid follicles/aggregates, therefore it is not recommended to value lymphocytes counts in these areas[51]. Moreover, a mild increase in IELs numbers without a clear epithelial injury and/or increased lamina propria inflammatory infiltrate may be nonspecific and may be related to different conditions. Consumption of drugs, celiac disease, infectious colitis in remission[52] immunologic conditions (*e.g.*, Hashimoto's thyroiditis, common variable immune deficiency (CVID), autoimmune enteropathy, allergy, immune deficiency), and IBD in remission may lead to an increased number of IELs[12,52,53]. Therefore, knowledge of the clinical presentation, an endoscopic analysis, and anamnestic data will be necessary to differentiate between health and disease[54]. The lamina propria contains B cells (mainly plasma cells, 15%-40% of total mononuclear cells) and a vast majority of T cells (mainly $CD4^+$; 65%), with a few natural killer cells. Moreover, the lamina propria is composed of a not conspicuous number of monocyte-macrophagic cells, especially under the subepithelial collagenous layer, unlike the rectal mucosa, where macrophages are frequently found. The cut-off values for eosinophil normal counts have not been established either, and a proximal-to-distal gradient has been

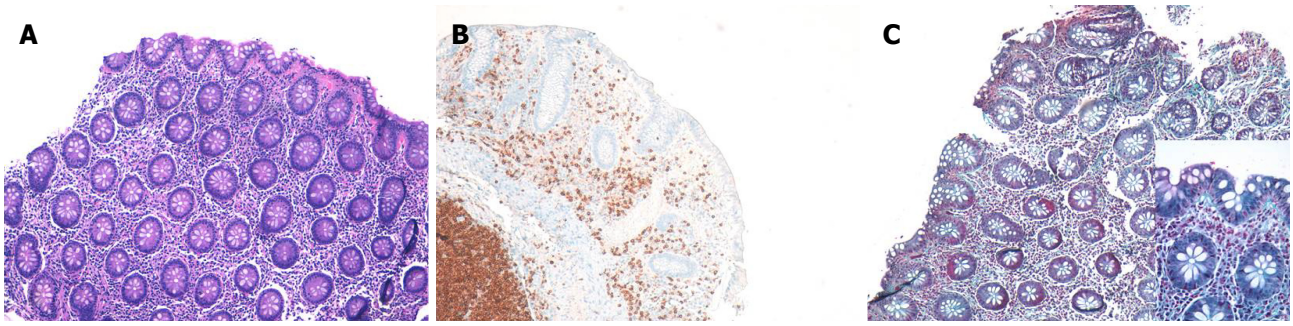

Figure 6 Photomicrographs of a colonic specimen from a healthy donor. Hematoxylin-eosin staining, magnification × 100 (A) showing low number (usually less than 5) of intraepithelial lymphocyte cells, which can be easily identified by CD3 immunohistochemistry marker (B, magnification × 100). The subepithelial collagen band is tiny and regular (C, Gomori's Trichrome staining, magnification × 10 and inset, Gomori's Trichrome staining, magnification × 400).

observed, with 37 to 9 cells per high-powered field in the right and left colon, respectively[55,56]. In the healthy mucosa, the basement membrane is composed of laminins, predominantly collagen Ⅳ, proteoglycans, calcium-binding proteins and other structural and adhesive proteins[56]. Its normal thickness is around 3-4 μm[25], reaching its thickest value in the rectum[56]. When analyzing biopsy specimens, the evaluation of lamina propria cellularity may be very subjective, as different studies have evidenced great inter-observer variability[57]. For this reason, the diagnosis of MC requires a full endoscopy, with collection of two or more biopsies from each segment of the large bowel and a clinical-histopathological correlation[57].

Mucosal immune mechanisms in MC

Variations in number and activation state of mucosal immune cells have been described in physiological and some pathological conditions, in association with intestinal dysfunction. However, the etiopathogenic mechanisms of inflammatory diseases such as MC are not completely known. It is generally accepted that MC is a multifactorial disease, probably secondary to an abnormal immune reaction in predisposed individuals, triggered by different luminal factors[58-60]. Several studies showed that an impaired adaptive immune response through aberrant T-cell responses leads to chronic gut inflammatory conditions in MC patients[58]. In particular, several studies showed a heavy infiltration of $CD8^+$ cytotoxic T-lymphocytes (CTLs) in the colonic mucosa of MC patients due mainly to an increased expansion of resident T-cells[61] with a mixed Th17/Tc17 and Th1/Tc1 mucosal cytokine profile[62-65]. Moreover, other studies showed an impaired epithelial barrier dysfunction in MC that worsened in the case of active disease, which persisted despite effective treatment with the first line budesonide therapy[66]. All these available data derive from studies performed in patients with classical MC without identification of a MCi subgroup, with the exception of paucicellular LC. In fact, one study showed a lack of expression of $CD25^+FOXP3^+$ cells in paucicellular LC compared to higher expression in both LC and CC[9]. Despite recent research, the mechanisms through which mucosal immunity generates colonic dysfunction in MC without the development of significant macroscopic mucosal damage is still unknown. Due to similar histological findings and clinical outcome, a proximity to the pathophysiology of diarrhoea-predominant IBS (IBS-D) has been suggested. In fact, from the clinical point of view, there is significant overlap between MC and IBS. In particular, the pooled prevalence of any type of functional bowel disorders in patients who present diagnostic criteria of MC is 39.1% (95%CI: 22.8-56-6, I^2: 97%), as reported in a recent meta-analysis without any data available in MCi patients[67]. Moreover, from the pathogenetic point of view, low-grade mucosal inflammation has been demonstrated not only in MC but also in IBS-D although to a lesser extent than that in MC[59,68-70]. In particular low-grade inflammation has been demonstrated in association with disease severity in both, IBS and MC[63,71], suggesting immune activation as a key mechanism in both entities. Despite a clear overlap in the pathogenesis and clinical manifestations between IBS and MC, the common pathophysiological pathways between the two conditions remain poorly understood, especially considering the incomplete and variant forms of MC.

EPIDEMIOLOGICAL DATA

MC was initially considered a rare disease with only 446 cases of CC described at the end of 1992[72]. Over time, an increasing number of studies has explored the incidence and prevalence of MC and has evidenced significant geographic variations. To date, the pooled incidence rate of CC was 4.14 (95%CI: 2.89-5-40) per 100000 person-year and 4.85 (95%CI: 3.45-6.25) for LC as shown in a recent meta-analysis, observing a north-south gradient only for CC[73]. An increasing incidence in classical MC has been reported in several studies, whereas other analyses have suggested a more stable incidence in some geographic regions and especially after the year 2000[73]. However, it is unknown whether the increasing incidence is genuine

Table 2 Incidence of incomplete microscopic colitis and paucicellular lymphocytic colitis

Ref.	Study period	Country	MCi subtype	Incidence
Fernández-Bañares *et al*[9]	2006-2009	Spain	Pacicellular LC	3.24 (2-4.48)
Bjornback *et al*[16]	1999-2010	Denmark	MCi	4 (NS)
Rasmussen *et al*[74]	2000-2014	Denmark	MCi	5 (NS)

Incidence per 100000 inhabitants and year. MCi: Incomplete microscopic colitis; Paucicellular LC: Paucicellular lymphocytic colitis; NS: Not specified.

or is the result of greater awareness of the disease.

Incomplete microscopic colitis

In contrast to classical MC, the real epidemiological impact of MCi and variant forms remains unknown, because only a few epidemiological studies and case reports have been described. In fact, as an emerging disease, the definition of MCi and variant forms has evolved over time and it is still under discussion. This has yielded, unfortunately, non-comparable results due to variations in the different cut-off values to define its diagnosis.

The incidence of MCi has been described in only three retrospective population-based studies using a more recently described histological definition for MCi or one of its sub-groups[9,16,74] (Table 2). Firstly, one Spanish study demonstrated the incidence of pauciclellular LC, a subtype that is now considered as synonymous of LCi[5,9]. The mean annual incidence of paucicellular LC was 3.24/100000/year (95%CI: 2-4.48) compared to the mean annual incidence of 2.37/100000/year (95%CI: 1.3-3.43) for CC and LC[9]. However, in the paucicellular LC group the study also included the patients with IELs > 20 IELs per 100 surface epithelial cells but with patchy distribution of epithelial lymphocytosis in more than one biopsy sample, though not in all, possibly generating a slight bias risk in the estimated incidence of this entity[9]. In all, 26 patients with paucicellular LC were identified, showing that they were younger than LC patients with a slightly higher prevalence in female sex (61.5%)[9]. Moreover, 5/26 patients firstly diagnosed as paucicellular LC were finally diagnosed as IBS-D during follow-up, as their condition did not improve after MC conventional therapy[9].

Subsequently, one Danish retrospective population-based cohort study estimated a mean incidence rate of 4/100000/year for MCi compared to 6.7/100000/year for LC and 10.8/100000/year for CC. The incidence of MCi as well as LC increased seven-fold from 1999-2001 to 2008-2010 as compared to a three-fold increase for CC[16]. However, the cut-off values used to establish the MCi diagnosis were not completely equivalent to the more recently accepted ones[5]. In fact, LCi was defined in this study as the existence of an abnormal IELs counts, with a minimal cut-off value of > 5 per 100 epithelial cells compared to the current recommended minimal cut-off value of > 10 per 100 epithelial cells, possibly generating a tendency towards overdiagnosis of LCi in this cohort of patients[16]. Nonetheless, 101 patients with MCi were identified in a consecutive cohort of 539 MC patients, with a mean age at diagnosis of 62 years and with higher prevalence in female sex (82% of women)[16].

Recently, another Danish retrospective consecutive study estimated an incidence of MCi of 5/100000/year compared to 14.5/100000/year for CC and 14.9/100000/year for LC in 2014. However, also in this study, a minimal cut-off value of IELs infiltration was > 5 per 100 epithelial cells compared to the current recommended minimal cut-off value of > 10 per 100 epithelial cells, possibly generating a slight overdiagnosis of LCi in this cohort of patients. This study described 226 cases of MCi in non-selected patients presenting, in the majority of cases, chronic watery diarrhoea, with a median age of 61 years-old and a 69% of women prevalence[74]. Discrepancy between histopathology in the right and left colon was rare and only in one patient MCi was found in the right colon and LC in the left colon. The diagnostic sensitivity of biopsies from the right and left colon did not differ among MC subgroups including MCi, the latter having a sensibility of 91% (95%CI: 84-96) for the right colon and of 97% (95%CI: 91-99) for the left colon[74]. MCi persisted histologically for up to one year in 45% of patients compared to 77% of CC patients and 64% of LC patients. While these differences did not reach statistical significance, these data showed that the pathological changes in CC and LC were more persistent than those in MCi[74].

Moreover, other studies described the prevalence of CMi in patients with chronic diarrhoea, although heterogeneous definitions are used to defining them, using several unspecific names [nonspecific microscopic colitis (NSMC) or NOSMC or uMC]. In particular, in one study the authors described NSMC prevalence of 46.7% (28/60) in patients with IBS-D defined using the Rome Ⅱ criteria[75]. However, the term NSMC was used to define IELs infiltration of < 20 per 100 epithelial cells without defining the minimal cut-off value. This group of patients could be considered as having an incomplete form of LC because the mean reported number of IELs per 100 epithelial cells was 11.71 (± 1.83)[75]. Another study used the term NOSMC to define the presence of an increased number of IELs > 7/100 but < 20/100 per epithelial cells with the absence of both a thickened sub-epithelial collagen layer and flattening of epithelial cells, epithelial loss and detachment[76]. Of 613 patients with watery chronic diarrhoea and normal finding at colonoscopy, 64 cases (10%) of NOSMC were diagnosed without describing any clinical characteristics of these patients sub-group in the article[76]. Another recent multicenter prospective case-control study used

the term undetermined MC to define the abnormal IELs infiltration and/or sub-epithelial collagen thickening without reaching the diagnostic thresholds for CC and LC but without specifying the cut-off values used to define these entities[77]. They described 8 undetermined MC cases of 433 (1.8%) patients with watery chronic diarrhoea with normal colonoscopy[77]. Finally, in a nationwide population-based cohort from The Netherlands, the authors classified MC patients in CC, LC and undefined MC (uMC), the latter was used to define the cases in which no information for further sub classification was available[78]. The authors showed a relatively high frequency of uMC (10%) with general and sex specific mean annual incidence rates between 2000 and 2012 ranging around 0.4 (95%CI: 0.3-0.5) per 100000 inhabitants and year and which changed only marginally over time[78]. Although the term uMC was chosen to avoid any confusion with the term MCi, the same authors proposed that it was plausible that the majority of the uMC cases identified were either LC or cases not fully meeting the criteria for either CC or LC, stressing the need to improve the knowledge on MC variant and incomplete forms in both gastroenterologists and pathologists[78].

CLINICAL FINDINGS

All three entities LC, CC and MCi present indistinguishable clinical findings, with some differential features associated with chronic watery diarrhoea[16]. In MC the chronic diarrhoea seems to be associated with nightly defecation in 25%-50% of cases, high frequency of defecation urgency (70%) and fecal incontinence (40%) that particularly affect patient's quality of life[4]. Moreover, MC can be associated with other additional symptoms such as abdominal pain, abdominal bloating, fatigue and weight loss (described in up to 50% of cases). However, in a retrospective cohort MCi patients were less likely to report nightly defecation (31% *vs* 39% of LC and 57% of CC), watery stools (68% *vs* 88% in LC and 92% of CC) and fecal incontinence (22% *vs* 34% of LC and 43% of CC)[16]. In contrast to that, the frequency of other associated symptoms was similar in the three groups included[16]. Regarding paucicellular LC patients, a study evidenced less likely reported significant weight loss compared to MC patients[9]. Moreover, routine laboratory parameters were generally normal in all MC subtypes except for a slightly increased C reactive protein (CRP), respect to reference values[16]. Unfortunately, serological or fecal markers are not available yet, so neither diagnosis nor monitoring can be performed in any MC subtypes without biopsies collection. In view of the few data available, we can assume that MCi patients expressed a milder clinical activity with less alarm symptoms (such as weight loss and nocturnal diarrhoea) compared to classical MC. Since a subtle clinical expression of the disease reflects a possible more benign evolution course, in the absence of correct awareness of this mild clinical expression there could be increased risk of misdiagnosis or delayed diagnosis.

Risk factors

Several risk factors have been identified in MC patients such as the use of certain drugs and the association with autoimmune diseases. In particular, the use of NSAIDs, including low-dose of aspirin, proton pump inhibitors (PPIs), selective serotonin reuptake inhibitors (SSRIs) and other drugs, has been associated to an increased risk of developing MC[4]. However, only a few studies evaluated drug consumption as a risk factor in MCi. In particular, the consistent ingestion of NSAIDs showed a similar risk for complete LC and paucicellular LC, but lower than CC[9,10]. In MCi the use of NSAID, salicylic acid and PPI was similar compared to CC and LC patients, except for a slightly higher use of statin in MCi compared to CC and LC groups (24% *vs* 19% *vs* 18%, respectively)[16]. However, it is important to highlight that no universally accepted methods are available for assessing cause-effect relationships in adverse drug reactions[79]. In fact, the association between drugs intake and MC or MCi derived primarily from case control studies and drug prescription registry data, and only a few drugs show causal relationship in MC but not in MCi.

Other associated diseases

The presence of autoimmune diseases has been described to be associated with MC in over 30%-50% of cases, with OR 11 (95%CI: 5.1-23.8) for CC ($P < 0.001$) and OR 16.6 (95%CI: 6.4-43.1) for LC ($P < 0.001$), especially for celiac disease, type 1 diabetes, autoimmune thyroiditis, seropositive/seronegative rheumatoid arthritis and others[4,80]. There are few data available about the association between autoimmune disease and MCi. In particular, in paucicellular LC less association with autoimmune diseases was observed showing a prevalence of 15.4% compared to 38.6% in LC and 32.4% in CC. Moreover, a lower frequency of HLA-DQ2 genotype was observed in paucicellular LC compared to LC (30.4% *vs* 48%)[9]. Only a few cases of CMi were described to be associated with concomitant celiac disease in one Danish cohort (2/176 cases) but not in another study (0/101 cases). However, the new association between MC and Takayasu's arteritis (TAK) has been recently reported with especially higher frequency in MCi than in complete MC cases (20% *vs* 10%)[81]. Moreover MC (complete and incomplete forms) was found to be significantly higher in active TAK patients than in the active group (67% *vs* 14%, $P \leqslant 0.03$, OR 7.9)[81]. TAK is a chronic granulomatous vasculitis, mainly affecting the aorta and its branches especially in middle-aged females. It has been found as the most common subtype in vasculitis patients with IBD[82]. Its etiopathogenesis is still unknown, but upregulated innate and adaptive immune response

has been observed in this disease[81].

Moreover, MC has been associated with bile acid malabsorption (BAM; 44%) in both CC and LC patients as shown using the tauroselcholic (selenium-75) acid technique (SeHCAT)[4]. Whereas bile acid chelating agents have shown effectiveness in CC, these data come from only noncontrolled studies and do not show any significant improvement of the colon histology. Also in the MCi Danish cohort, BAM was very common with higher prevalence (48%) compared to CC and LC (26% and 18%, respectively)[16]. However, in paucicellular LC this association was less frequent[9]. Although these data suggest the possible implication of bile acids in the pathogenetic mechanisms of MC, its influence is not completely understood.

Finally, MCi patients presented a higher rate of lactose malabsorption (9% *vs* 3% in CC and 1% in LC), as diagnosed by oral testing and later by C/C genotype by the lactase-phlorizin hydrolase 13910 polymorphism gene analysis, suggesting a possible involvement of the small bowel to induce a secondary lactose malabsorption, especially in MCi[16]. Although about two thirds of the World's population undergoes a genetically programmed decrease in lactase synthesis after weaning[83], symptoms of lactose intolerance generally do not occur until there is less than 50% of lactase activity. However, IBS patients show an increased risk of developing symptoms especially for IBS-D subtype[84]. In fact, visceral hypersensitivity and anxiety are associated with symptoms after ingestion of only a modest dose of lactose. Moreover, IBS patients with lactose intolerance also show heightened activity of the innate mucosal immune system with increased counts of mast cells, IELs and enterochromaffin cells in the terminal ileum and right colon[85]. We can speculate that other mechanisms such as visceral hypersensitivity could be implicated in symptoms development in MC and MCi, similarly to IBS, however studies designed to test this hypothesis are needed.

THERAPY

Several therapeutic interventions have been developed to achieve clinical and histological remission of MC and, in recent years, a number of randomized-controlled trials have provided a more evidence-based approach to treating this disease. Oral budesonide is currently the first line of treatment for the induction of clinical relief in moderate-severe MC, with a 76%-88% clinical response in CC and LC patients[4]. However, few drug efficacy data coming from retrospective cohorts of MCi patients are available. Noteworthy, there is a phase Ⅲ randomized trial to test the efficacy and safety of budesonide in MCi. This study is already registered and is in the process of subject recruitment (www.clinicaltrials.gov, Identifier NCT02142634). In one retrospective cohort budesonide was prescribed as short-term therapy of 2-4 mo, leading to normalization of stool consistency and frequency in 84% (95%CI: 66%-95%) for MCi, 84% (95%CI: 77%-89%) for CC and 88% (95%CI: 80%-98%) for LC, respectively[16]. Moreover, another retrospective study showed the effectiveness of budesonide treatment in MCi, while only a minor number of patients were treated with colestyramine and mesalazine[74]. The response to budesonide was independent of the SeHCAT results. However, in only three patients with paucicellular LC budesonide therapy did not achieve clinical response[9]. Therefore, the effect of budesonide in the MCi-group seems to be similar to that of the other MC sub-groups. These findings further support the hypothesis that inflammed lamina propria may well be more important for the diarrhoea than specific IELs infiltration in all MC subtype entities. In fact, a recent study has evaluated the contribution of inflammatory mediators to water secretion in the sigmoid colon of patients with LC. The key effector cytokines TNFα, IFNγ and IL-15 inhibited γ-ENaC upregulation in response to aldosterone through a MEK ½-mediated pathway, preventing ENaC from reaching its maximum transport capacity, leading to Na malabsorption, which directly contributes to diarrhoea[86]. Other treatments were also used in MCi patients and its sub-groups. The efficacy of cholestyramine was 100% (95%CI: 75%-100%) in MCi associated with BAM, compared to 56% (95%CI: 35%-75%) in CC patients and 82% (95%CI: 48%-98%) in LC patients. Paucicellular LC seems to respond well to loperamide or cholestyramine treatment, and 6/10 patients achieved clinical remission with mesalazine treatment[9]. It is important to stress that in MCi, some cases of spontaneous remission such as those observed in the classical subtypes of MC have also been reported, however this was particularly evident for patients with MCi[74].

Although a number of medical treatments have been evaluated in MC patients, currently there is no casual intervention for MC and MCi, and current therapy for MC and MCi mainly follows an empirical approach. Further studies are needed in order to better understand the molecular mechanisms behind the origin of the disease and to develop specific therapies for each MC subtype.

CONCLUSIONS AND FUTURE PERSPECTIVES

MCi has clinical and histological features that support its classification as a form of MC. In fact, MCi presents clinical characteristics indistinguishable from complete MC and it shows a good response to budesonide and cholestiramine treatment. Therefore, introducing the histological diagnosis of MCi could reduce the risk of missing patients with a treatable cause of chronic diarrhoea. Of note, variant forms of MC are extremely rare disorders with still unclear clinical significance and which probably do not represent real specific entities[13]. Further investigations on the prevalence of

MCi and its sub-groups and detailed studies that can better define its natural history and etiopathological characteristics are warranted.

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MINIREVIEWS

Endocrine manifestations in celiac disease

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Abstract

Celiac disease (CD) is an autoimmune small intestinal mucosal disorder that often presents with diarrhea, malabsorption and weight loss. Often, one or more associated endocrine disorders may be associated with CD. For this review, methods involved an extensive review of published English-language materials. In children and adolescents, prospective studies have demonstrated a significant relationship to insulin-dependent or type 1 diabetes, whereas in adults, autoimmune forms of thyroid disease, particularly hypothyroidism, may commonly co-exist. In some with CD, multiple glandular endocrinopathies may also occur and complicate the initial presentation of the intestinal disease. In others presenting with an apparent isolated endocrine disorder, serological screening for underlying subclinical CD may prove to be positive, particularly if type 1 diabetes, autoimmune thyroid or other autoimmune endocrine diseases, such as Addison's disease are first detected. A number of reports have also recorded hypoparathyroidism or hypopituitarism or ovarian failure in CD and these may be improved with a strict gluten-free diet.

Key words: Pituitary insufficiency; Ovarian infertility; Celiac disease; Gluten-sensitive enteropathy; Endocrine disorders; Thyroiditis; Hypothyroidism; Diabetes; Adrenal insufficiency

Core tip: Celiac disease (CD) is an immune-mediated intestinal disorder that may be closely linked to a number of extra-intestinal disorders, particularly endocrine diseases. These include thyroiditis, particularly in adults, and insulin-dependent diabetes mellitus, particularly in children and adolescents. Other endocrine disorders have also been recorded, including adrenal insufficiency and pituitary disease. Usually, only a single endocrine gland is involved in CD, but changes in multiple different glands has also been recorded. If an endocrine disorder is present, screening for CD, even without gastrointestinal symptoms, has been recommended. In established CD, regular follow-up and evaluation for the possible appearance of an occult endocrine disorder may also be appropriate.

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INTRODUCTION

Celiac disease (CD) is an immune-mediated small intestinal disorder that occurs in genetically susceptible people and is characterized by an intolerance to gluten-containing proteins found in wheat, rye and barley grains. Most often, symptomatic persons present with diarrhea, nutrient malabsorption and weight loss associated with a mucosal inflammatory process in the proximal small intestine. Mucosal architecture may be severely altered in the duodenum and, with increasing severity, may extend for variable distances into more distal jejunum and ileum. It may be that the severity of the individual inflammatory response, the timing of its appearance as well as the extent and localization within the small intestine are genetically-programmed[1].

In recent years, the disorder has become increasingly appreciated even without significant gastrointestinal symptoms, being documented in up to 2% of the serologically-studied populations, and perhaps, higher in referred patients using endoscopic screening biopsies[2]. The disorder is not only common, but has been increasingly recognized as a phenotypically heterogeneous disorder. Increased clinician awareness as well as widespread use of serological testing for case-finding have been important factors in the emergence of this disorder, not only in the scientific community, but also in popular press.

As a result, clinical features attributed to celiac disease or its complications have been noted in other extra-intestinal sites, including endocrine manifestations. Of these, two endocrine disorders are particularly prominent, thyroiditis, especially, but not exclusively, in adults, and insulin-dependent diabetes, particularly, but also not exclusively, in children. This manuscript aims to increase awareness of endocrine changes in CD, especially if there are few or no gastrointestinal symptoms, discuss screening opportunities and provide added insight related to a long and personal experience in CD diagnosis and management.

THYROID DISEASE

Early reports with CD

Autoimmune thyroid diseases were associated with CD in some initial descriptive studies for both children and adults[3,4]. Some early case reports also noted a link with hyperthyroidism[5-7] while others reported an association with hypothyroidism[8-10]. Interestingly, the simultaneous occurrence of adult CD and lymphocytic thyroiditis was also noted and hypothesized to be more than coincidential[11]. In a later report[12] from a defined area in Scotland, studies for thyroid autoantibodies and measurements of thyroid function suggested that the risk of even clinically overt thyroid disease, especially hypothyroidism, was increased in CD. Some suggested that this may be genetically determined owing to the common detection of human lymphocyte antigen (HLA) haplotypes in most with autoimmune thyroid disease and CD compared to the general population[13-16].

Later prevalence studies in CD

An evaluation of 96 consecutive adults (70 females and 26 males) with biopsy-defined CD (*i.e.,* severe lesion, Marsh 3, crypt hyperplastic villous atrophy, followed by a clinical and biopsy-defined response to a gluten-free diet, average age diagnosis of CD, 47.3 years) revealed 16 with autoimmune thyroid disease (including 11 females and 5 males, overall average age of CD in this group with thyroid disease, 57.1 years). Of these, 16 had hypothyroidism, but 4 had previously received radio-iodine ablation or thyroidectomy for Grave's (hyperthyroidism) disease. Interestingly, almost half also had dermatitis herpetiformis, an autoimmune dermatological disorder closely linked to adult CD. None had familial CD or a familial thyroid disorder. Diagnosis of thyroid disease preceded diagnosis of CD in 13 patients or was made concurrently in 2 patients. Only 1 had thyroid disease detected about a decade after CD was first diagnosed and treated with a gluten-free diet. Of note, 4 also developed a small intestinal lymphoma or adenocarcinoma, both known complicating malignant disorders in adult CD.

These findings are similar to a more recent and larger prospective evaluation of 242 celiacs[17]. In this study hypothyroidism was present at a similar rate of 12.9%, 3-fold higher than controls of 4.2%. Most interesting were the results in those treated with a strict gluten-free diet for at least 1 year. In these, there was an apparent normalization of subclinical hypothyroidism. In 5 of 91 celiacs with normal thyroid function development of thyroid disease occurred. Others have noted autoimmune thyroid disease in 13.9% of 79[18] and 30.5% of 36 adult celiacs[19], respectively, similar in prevalence to earlier studies.

Pathogenetic linkages with CD

This linkage between adult CD and autoimmune thyroid disease is not entirely novel, particularly as HLA (human leukocyte antigen) haplotypes B8 and DR3 were noted to occur with increased frequencies in adults and children with CD as well autoimmune thyroid disease. Interestingly, HLA DR antigen was also demonstrated in other epithelial glandular structures in children and adults with autoimmune disorders (*e.g.,* salivary glands in Sjogren's syndrome)[20,21]. An alternate, but not necessarily entirely exclusive hypothesis is also possible. The thyroid gland shares a common embryonic origin during fetal development,

being derived from the pharyngeal gut on the 17th day. Some autoimmune disorders may also require time to evolve, perhaps increased intestinal permeability may allow excessive amounts of antigen to enter the circulation and cross-react with other tissues, including the thyroid gland.

Clinical implications

The linkage between these two disorders may have important clinical implications. A relatively high prevalence of autoimmune thyroid disease, particularly hypothyroidism, in elderly adults may make clinical recognition of CD, at times, difficult. For example, the severity of the diarrhea or weight loss may be more limited with reductions of circulating thyroid hormone leading to increased time for intestinal transit or fluid retention with myxedema. In addition, some with an altered bowel habit may have impaired absorption, particularly with an increased transit rate in hyperthyroidism. As a result, an apparent failure to respond to a gluten-free diet may be considered. In contrast, hypothyroid patients may fail to respond to oral thyroid replacement therapy because of reduced small intestinal surface absorptive area associated with unrecognized or occult CD.

Serological CD screening in thyroid disease

A number of recent studies have explored the role of serological screening for celiac disease in patients with autoimmune thyroid disease. In a study from Finland of 83 patients[22], 3 asymptomatic cases and 1 previously diagnosed celiac patient were defined for an overall frequency of 4.8%. In an Italian study of 152 patients, 5 new cases were detected using endomysial antibodies and duodenal biopsy confirmation[23]. Similar results were reported later by other investigators in both children and adults with CD[24-29]. Far less information is available in Grave's hyperthyroidism. In 115 consecutive patients with Grave's hyperthyroidism[30], gliadin and tissue transglutaminase antibodies were used to screen for CD. 5 patients were detected, although 2 were already known to have CD. All 5 (*i.e.,* 4.5%) were free of symptoms. On the basis of these studies, these investigators have suggested that patients with hypothyroidism or hyperthyroidism should have serological screening for CD.

Other thyroid disorder in CD

Finally, other thyroid disorders have been recorded in CD. Most intriguing are reports of malignant thyroid lymphomas with CD, given the increased risk of lymphoma, particularly T-cell enteropathy, in CD. In an early retrospective evaluation of 12 cases of malignant thyroid lymphoma, 2 had intra-abdominal lymphoma and 1 had documented adult CD[31]. In a later report, a thyroid mass from a lymphoma was described in adult CD[32]. In this instance, the lymphoma was noted to be a rare T-cell lymphoma, indicating another site of extranodal lymphoma that may complicate the clinical course of CD, possibly due to its shared embryological developmental links with the gastrointestinal tract.

DIABETES

Early reports in CD

A number of early reports first described the association between CD and diabetes mellitus, sometimes co-existing with thyroid disease[6,33]. Although most were noted in pediatric-aged patients, some older clinical series also described adults with this association[34-36].

Recent prevalence and screening studies

In recent years, a number of studies from North America[37,38] and Europe[39] reported an increased prevalence of type 1 diabetes in CD thought to be due to an autoimmune process targeting the insulin-producing islet cells of the pancreas[40]. In our investigations, 233 children and adolescents with type 1 diabetes were prospectively screened with serological markers for CD[41]. Sera were blinded and IgA endomysial (EMA) as well as IgA tissue transglutaminase (tTG) assays were done. Among these, 19 were positive for EMA and also had elevated tTG levels. Of these, 1 was already known to have CD while 18 others had minimal or no symptoms and small intestinal biopsies performed. Of these, 14 had moderate to severe morphological changes consistent with CD (along with 1 having normal biopsies and 3 with epithelial lymphocytosis), yielding an overall biopsy-confirmed prevalence of CD of 7.7%, a rate later confirmed by reports from different countries[42-47]. A recent meta-analysis pursued these observations further by urging screening for CD in type 1 diabetes, particularly in children[48].

Pathogenetic linkages

CD and type 1 diabetes mellitus are complex disorders with shared genetic components. The major histocompatibility complex (MHC) is known to be involved in the presentation of peptide antigen from gluten-containing foods to T-cells. MHC class Ⅱ DQ peptides may be associated with both celiac disease and type 1 diabetes. Specific genetic determinants, HLA-DQ2, *i.e.,* haplotypes (DR3-DQ2), occur in about 90% or more of patients with CD and over 50% with type 1 diabetes, while HLA-DQ8 has been reported to occur in about 10% of patients with CD and about 70% of type 1 diabetes[49]. There are several HLA and non-HLA loci in type 1 diabetes mellitus shared in CD[50,51]. Non-HLA genes, such as CTLA4, have also been noted in both CD and type 1 diabetes. For several of these, multiple genes are present[52]. In addition, other non-genetic or environmental factors likely play a role in pathogenesis of these autoimmune disorders[53].

Most intriguing are recent observations related to the intestinal luminal organisms. Often, viruses such

as enteroviruses and rotavirus have been noted[53-58]. Changes in the luminal environment have been hypothesized to alter the intestinal immune system, its regulation or intestinal permeability. The intestinal microbiome may also play a critical environmental role in genetically-predisposed individuals. An altered composition of the gut microflora has been reported in CD, including a decreased ratio of *Firmicutes* and *Bacteroidetes*[59,60]. As reviewed elsewhere, alterations in the intestinal microbiota have also been documented with dietary removal of gluten[61].

Although the precise mechanism for a possible pathogenic effect of gluten in type 1 diabetes is not known, the effects of a gluten-free diet on control of insulin-dependent diabetes mellitus have been explored. Most reported investigations involve studies with animal models. However, in a case report, remission was achieved in a male child with insulin-dependent diabetes without insulin therapy on a gluten-free diet alone[62].

Clinical implications

Time course studies of diagnosis of type 1 diabetes and CD suggests that diagnosis of type 1 diabetes usually occurs first, followed by CD[63-66]. In patients with type 1 diabetes and celiac disease, additional autoimmune diseases may later develop, particularly autoimmune thyroid disease[67-69]. Moreover, risk of disease complications, including bone disease, retinopathy or nephropathy may occur, particularly if concomitant CD is present[67-71], and symptoms associated with CD may be more difficult to resolve if type 1 diabetes is also present[69]. Finally, a gluten-free diet (if CD is present) may lead to better glycemic control and protect patients against development of diabetes-related vascular complications[70]. Clearly, further studies are needed to determine the impact of a gluten-free diet in patients with both diagnoses. In addition, added studies in pediatric and adolescent patients with both diagnoses may be important because of concerns related to compliance, especially in those with limited symptoms[71].

OTHER ENDOCRINE DISORDERS

Early and recent prevalence reports

Adrenal insufficiency and CD may occur in some patients. Indeed, a high frequency of adult CD may be present in association with autoimmune adrenocortical failure (autoimmune Addison's disease). Many occur in the setting of polyendocrine failure that may include Addison's disease, thyroiditis, ovarian failure and CD[72]. In a study of 76 patients (44 females) with Addison's disease from Norway, 5 had biopsy-confirmed changes of CD and 1 additional patient had a previous diagnosis of CD[73]. All had HLA haplotype DR3-DQ2 with a total prevalence of CD of 7.9%. Although 52% had evidence of polyglandular endocrine failure, the investigators recommended that Addison patients be screened for CD and suggested that causes of failure of substitute hormonal treatment may include CD. In a separate Swedish national registry study[74], both children and adults with CD had a significant positive association with Addison's disease. As there was no apparent temporal sequence in diagnosis of either disorder, it was recommended that cases with adrenal insufficiency be screened for CD, and that CD patients have increased awareness of adrenal insufficiency.

Autoimmune polyglandular syndromes

More recent reports have emphasized the significance of recognition of autoimmune polyglandular syndrome[75] in different age groups permitting definition of 2 major subtypes. A juvenile form (APS, type I) usually develops in early adolescence or infancy and appears to be characterized by multiple endocrine deficiencies, mucocutaneous candidiasis, ectodermal dystrophy and different endocrine disorders, including hypoparathyroidism and usually Addison's disease, type 1 diabetes, hypogonadism and thyroid disease. Another form usually occurs later in the 3rd or 4th decade (APS, type Ⅱ), with a female predominance. Endocrine diseases that occur commonly include autoimmune thyroid disease, type 1 diabetes and Addison's disease while hypoparathyroidism is rare and no mucocutaneous candidiasis develops[76]. Although hypoparathyroidism has been rarely recorded with coincident CD[77], the endocrine pattern in adult CD most often fits the type Ⅱ pattern[76]. In a very recent report, however, it was noted that in those with concurrent celiac disease and hypoparathyroidism, a gluten-free diet had a beneficial effect on calcium regulation[78].

In another report, the evolving nature of these autoimmune polyendocrine syndromes with CD was further emphasized[79]. The authors confirmed that APS Ⅰ most often developed in childhood and included hypoparathyroidism, mucocutaneous candidiasis and Addison's disease. The more common APS Ⅱ type was often diagnosed if Addison's disease occurred together with thyroiditis (Schmidt's syndrome) or type 1 diabetes (Carpenter's syndrome). Another form, APS 3, may be seen if there is no adrenal cortical defect. A further type 4 may occur if other less common autoimmune endocrine failure develops, specifically autoimmune hypophysitis, with CD[79]. The authors noted that the detection of a monoglandular endocrinopathy may only be part of an evolving and dynamic process with the appearance of other endocrinopathies at a later stage in CD. In contrast, an Italian study of children and adolescents noted a high detection rate (42%) of anti-pituitary antibodies in newly diagnosed celiacs[80]. Interestingly, high antibody levels were associated with height impairment, possibly mediated by a reduction in insulin-like growth factor, and suggesting that an autoimmune pituitary process may be important

in the induction of linear growth impairment in CD. Further mechanisms may be at play in growth failure in CD. A gluten-free diet has been reported to result in rapid catch-up growth and normalization of pituitary function[81] and some have suggested a possible role for growth hormone replacement in children with short stature, despite a gluten-free diet over a 1 year period[82]. Other evidence has accumulated that the pituitary gland may be altered in CD. For example, prolactin is produced by the anterior pituitary gland, may be important in breast glandular development and may play a role in autoimmune regulatory mechanisms. In some studies, prolactin levels were increased in recently diagnosed CD in pediatric patients and these levels decreased over a few months with a gluten-free diet[83,84].

Ovarian failure causing infertility is also becoming increasingly recognized in adult CD and has recently been reviewed[85]. Indeed, in some prospective serologically-based studies, over 4% of infertile females may prove to have CD. If positive, subsequent biopsy studies have confirmed the presence of adult celiac disease. In some of these, treatment with a gluten-free was associated with later subsequent successful pregnancy. A recent meta-analysis of relevant studies indicated that CD was more prevalent in women with "all-cause" and "unexplained" infertility compared to the general population[86].

CONCLUSION

In CD, a number of autoimmune endocrine disorders may occur. Often, these clinically present with monoglandular involvement, usually the thyroid gland in adults or with insulin-dependent (type 1) diabetes in children and adolescents as well as adults[87]. Similar observations have also recently been reported from China[88]. Polyglandular disease has also become increasingly recognized, sometimes in association with a long clinical course of undiagnosed CD prior to detection and institution of a gluten-free diet. Diagnosis of CD may eventually also lead to definition of a single or multiple gland autoimmune syndrome that may only result after repeated evaluations of the patient with CD.

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MINIREVIEWS

Circulating tumor DNA as a liquid biopsy target for detection of pancreatic cancer

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Abstract

Most pancreatic cancer patients present with advanced metastatic disease, resulting in extremely poor 5-year survival, mainly because of the lack of a reliable modality for early detection and limited therapeutic options for advanced disease. Therefore, there is a need for minimally-invasive diagnostic tools for detecting pancreatic cancer at an early stage, when curative surgery and also novel therapeutic approaches including precision medicine may be feasible. The "liquid biopsy" addresses these unmet clinical needs based on the concept that simple peripheral blood sampling and detection of circulating tumor DNA (ctDNA) could provide diagnostic information. In this review, we provide an overview of the current status of blood-based tests for diagnosis of pancreatic cancer and the potential utility of ctDNA for precision medicine. We also discuss challenges that remain to be addressed in developing practical ctDNA-based liquid biopsy approaches for early diagnosis of pancreatic cancer.

Key words: Circulating tumor DNA; Pancreatic cancer; Biomarker; Precision medicine; Liquid biopsy

Core tip: There is a need for minimally-invasive diagnostic tools for detecting pancreatic cancer at an early stage and also novel therapeutic approaches including precision medicine may be feasible. The "liquid biopsy" addresses these unmet clinical needs based on the concept that simple peripheral blood sampling and detection of circulating tumor DNA (ctDNA) could provide diagnostic information. In this topic, we provide an overview of the current status of blood-based tests for diagnosis of pancreatic cancer and the potential utility of ctDNA for precision medicine. We also discuss challenges that remain to be addressed in developing practical ctDNA-based liquid biopsy.

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer, representing about 80% of all cases. It is a devastating disease with a 5-year survival rate of only approximately 4%-7% (http://seer.cancer.gov/statfacts/html/pancreas.html), and this figure has not been improved in recent decades. Although surgical resection is the only curative treatment for PDAC, only 15% to 20% of patients present with resectable disease and the majority are diagnosed with locally advanced or metastatic cancer[1]. This situation is mainly a consequence of the aggressive nature of this disease and the lack of an efficient method for detection of early-stage lesions. In addition, very early stage organ metastasis is often observed in patients which are treated with potentially curative surgery. This suggests that occult tumor cells may be present in blood even with small lesions

Currently the detection and diagnosis of pancreatic cancer largely rely on imaging modalities, including ultrasonography, computed tomography, positron emission tomography, magnetic resonance imaging and endoscopic ultrasonography[1]. However, early-stage pancreatic cancers and very small metastases are difficult to detect even if combinations of these modalities are employed. In addition, these modalities require expensive equipment and specialist technicians. Although blood-based tumor biomarkers, such as carcinoembryonic antigen and carbohydrate antigen (CA) 19-9, are much cheaper, simple and minimally invasive alternatives, the sensitivity and specificity of these currently used tumor biomarkers are not sufficient for effective early detection of pancreatic cancer. Despite recent progress in understanding of the disease at the molecular level, no reliable blood-based biomarker for screening of pancreatic cancer has yet become clinically available.

The problem is compounded by the few viable therapeutic options for patients with advanced pancreatic cancer who are not eligible for resection. Chemotherapy for pancreatic cancer patients is limited, and cytotoxic drugs, such as gemcitabine, which have been the standard chemotherapeutic drugs for patients with advanced disease for many years, provide limited survival advantage[1]. Personalized therapies based on cancer-specific alterations are currently not conducted in clinical practice for pancreatic cancer.

In this context, new effective biomarkers are required to improve diagnosis, disease monitoring and process of therapeutic choice of PDAC. As tumor-derived somatic gene alterations can be detected in circulating tumor DNA (ctDNA) from cancer patients, ctDNA could provide a less-invasive diagnostic tool based on the concept of "liquid biopsy". In this review, we summarize the current status of ctDNA analysis, and discuss the potential clinical utility of liquid biopsy for pancreatic cancer.

OVERVIEW OF LIQUID BIOPSY IN CANCER

The concept of detecting tumor-specific molecular alterations by analysis of bodily fluids, including peripheral blood, of cancer patients is termed "liquid biopsy" (Figure 1). Cell-free DNA (cfDNA) consists of small double-stranded DNA fragments found in blood. In 1948, Mandel and Metais firstly reported the presence of cfDNA in the circulation[2]. Tumor-derived cfDNA, now commonly known as ctDNA, was described in 1989[3]. The clinical utility of cfDNA in plasma and serum has been an active area of research in a variety of clinical settings. Indeed, evaluation of fetal cfDNA in the circulation of pregnant women is becoming a routine diagnostic test for high-risk patients in the clinic. To date, cfDNA has been the main target of liquid biopsy for cancer detection, together with exosomes, micro-RNA and circulating tumor cells. In the oncology setting, ctDNA is expected to provide a minimally-invasive approach for cancer diagnosis, monitoring of chemotherapy-resistant mutations, and overcoming the problem of tumor heterogeneity[4-6].

It has been suggested that cancer patients have higher levels of cfDNA than healthy individuals[7], although an increase is also observed with a variety of other physiological and pathological conditions, including exercise, inflammation, exposure to smoking, sepsis and trauma[8]. cfDNA is shed into the bloodstream *via* apoptosis, necrosis, direct release from viable cells, and lysis of circulating cells, but the major sources are now thought to be apoptotic and necrotic cells. In fact, the length of cfDNA fragments in the circulation often shows a characteristic laddering pattern with multiples of 170-180 base pairs, which is a well-known feature of apoptosis[7]. Apoptosis is programmed even for many normal cells on a daily basis and it has been suggested that a large fraction of cfDNA is derived from bone marrow and liver in healthy individuals[9]. However, in a tumor mass, hyperproliferation and rapid cellular turnover of cancer cells can lead to very greatly increased programmed cell death. Features of intratumoral microenvironments such as hypoxia may also lead to necrosis. Cellular debris from apoptotic or necrotic cells is normally phagocytozed by infiltrating macrophages and the cellular components are cleared. However, this clearance mechanism does not proceed effectively in a tumor mass, leading to accumulation of cellular debris, including DNA, and its release into the circulation[5,7,10].

Although total cfDNA may be generally increased in patients with cancer, the sensitivity and specificity for cancer detection are low, and the utility as a cancer

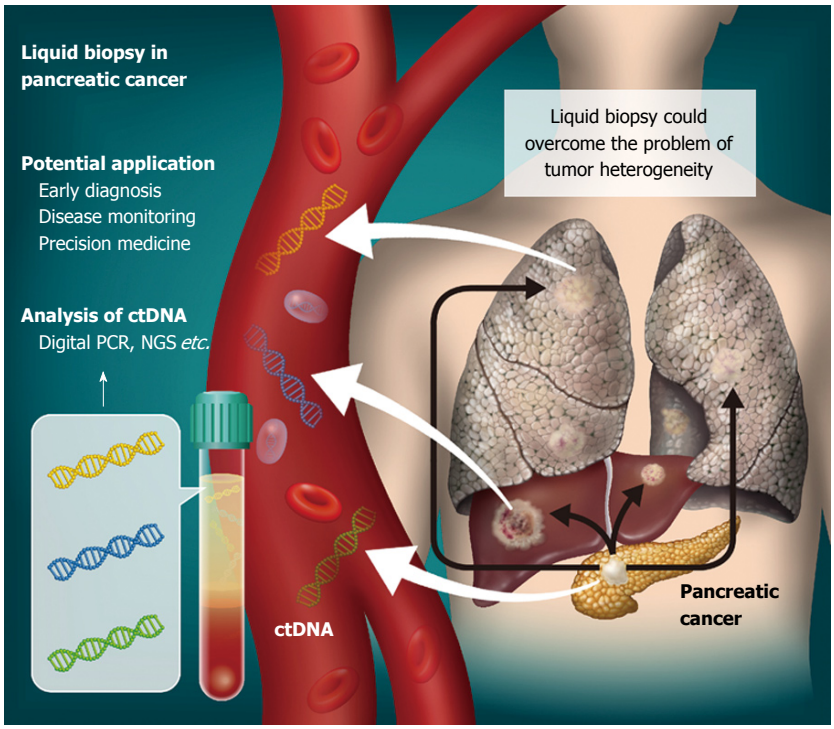

Figure 1 Liquid biopsy for pancreatic cancer. Circulating tumor DNA (ctDNA) can be isolated from plasma as a liquid biopsy approach. Genomic alterations detected could then have various clinical applications in pancreatic cancer cases. As ctDNA is released not only from primary tumors, but also metastases, its analysis might overcome the problem of tumor heterogeneity.

biomarker is questionable. On the other hand, tumor DNA can be discriminated from normal cfDNA by detecting tumor-specific somatic mutations that exist only in the genomes of cancer or precancerous cells, but not in the genomes of their normal counterparts. This assures the specificity of ctDNA as a cancer biomarker.

However, detection of ctDNA has in practice proven challenging, since the percentage of ctDNA may be very low (< 1.0% in many cases) in total cfDNA[5,11]. Traditional methods such as Sanger sequencing or pyrosequencing can detect mutated tumor-derived DNA fragments only in patients with a high tumor burden and a large amount of ctDNA. However, recent advances in sequencing technologies, including the digital polymerase chain reaction (dPCR) and next-generation sequencing (NGS), have made it possible to detect ctDNA present at relatively low frequencies in blood, and there has been an explosive increase of studies of clinical utility of ctDNA[12-14].

METHODS FOR DETECTION OF CTDNA

The dPCR is now one of the major methods to sensitively detect genomic alterations in cfDNA. In 2003, a PCR-based digital approach, named BEAMing (Beads, Emulsion, Amplification, and Magnetics) was first described[15]. Using emulsion PCR and flow cytometry, BEAMing can efficiently identify rare mutations with allele fractions as low as 0.01%[16].

Nowadays several dPCR systems, including droplet-based platforms, are commercially available. Generally, the sensitivity of a droplet dPCR system depends on the number of droplets. One of the most widely used droplet dPCR devices, the QX200 Droplet Digital PCR System (Bio-Rad Laboratories) generates 20000 nanoliter-sized droplets. The RainDrop Digital PCR System (RainDance Technologies) can perform "single-molecule" PCR for up to 10 million picoliter-sized droplets, and therefore possesses very high sensitivity. In addition, multiplex assays are possible in the RainDrop system by using combinations of two color probes (up to 10 targets)[17].

NGS is also widely applied to analyze genomic alterations in cfDNA. Unlike dPCR, NGS techniques can analyze multiple, broad regions of interest. Even whole-genome sequencing or whole-exome sequencing of cfDNA from advanced cancer patients has been reported, and various alterations, including single nucleotide variants (SNV), copy number alterations (CNA) and structural alterations of DNA, were detected[13,18,19]. However, only genomic alterations with high allele frequencies may be appropriate with these platforms, since deep genome-wide analysis, especially whole-genome sequencing, is quite costly and not feasible in the routine clinical context. Therefore, global genomic analyses can be applied for only cfDNA samples from advanced cancer patients with high tumor burden. On the other hand, targeted sequencing can be performed at relatively low cost. By focusing on clinically important genes, mutations can be detected with higher sensitivity compared to genome-wide

analyses.

Amplicon sequencing is one of the major techniques for analyzing mutations in specific genomic regions. Ion AmpliSeq Technology (Thermo Fisher Scientific) is a widely used targeted sequencing platform. Highly multiplex PCR followed by NGS, such as Ion Personal Genome Machine (Ion PGM), allows deep sequencing of target regions with as little as 10 ng input DNA at low cost and with a short turnaround time. However, the Ion Ampliseq system has issues such as a relatively high error rate in detection of small insertions and deletions (indels)[20].

Target enrichment techniques, namely target capture-based platforms, are widely used for analyzing gene alterations of cancer. In principle, fragmented genomic DNA is hybridized with DNA/RNA probes designed for capturing targeted regions, and the enriched DNA libraries are analyzed by NGS. The SureSelect Target Enrichment System (Agilent Technologies) is widely employed for targeted sequencing in combination with the Illumina paired-end sequencing platform, which has a relatively low error rate amongst high-throughput sequencing instruments[21]. Although the manufacturer's protocol for the SureSelect Target Enrichment System requires at least 200 ng of input DNA, the amount can be reduced by using particular library preparation kits, such as the KAPA Hyper Prep Kit (KAPA Biosystems)[22].

In addition to these commercially available technologies, various highly sensitive sequencing methods have been developed for detecting ctDNA. In an amplicon-based system, Safe-Sequencing System, individual DNA molecules are tagged with a unique identifier, then amplified and sequenced. According to the original paper, the error rate could be lowered to 9×10^{-6} by taking into account unique identifiers[23]. Forshew *et al*[24] reported a method termed Tagged-Amplicon deep Sequencing in 2012. They detected somatic mutations in cfDNA at a 2% allele frequency. In the case of non-small cell lung cancer, another method for profiling ctDNA, Cancer Personalized Profiling by deep Sequencing, has been described[25]. In addition to methods for detecting SNVs, Personalized Analysis of Rearranged Ends identifies cancer-specific genome rearrangements, and it has been shown that such alterations can be used as personalized cancer biomarkers[26].

CTDNA AS A BIOMARKER FOR PANCREATIC CANCER

Clinical utility of ctDNA has been investigated in various types of cancer. For diagnosis, the most commonly mutated genes are considered to be best suited for analysis as blood-based biomarkers. However, even within a single tumor type, the mutation profile generally varies from patient to patient. Even if a single gene is commonly mutated in a particular cancer type, the altered locus can vary, especially in tumor suppressor genes such as *TP53*. For this reason, among others, it is not simple to utilize tumor-derived DNA in plasma for the diagnosis of many cancer types without information about actual mutations in the tumor tissues themselves.

The molecular genetics landscape of PDAC has been studied by whole-genome or exome sequencing and somatic alterations associated with this disease have been identified[28-31]. Four genes, *KRAS*, *CDKN2A*, *TP53* and *SMAD4*, are commonly mutated or modified epigenetically in PDAC, and dozens of candidate driver genes are altered at low frequency (< 5%)[27-30]. Clonal evolution of pancreatic cancer has also been investigated and it has been exhibited that genetic heterogeneity arise during subclonal evolution[4]. Since point mutations of *KRAS* are particularly commonly observed in PDAC and 90% of all *KRAS* mutations occur in codon 12 or 13, these have been a focus of attention. To date, many studies have confirmed that mutant *KRAS* can be detected in plasma or serum from patients with PDAC, although detection methods applied were diverse[22,31-36].

In the early 21st century, several research groups investigated the potential use of *KRAS* mutation in cfDNA as a biomarker of pancreatic cancer and demonstrated that such mutations were more frequently detected in the blood of PDAC patients than in individuals suffering from chronic pancreatitis[31,32]. It has also been suggested that sensitivity and specificity for detection of PDAC can be improved by combining *KRAS* mutations in blood with increase in the serum CA19-9 level[32,33]. Maire *et al*[31] reported that the sensitivity and specificity of serum *KRAS* mutations for the diagnosis of pancreatic cancer were 47 and 87%, respectively, whereas the combination of serum *KRAS* mutations and CA19-9 had a sensitivity and specificity of 98 and 77%, respectively. Analysis by Däbritz *et al*[32] also suggested that detectable *KRAS* mutations in the plasma were associated with progressive disease (75%), whereas the association was more evident when combining plasma *KRAS* mutations and elevated CA19-9 (92%). Furthermore, it has been reported that the presence of mutant *KRAS* in the circulation is associated with poor prognosis of patients with pancreatic cancer[23,33-36]. Multivariate analysis also showed that *KRAS* mutations in plasma DNA were stronger prognostic factor for survival (HR = 7.39, $P < 0.001$) than elevated CA19-9 (HR = 2.49, $P = 0.087$)[33]. Thus, *KRAS* mutant cfDNA could be useful as a predictive biomarker for treatment decisions.

One of the major potential applications of ctDNA is disease monitoring. Tjensvoll *et al*[36] reported that changes in mutant *KRAS* levels in the circulation correlated with radiological imaging data and CA19-9 levels during the course of chemotherapy. They suggested the utility of *KRAS* mutant cfDNA for monitoring treatment efficacy and tumor progression

in pancreatic cancer patients. Our own experiments further suggested that the detectability of *KRAS* mutant cfDNA is associated with the presence of distant organ metastasis, and thus ctDNA might be also useful to monitor tiny distant metastases that are hard to detect by routine imaging tests[22].

As mentioned above, currently available tumor biomarkers, such as CA19-9, are insufficient to detect PDAC due to low sensitivity and low specificity. Somatic mutations, on the other hand, are highly specific to DNA derived from cancer or precancerous cells. Especially, *KRAS* is the most frequently mutated gene in PDAC and the mutations occur at the very early stage of carcinogenesis. As technology advances, ctDNA discriminated by *KRAS* mutation may have great potential as a blood-based biomarker for PDAC.

DETECTING TARGETABLE GENOMIC ALTERATIONS IN CTDNA

It is noteworthy that various other cancer-related genes are mutated at relatively low frequencies in PDAC. Importantly, it has been indicated that 20% of patients with pancreatic cancer have somatic alterations in genes that are potential targets of therapies approved by the U.S. Food and Drug Administration for oncologic indications or therapies in published prospective clinical studies[37]. This suggests that genomic profiling in pancreatic cancer could be useful to design precision treatment strategies. Due to improvements of sequencing technologies, global or highly multiplexed genomic analysis of ctDNA is becoming feasible using NGS. Analyzing ctDNA has also been proposed as an alternative method to tissue biopsy in the setting of precision medicine, which relies on the presence of specific targets. Although tumor tissue biopsy is the gold standard for molecular screening of cancer, some patients are precluded from molecular screening because of difficulty in obtaining a tissue biopsy or insufficient tumor content in the available specimens[38]. Indeed, adequate biopsy tissues for molecular diagnosis are often difficult to acquire in pancreatic cancer patients. Very importantly, taking tissue biopsies is invasive and therefore not without clinical complications. Zill *et al*[39] analyzed 54 genes in tumor tissues and cfDNA samples using a commercially available gene panel, and demonstrated that a large proportion of mutations in pancreatic and biliary cancer could be detected in both. Although 35% of patients had an insufficient quantity or quality of tissue biopsy samples for sequencing analysis in their cohort, sequencing of cfDNA identified somatic mutations in many of these cases. We also have reported targeted deep sequencing analysis of cfDNA using a modified SureSelect-Illumina platform and an original gene panel for pancreatic cancer[22]. Our gene panel consisted of 60 genes, including 17 potentially actionable examples. In order to apply the SureSelect Target Enrichment System for the small amounts of cfDNA samples, we modified the library preparation conditions by combination with a KAPA Hyper Prep Kit. In our protocol, input cfDNA could be reduced to as little as 5 ng. As prescreening for sequencing analysis, dPCR assays were first performed to determine the mutational status of *KRAS* in plasma cfDNA of 259 patients with PDAC. We then carried out targeted deep sequencing in 48 patients, including 43 cases that were considered to have $\geqslant$ 1% tumor DNA in total cfDNA based on dPCR *KRAS* assay and 5 patients with obvious distant organ metastasis, even though they were negative for *KRAS* mutation in plasma on dPCR assay. We found somatic mutations in potentially targetable genes in 14 of 48 patients (29.2%). In addition, we analyzed somatic CNA using targeted sequencing data for cfDNA, and potentially targetable gene amplifications, such as in *CCND1* and *ERBB2*, were also detected. At present, as NGS assays are still costly and the sensitivities of standard sequencing technologies are limited, targeted deep sequencing of cfDNA may not be practical in clinical settings for all patients. Since *KRAS* mutation is a good cancer biomarker in pancreatic cancer patients, our two-step approach combining dPCR and NGS could be cost-effective and applicable in the clinic. It may be possible to apply such ctDNA assays to broader range of patients by using a larger volume of plasma because the sensitivities of these assays should depend on the amount of input cfDNA. In addition, the use of novel techniques, including molecular barcoding, and error reduction methods by bioinformatics approaches could improve the sensitivities of sequencing analysis[40,41]. Thus, the available data indicate that liquid biopsy has great potential for diagnosis and treatment design in pancreatic cancer in diverse clinical settings.

EARLY DETECTION OF PANCREATIC CANCER BY LIQUID BIOPSY

Although PDAC is a highly aggressive disease, the investigation of clonal evolution of this disease and mathematical modeling of the rate of mutation acquisition suggests that there is an 11.7-year period from acquisition of the initiating mutation to full transformation in a pancreatic cell, and another 6.8 years are needed to develop the first metastatic subclone[4]. This model implies that there is a substantial time window for early detection of PDAC. Early diagnosis could have a major impact on patient survival, and therefore new effective biomarkers are urgently needed to improve prognosis.

At present, clinical screening for early detection of PDAC has only limited effectiveness, and liquid biopsy appears to be a promising approach to overcome this problem. In general, however, detection of ctDNA is still challenging in early-stage cancer patients because of the high background levels of normal cfDNA. *KRAS*

mutation has been proposed as a biomarker in cfDNA for detection of PDAC, but in early-stage malignant disease (and also in some metastatic cancers), ctDNA may be extremely rare in total cfDNA (0.01% or less)[23,25,39]. Although many analyses of ctDNA have been reported in various cancer types, the vast majority of those studies were analyses of advanced-stage cancer patients, with metastasis or high tumor burden, and the utility of detecting ctDNA in patients with early-stage lesions has been poorly investigated[14,25,42].

A multicenter study of liquid biopsies in 846 patients with 15 cancer types (including PDAC), using digital technologies and approximately 5 mL plasma, reported a detection rate of ctDNA of 80% in patients with advanced cancer, but only 47% in cases of localized cancer[12]. This finding implies that current technologies for ctDNA analysis are still insufficiently sensitive for reliable detection of early-stage cancers. Novel detection methods with much higher sensitivity are required. The same study also demonstrated that detection rates for ctDNA differ depending on the type of cancer[12]. The factors determining ctDNA levels are still not completely understood, but may include tumor burden and spatial proximity to the vasculature, in addition to type. Detailed analyses and accumulation of larger numbers of experimental data for patients with pancreatic cancer in various clinical situations are needed to develop ctDNA analysis that would be practical for early diagnosis.

In addition to peripheral blood, other body fluids such as pancreatic juice may be a secondary source of tumor DNA for liquid biopsy. While collection of pancreatic juice is invasive, as it requires endoscopic techniques which are much more intricate than simple drawing of blood, pancreatic juice would be expected to contain a much higher concentration of tumor DNA. Indeed, mutant *KRAS* has been detected in pancreatic juice from pancreatic cancer patients[43,44].

Not only genetic alterations, but also epigenetic aberrations, such as DNA hypermethylation, occur during pancreatic carcinogenesis. Aberrant DNA methylation seems to occur in early-stage tumors, resulting in inactivation of tumor suppressor genes or gain-of-function of oncogenic signaling pathways[45,46]. Genes that are aberrantly methylated in a high proportion of pancreatic cancer patients could thus be biomarkers for cancer screening. Methylation of several genes (including *NPTX2*, *SFRP1* and *SPARK*) has been detected in pancreatic juice samples, and allow distinction of patients with chronic pancreatitis or normal individuals from cancer cases[47]. Detecting tumor-specific epigenetic alterations in cfDNA could be an attractive option for diagnosis of pancreatic cancer by means of a liquid biopsy approach, since epigenetic markers, including aberrant DNA methylation, can be also found in ctDNA. Indeed, Yi *et al*[48] demonstrated this possibility with promoter methylation of *BNC1* and *ADAMTS1*. In the future, it may be worth investigating the feasibility of utilizing combinatorial approaches with multiple blood-based biomarkers, including genomic mutations in ctDNA and epigenetic alterations in ctDNA, as a strategy to improve sensitivity and specificity in the diagnosis of early-stage pancreatic cancer.

CONCLUSIONS AND FUTURE DIRECTIONS

Although pancreatic cancer is a highly lethal disease with limited treatment options, a novel diagnostic test able to accurately detect the disease at an early stage, when curative surgery may be feasible, should greatly improve the prognosis. Minimally-invasive blood tests might also be useful for cancer screening. A number of studies have already detected genomic alterations in blood from patients with pancreatic cancer, confirming the potential value of liquid biopsy approaches. In addition, detecting actionable genomic alterations in ctDNA might provide a less-invasive approach for precision medicine even in PDAC, which is often inaccessible for tumor tissue biopsy.

However, at present there is still insufficient concrete evidence of the utility of ctDNA analysis regarding treatment of pancreatic cancer, and several issues need to be addressed. One of the most urgent is improvement of sensitivity. While the prospects for technological development and analytical advances seem promising, implementation of new ctDNA analyses for pancreatic cancer screening will depend on demonstration of clinical validity in large prospective studies. Especially for investigating the feasibility of utilizing ctDNA for early diagnosis, it is particularly important to analyze samples from patients with early-stage disease, although this will presumably only be possible with a generalized screening approach. Prospective follow-up and sequential blood sampling of individuals at high risk of pancreatic cancer (*e.g.*, those with a family history of pancreatic cancer or chronic pancreatitis) might thus be essential. Another issue is the diverse range of methods used so far for processing of blood samples and extraction of cfDNA. It will be important to standardize preanalytical processes for cfDNA analysis, such as blood sample acquisition, plasma separation, sample storage, cfDNA extraction and quantification. This issue has only just begun to be discussed. Recently, there are an increasing number of new products for cfDNA processing including blood collection tubes [*e.g.*, Cell-Free DNA BCT® (Streck) and Cell-Free DNA Collection Tube (Roche)] and cfDNA extraction kits [*e.g.*, Quick-cfDNA™ Serum & Plasma Kit (Zymo Research), Maxwell® RSC ccfDNA Plasma Kit (Promega), and MagMAX™ Cell-Free DNA Isolation Kit (Thermo Fisher Scientific)]. For sequencing of cfDNA, new library preparation kits optimized for small amounts of fragmented DNA, such as Accel-NGS® DNA Library Kits (Swift Biosciences) and ThruPLEX® Plasma-

seq Kit (Rubicon Genomics), have also been available. It is worth evaluating the new products to establish standardized methods of ctDNA analysis. In view of the potential benefit to patients of a liquid biopsy approach using ctDNA for early detection of pancreatic cancer, we believe work to address these issues should be a high priority.

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MINIREVIEWS

Elucidation of the early infection machinery of hepatitis B virus by using bio-nanocapsule

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Abstract

Currently, hepatitis B virus (HBV), upon attaching to human hepatocytes, is considered to interact first with heparan sulfate proteoglycan (HSPG) *via* an antigenic loop of HBV envelope S protein. Then, it is promptly transferred to the sodium taurocholate cotransporting polypeptide (NTCP) *via* the myristoylated N-terminal sequence of pre-S1 region (from Gly-2 to Gly-48, HBV genotype D), and it finally enters the cell by endocytosis. However, it is not clear how HSPG passes HBV to NTCP and how NTCP contributes to the cellular entry of HBV. Owing to the poor availability and the difficulty of manipulations, including fluorophore encapsulation, it has been nearly impossible to perform biochemical and cytochemical analyses using a substantial amount of HBV. A bio-nanocapsule (BNC), which is a hollow nanoparticle consisting of HBV envelope L protein, was efficiently synthesized in *Saccharomyces cerevisiae*. Since BNC could encapsulate payloads (drugs, genes, proteins) and specifically enter human hepatic cells utilizing HBV-derived infection machinery, it could be used as a model of HBV infection to elucidate the early infection machinery. Recently, it was demonstrated that the N-terminal sequence of pre-S1 region (from Asn-9 to Gly-24) possesses low pH-dependent fusogenic activity, which might play a crucial role in the endosomal escape of BNC payloads and in the uncoating process of HBV. In this minireview, we describe a model in which each domain of the HBV L protein contributes to attachment onto human hepatic cells through HSPG, initiation of endocytosis, interaction with NTCP in endosomes, and consequent provocation of membrane fusion followed by endosomal escape.

Key words: Bio-nanocapsule; Endosomal escape; Hepatitis B virus; Heparan sulfate proteoglycan; Sodium taurocholate cotransporting polypeptide

Core tip: Owing to the poor availability and the difficulty of manipulations of hepatitis B virus (HBV), it has been

difficult to analyze its early infection events in human hepatocytes. Using a bio-nanocapsule, a unique model of HBV, we could study these events by biochemical and cytochemical methods, and finally identify a low pH-dependent fusogenic domain in HBV pre-S1 region, which might play a pivotal role in the endosomal escape of HBV. We hereby postulate a model in which each domain in HBV envelope L protein participates in cell attachment, endocytosis, membrane fusion, and consequent endosomal escape (*i.e.*, uncoating process of HBV).

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INTRODUCTION

Hepatitis B virus (HBV) is an approximately 42 nm envelope virus containing a nucleocapsid with an approximately 3.2 kilobase double-stranded DNA genome (Figure 1). There are three types of envelope proteins on the surface of HBV virion: The small (S) protein, middle (M, pre-S2 + S region) protein, and large (L, pre-S1 + pre-S2 + S region) protein[1] (Figure 2). Although nearly half a century has passed since the discovery of HBV, it has been difficult to obtain HBV in substantial amounts for biochemical analyses and establish an efficient *in vitro* system covering infection, replication, and virion release. As a first generation system, HBV was purified from chronically infected patient's plasma, and primary human hepatocytes (PHH) were used as target cells. Since this system completely depends on clinical specimens, many researchers have reluctantly utilized human hepatoma-derived cell lines for a long time, but these often neither accept HBV infection nor reproduce it. Next, as a second generation system, primary Tupaia hepatocytes (PTH) were found to accept HBV infection efficiently in the presence of dimethyl sulfoxide (DMSO) and allow HBV reproduction[2]. Furthermore, as a third generation system, one human hepatoma cell line, HepaRG, was recently found to accept HBV infection in the presence of DMSO as well as PHH and PTH[3], providing a new possibility for studying HBV early infection. Although the aforementioned progress in HBV target cells has been made for the long time, it is still necessary to obtain a substantial amount of HBV exclusively from patient's plasma. These situations have hampered the comprehensive elucidation of early infection machinery of HBV.

HBV AND ITS RECEPTORS

To elucidate the mechanism how HBV recognizes human hepatocytes, many parts of the HBV envelope L protein have been proposed to be indispensable for early infection, especially for the initial attachment. In 1986, the N-terminal part of pre-S1 region (from Pro-10 to Pro-36; see Figure 2) was shown to interact with PHH[4]. Monoclonal antibody against the pre-S1 region, MA18/7, could neutralize the *in vitro* infectivity of HBV to PTH, whereas antibodies against pre-S2 and S regions could not[5,6]. N-terminal myristoylation of pre-S1 region is also indispensable for HBV to infect PHH[7]. Furthermore, since the addition of heparin could efficiently inhibit *in vitro* infection with HBV, attachment to heparan sulfate proteoglycan (HSPG), a protein abundant in the extracellular matrix, is a prerequisite for infection of HepaRG cells[8] and PTH[9]. Highly conserved residues (Gly-282, Pro-283, Cys-284, Arg-285, Cys-287, and Lys-312; see Figure 2) in the antigenic loop (AGL) of S region could directly contact with HSPG[10-12]. It is interesting that other viruses, including herpes simplex virus-1 and human immunodeficiency virus-1 (HIV-1), also require interaction with HSPG for their entry[13]. These results strongly suggest that HSPG is a low-affinity receptor for HBV entry. In case of HIV-1, following attachment to HSPG, the virus interacts with CD4 receptor, leading to conformational changes in the viral envelope protein, and subsequently enters the cell using the CCR5 co-receptor[14]. However, it remains unclear what events occur during infection and how HBV exhibits such stringent specificity to human hepatocytes.

In 2012, a transmembrane protein, sodium taurocholate cotransporting polypeptide (NTCP), also known as SLC10A1[15], was indicated as a functional receptor responsible for HBV infection[16]. NTCP is predominantly expressed on the sinusoidal membrane of hepatocytes and is responsible for the majority of sodium-dependent bile acid translocation, playing an essential role in the enterohepatic cycle of bile acids[17]. Knockdown or overexpressed NTCP in human hepatocytes prevented or facilitated HBV infection, respectively[18]. Most importantly, an N-terminally myristoylated pre-S1 (2-47) peptide could inhibit both the transporter function and HBV interaction of NTCP efficiently[19] (Figure 2). These results indicate that NTCP is a high-affinity human liver-specific HBV receptor and would open up new avenues for understanding the early infection machinery of HBV.

BIO-NANOCAPSULE AS A VACCINE IMMUNOGEN

Large fraction of the world's population suffers from HBV infection. Since HBV can be transmitted through blood and body fluids, and there has not been any effective anti-HBV drug, vaccination against hepatitis B (HB) is essential for protection from blood-borne infection. Initially, subviral particles consisting of HBV envelope S protein [*i.e.*, HBV surface antigen (HBsAg)]

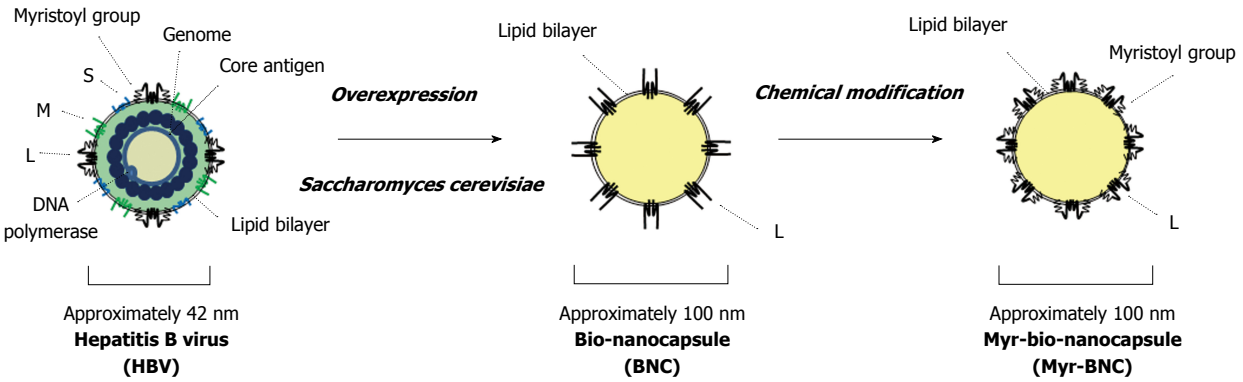

Figure 1 Schematic representation of hepatitis B virus, bio-nanocapsule, and myristoylated bio-nanocapsule.

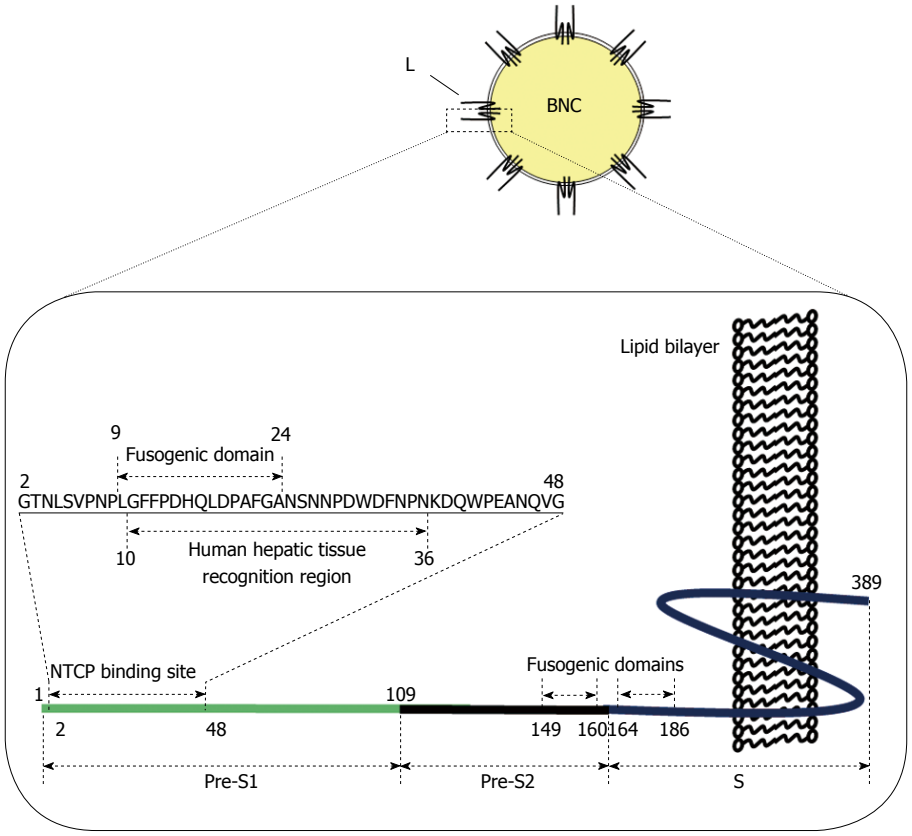

Figure 2 Functional domains in the hepatitis B virus and bio-nanocapsule L protein. BNC: Bio-nanocapsule; NTCP: Sodium taurocholate cotransporting polypeptide.

were purified from HBV e antigen negative patient's plasma and were formulated as first generation HB vaccines. For eliminating the risk caused by contamination with HBV, HBV envelope S protein was expressed in yeast cells in a particle form, and used as the second generation HB vaccine[20]. However, even after repetitive injection with these vaccines, approx. Five percent of vaccinees could not be seroconverted (*i.e.*, low and non-responders)[21]. The finding that pre-S2 region could elicit HBV-neutralizing antibodies in chimpanzees[22] led us to synthesize the HBV envelope M protein in particle form in yeast cells. This third generation HB vaccine candidate could effectively induce protective level of anti-pre-S2 antibodies even in low and non-responders[23]. Thereafter, owing to the recognition that pre-S1 region could elicit additional HBV-neutralizing antibodies[4], many researchers have attempted to synthesize the full-length HBV envelope protein (L protein) in particle form in eukaryotic cells, but the N-terminal part of pre-S1 region showed strong inhibitory effect on its synthesis. In 1992, fusion of the N-terminus with a chicken lysozyme-derived signal peptide could overcome the inhibitory effect and facilitated overexpression of L particles in yeast cells (up to approx. 42% of the total soluble protein)[24]. The particles could be purified by heat-treatment,

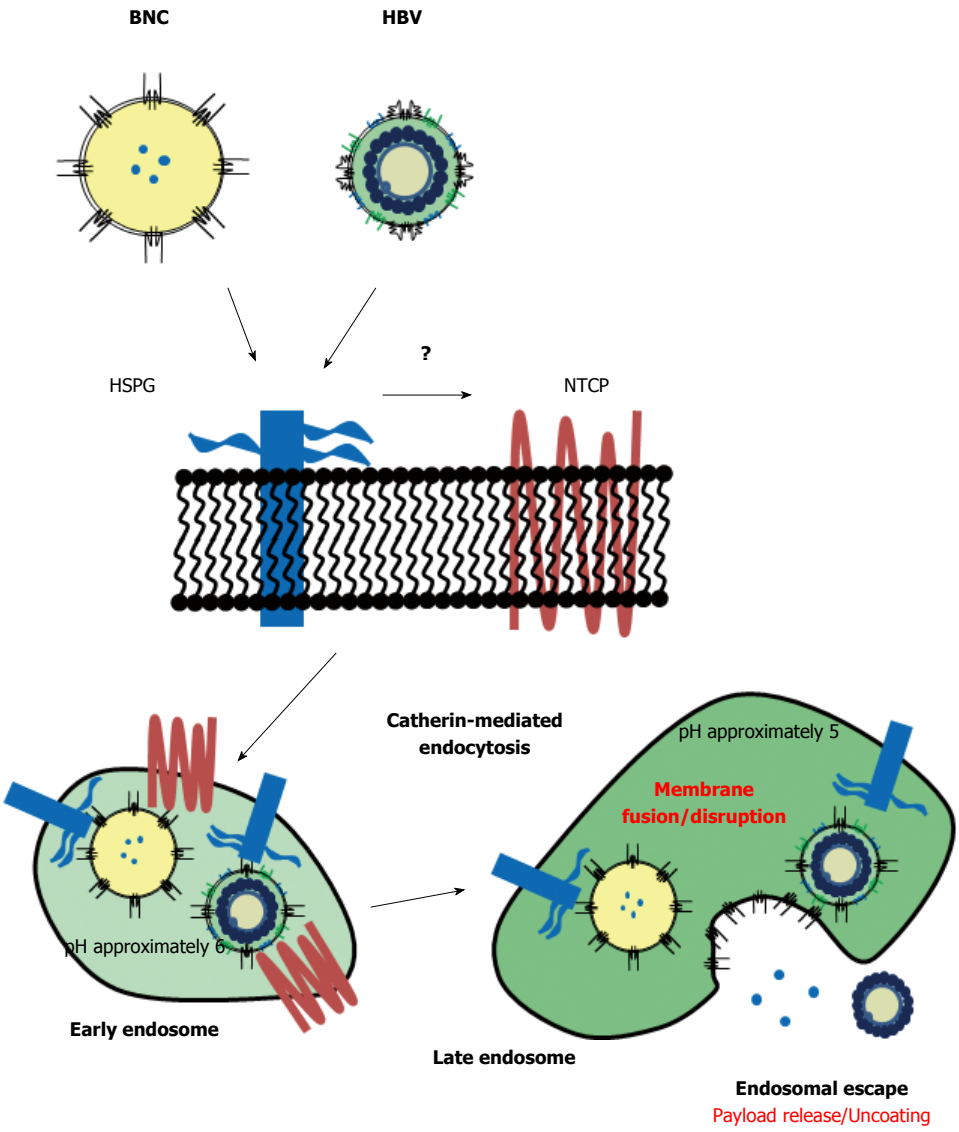

Figure 3 Model of early infection machinery for hepatitis B virus and bio-nanocapsule L in human hepatocytes. HSPG: Heparan sulfate proteoglycan; NTCP: Sodium taurocholate cotransporting polypeptide.

affinity column chromatography, and size exclusion column chromatography[25]. Unlike the approx. 42-nm HBV virion, the particle exhibited approx. 100-nm spherical hollow structure, consisting of about 110 L proteins embedded in an yeast endoplasmic reticulum membrane-derived liposomal structure, whereas the stoichiometric ratio of L/M/S envelope proteins of HBV virion is approx. 1:1:4 (Figure 1)[26,27]. Furthermore, the density of particle is approx. 1.22 g/cm^3[28], which is similar to that of HBV virion (approx. 1.17 g/cm^3)[29]. The particle lacks the N-terminal myristoyl group and possesses additional sugar groups in the pre-S1+pre-S2 region. They could elicit anti-S, anti-pre-S1 and anti-pre-S2 antibodies effectively in mice[26]. According to proteinase protection assay, the pre-S1 region, pre-S2 region, and a part of S region (AGL) are deployed outwardly on the surface of L particle, similar to HBV[24]. This similarity in surface structure prompted us to utilize it as a bio-mimic of HBV in further studies. We therefore designated the HBV envelope L particle as a "bio-nanocapsule (BNC)"[30,31].

BNC AS NANOCARRIER

Human hepatic cell-specific targeting activity

Since HBV specifically infects human hepatocytes and delivers its genetic material and associated proteins into the cytoplasm, we examined whether BNC also exhibited a similar function. Following the introduction of a fluorophore or enhanced green fluorescence protein -expression plasmid into the hollow space of BNC by electroporation, the human hepatic cells receiving BNCs were found to exhibit fluorescence *in vitro*[32]. Furthermore, after an intravenous injection of these BNCs, fluorescence was emitted exclusively from human hepatic cell-derived tumors of xenograft mice[32] and in normal human liver tissues under the kidney skin of severe combined immunodeficiency

(SCID) mice[33]. These results strongly suggested that BNC target and enter human hepatic cells *in vitro* and *in vivo* using HBV-derived infection machinery, present in the L protein.

Liposome fusion activity

BNC was capable of fusing with liposomes (LPs), leading to the formation of BNC-LP complex[28]. Under high temperature (up to 70 ℃) and acidic conditions, the complex was found to transform into a smooth and spherical structure (namely virosomes), in which L proteins translocated across the membrane in the correct topology[34]. Following incorporation of beads and genes, these virosomes could efficiently deliver into the cytoplasm of human hepatic cells *in vitro*[28] and specifically to human hepatic cells *in vivo*[28,34]. Furthermore, after incorporating doxorubicin by remote loading method, the virosomes were intravenously injected into xenograft mice harboring human hepatic cell-derived tumors. The virosomes could retard the tumor growth more effectively than LPs containing doxorubicin, strongly suggesting that virosome could delivery their payload into human hepatic cell-derived tumor efficiently utilizing HBV-derived infection machinery[34]. These results indicate that the virosome is a promising nanocarrier for cytoplasmic delivery of drugs and genes.

Stealth activity

Intravenously injected nanoparticles were unexpectedly trapped by the reticuloendothelial system (RES) in liver, lung, spleen, and so on. However, some viruses can evade RES effectively and finally infect target cells and tissues *in vivo*, namely by virus-derived stealth activity. Meanwhile, it was demonstrated that HBV could associates with monomeric human serum albumin (HSA) and polymerized-HSA[35,36] through the polymerized-albumin receptor (PAR) domain in the pre-S2 region (120-129 aa)[37]. When LPs displaying the PAR domain-containing peptide were intravenously injected into mice, they could recruit albumins on their surface and evade RES effectively (Takagi *et al* personal communication). Indeed, in nude mice harboring human hepatic cell-derived tumors, intravenously injected BNC could target and enter the target tumors by evading RES[32]. Thus, BNC (presumably as well as HBV) could recruit albumin in blood stream by its PAR domain and then exhibit stealth activity.

BNC AS A MODEL OF HBV

As described in the above paragraph, it was demonstrated that BNC possesses HBV-derived infection machinery. Compared with HBV virion, BNC has the following advantages for the elucidation of early infection machinery of HBV. First, the substantial amount of purified BNCs could be easily obtained from recombinant yeast cells. Second, BNCs could be easily labeled with fluorophores and enzymes for cytochemical and biochemical analyses.

Cell attachment and endocytosis

As described above, HBV is considered to interact primarily with HSPG (a low-affinity HBV receptor), change its own structure, and then interact with NTCP (a high-affinity HBV receptor)[18,19]. HSPG is abundantly expressed in the extracellular matrix of various tissues, which could interact with AGL of S region[8,11]. The binding of BNC to human hepatic cells[32] was efficiently suppressed by heparin[38]. Since the membrane topology of BNC is similar with HBV, HSPG might act as a low-affinity receptor for BNC. After BNC attached onto human hepatic cells, it was internalized mainly by clathrin-dependent endocytosis, with a rate very similar to HBV[39] (Figure 3). These results led us to assume that both BNC and HBV utilize the same machinery for cell attachment and endocytosis. The N-terminally myristoylated pre-S1 region (from Gly-2 to Val-47; see Figure 2) is essential for interaction of HBV with NTCP[40], but the original BNC lacks the myristoylated N-terminus owing to the addition of a signal peptide for expression in yeast cells[24]. After chemical modification with myristoyl group, the myristoylated BNC (Myr-BNC) was confirmed to interact with NTCP by immunoprecipitation assay by using lysate of NTCP-overexpressing HepG2 cells (HepG2/NTCP cells), and inhibit the infection of HepG2/NTCP cells with HBV *in vitro*, whereas BNC itself neither[41]. For evaluating the contribution of NTCP to the cell attachment and entry of HBV, fluorophore-labeled form of BNCs, Myr-BNCs, and HB patient plasma-derived HBsAg particles (containing native L protein with N-terminal myristoylation) was incubated with either HepG2 or HepG2/NTCP cells, and then analyzed by flow cytometry. Each of three particle was found to be equally associated with both cell types and be finally localized in late endosomes at comparable level (approx. 20% of each particle)[41]. Furthermore, overexpressed NTCP in HepG2 cells (HepG2/NTCP cells) could neither enhance the cell-surface interaction nor the internalization of fluorophore-labeled form of Myr-BNCs or HBsAg particles, strongly suggesting that cell surface NTCP is not involved in the interaction with HBV (Figure 3)[41]. It is likely that other receptors participate in the human hepatic cell-specific interaction and internalization of BNC and HBV.

Endosomal escape and uncoating process

For the majority of viruses, entry into the cell is *via* the endocytic pathway. Upon reaching late endosomes, the subsequent uncoating process assists in endosomal escape; otherwise, viruses would be degraded in lysosomes[42]. As is the case with other enveloped viruses, it was postulated that HBV also escapes from endosomes using the membrane fusion machinery[43]. The following fusogenic domains have been identified

in L protein: C-terminal half of pre-S2 region (pH-independent; amino acid residues from 149 to 160)[44], N-terminal part of S region (low pH-dependent; amino acid residues from 164 to 186)[45], and the whole pre-S1 region (low pH-dependent)[46]. However, it has thus far remained controversial as to which domains are responsible for the uncoating process of HBV in endosomes. Recently, by lipid mixing assay using BNC or LPs displaying pre-S1-derived mutant peptide, we identified novel low pH-dependent fusogenic domain in the N-terminus of pre-S1 region (from Asn-9 to Gly-24; see Figure 2)[47]. When BNC lacking pre-S1 region was used for the lipid-mixing assay, the fusogenic activity was completely lost. Pre-incubation of BNC with anti-pre-S1 antibodies also inhibited fusion. These results indicated that the fusogenic activity of pre-S1 (9-24) peptide is dominant over those of other fusogenic domains (see above). Furthermore, upon mixing LPs containing fluorophore and quencher (model of endosomes) with BNC or LPs displaying pre-S1 (9-24) peptide, the fluorophore was immediately released at low pH. This suggested that the pre-S1 (9-24) peptide possesses low pH-dependent membrane disruption activity. When BNCs containing fluorophore were mixed with LPs, the fluorophore was released at low pH, suggesting that BNC as well as HBV rupture upon interaction with endosomal membrane at low pH. These results strongly suggested that the pre-S1 (9-24) peptide is essential for the endosomal escape of BNC payload as well as the uncoating process of HBV (Figure 3), which agreed well with Watashi *et al*[48]. More recently, the fusogenic activity of pre-S1 (9-24) peptide was shown to correlate with its hydrophobicity, which is significantly enhanced by the protonation of Asp-16 and Asp-20 under acidic conditions[49].

CONCLUSION

While many researchers have attempted to isolate functional HBV receptors since the last two decades, HSPG and NTCP were finally identified as low-affinity and high-affinity HBV receptors, respectively. However, it is still unclear whether both molecules by themselves support the stringent specificity of HBV to human hepatic cells. Since NTCP is not expressed in all HBV susceptible cells, other molecules may participate in the interaction of HBV with hepatic cells in a cell-specific manner. The mechanism of transfer of HSPG-bound HBV to NTCP, presumably in the endosomes, and the change in structure of HBV (pre-S1 region) at low pH for adaptation to NTCP remains to be elucidated (see Figure 3). Interestingly, the pre-S1 (9-24) peptide is well conserved among all HBV genotypes, and is located within the NTCP-binding site (from Gly-2 to Val-47) (see Figure 2)[16]. This positional relationship of the two domains implies that the uncoating process of HBV and the endosomal escape of BNC are initiated by interaction with NTCP in late endosomes. The elucidation of the molecular machinery of HBV early infection can open avenues for novel targets for anti-HBV drugs.

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ORIGINAL ARTICLE

Basic Study

Prolonged feeding with guanidinoacetate, a methyl group consumer, exacerbates ethanol-induced liver injury

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Author contributions: Osna NA, Ganesan M and Tuma DJ contributed to this paper with drafting and critical revision, editing, and approval of the final version; Feng D and Maillacheruvu PF were involved in the feeding regimens, data collection, analysis and the approval of the final version; Orlicky DJ contributed to this paper with the histopathological assessments, critical revision, editing and approval of the final version; French SW contributed to this paper with the histopathological assessments and approval of the final version; Kharbanda KK contributed to this paper with conception, literature review, drafting and critical revision, editing, and approval of the final version.

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Abstract

AIM

To investigate the hypothesis that exposure to guanidinoacetate (GAA, a potent methyl-group consumer) either alone or combined with ethanol intake for a prolonged period of time would cause more advanced liver pathology thus identifying methylation defects as the initiator and stimulator for progressive liver damage.

METHODS

Adult male Wistar rats were fed the control or ethanol

Lieber DeCarli diet in the absence or presence of GAA supplementation. At the end of 6 wk of the feeding regimen, various biochemical and histological analyses were conducted.

RESULTS

Contrary to our expectations, we observed that GAA treatment alone resulted in a histologically normal liver without evidence of hepatosteatosis despite persistence of some abnormal biochemical parameters. This protection could result from the generation of creatine from the ingested GAA. Ethanol treatment for 6 wk exhibited changes in liver methionine metabolism and persistence of histological and biochemical defects as reported before. Further, when the rats were fed the GAA-supplemented ethanol diet, similar histological and biochemical changes as observed after 2 wk of combined treatment, including inflammation, macro- and micro-vesicular steatosis and a marked decrease in the methylation index were noted. In addition, rats on the combined treatment exhibited increased liver toxicity and even early fibrotic changes in a subset of animals in this group. The worsening liver pathology could be related to the profound reduction in the hepatic methylation index, an increased accumulation of GAA and the inability of creatine generated to exert its hepato-protective effects in the setting of ethanol.

CONCLUSION

To conclude, prolonged exposure to a methyl consumer superimposed on chronic ethanol consumption causes persistent and pronounced liver damage.

Key words: Methyl balance; S-adenosylmethionine; S-adenosylhomocysteine; Guanidinoacetate; Alcohol

Core tip: We examined the role of a combined exposure to ethanol and guanidinoacetate (GAA) in the pathogenesis of liver injury. Exposure to either treatment lowers the hepatic methylation index which is defined as the ratio of the methyl donor, S-adenosylmethionine to its product S-adenosylhomocysteine. We observed a worsening of liver pathology with prolonged GAA and ethanol treatment compared to either treatment alone. These detrimental consequences were related to the profound reduction in the hepatic methylation index, an increased accumulation of GAA and the inability of creatine generated to exert its hepato-protective effects in the setting of ethanol.

Osna NA, Feng D, Ganesan M, Maillacheruvu PF, Orlicky DJ, French SW, Tuma DJ, Kharbanda KK. Prolonged feeding with guanidinoacetate, a methyl group consumer, exacerbates ethanol-induced liver injury. *World J Gastroenterol* 2016; 22(38): 8497-8508 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i38/8497.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i38.8497

INTRODUCTION

Alcoholic liver disease is one of the most serious medical consequences of chronic ethanol use[1,2]. Investigations into the mechanisms of ethanol-induced liver injury have revealed that chronic ethanol abuse causes alterations in the methionine metabolism[3-5]. In particular, ethanol primarily impairs the activity of a vital enzyme, methionine synthase[6,7]. This reduction increases homocysteine secretion[8], promotes hepatic S-adenosylhomocysteine (SAH) accumulation[7,9] and causes a significant lowering in the hepatocellular S-adenosylmethionine (SAM, a methyl donor) to SAH ratio[7,9]. The resulting impairments in several transmethylation reactions have been shown to play a causal role in the generation of many hallmark features of alcoholic liver injury including steatosis, apoptosis, accumulation of damaged proteins and proteasome inhibition[3-5,7,10-14].

The above mentioned changes in methionine metabolism and significant hepatic steatosis are also evident as early as 2 wk of ethanol feeding[15]. Further, feeding rats a diet containing guanidinoacetate (GAA, a potent methyl group consumer) for only 2 wk depleted hepatic SAM levels and lowered SAM:SAH ratio resulting in macrovesicular steatosis[15]. This methylation stress occurs due to the increased utilization of SAM by the enzyme, guanidinoacetate methyltransferase (GAMT) that converts the administered GAA to form the methylated product, creatine[12,15-20]. Additionally, GAA and ethanol appeared to exert synergistic stress following 2 wk of feeding[15]. These GAA-supplemented ethanol diet-fed rats displayed marked decrease in the methylation index (*i.e.*, SAM:SAH ratio), significantly increased triglyceride accumulation, inflammatory changes and liver toxicity compared to the GAA or ethanol-fed rats[15].

Based on the above considerations, we hypothesized that feeding a methyl group consumer in conjunction with ethanol for a longer period than 2 wk will stimulate the progression of disease to produce more serious liver damage. To test this premise, we again chose GAA as a surrogate for methyl group consumers whose ingestion has considerably increased in the last decade[21]. We planned the study exactly as our previous 2-wk study[15] except the time of exposure to GAA, ethanol or combined treatment was extended to 6 wk.

We report the tremendous resilience and adaptation of the liver to continuous insult by the methylation stressor, GAA. However, rats exposed to the ethanol and GAA-treatment combined displayed persistence of liver injury that appeared more pronounced at 6 wk compared to the other groups in this study and to the pathology seen at 2 wk of the combined regimen.

MATERIALS AND METHODS

Feeding procedure

Lieber-DeCarli control and ethanol liquid diets[22] were purchased from Dyets, Inc. (Bethlehem, PA). Male Wistar rats weighing 180 to 200 g purchased from Charles River Laboratories, Wilmington, MA) were weight-matched and divided into four groups. Each group consisted of 5 rats that were fed the control diet (Group 1), control diet supplemented with 0.36% GAA (w/v) (Group 2), ethanol diet consisting of 36% of total energy (Group 3) or ethanol diet supplemented with 0.36% GAA (Group 4) for a 6 wk period. Rats in Groups 1-3 were fed the amount of diet consumed by rats in Group 4. The care, use and procedures performed on these rats complied with NIH guidelines and all procedures were approved by the Institutional Animal Care and Use Committee at the Omaha Veterans Affairs Medical Center.

At sacrifice, the following tissues were collected and processed as indicated. Serum was prepared by centrifuging the serum separator tube containing the collected blood at 13000 × *g* for 5 min. A portion of the liver was processed for the preparation of a deproteinized extract using perchloric acid as previously described[7]. Another portion of the liver was immediately fixed in formalin for histology. A third portion of the liver was used to prepare the cytosol fraction as detailed[11] on the day of sacrifice. The remainder of the liver was freeze-clamped and stored at -70 ℃ for subsequent biochemical assays.

Histopathological evaluation

Hematoxylin and eosin stained liver sections slides were independently evaluated (by Orlicky DJ and French SW) using published criteria[23,24] in a blinded fashion.

Mallory trichrome staining was performed as detailed before. Briefly, the sections were treated with 1% fuchsin acid solution for 2 min, washed and stained with 1% phosphomolybdic acid solution. The sections were washed again and then incubated in a solution containing Methyl Blue (0.5%), Orange G (2%) and oxalic acid (2%) for 15 min. Slides were then washed thoroughly, dehydrated with ethanol, cleared with xylenes and mounted.

Olympus BX51 microscope equipped with a 4 megapixel Macrofire© digital camera (Optronics, Goleta, CA) was used to capture the images using the PictureFrame© Application 2.3 (Optronics). All images in each composite were processed by Photoshop© (Adobe Systems Inc., Mountain View, CA) and handled identically.

Hepatic SAM, SAH, GAA, creatine, triglycerides, cholesterol and non-essential fatty acid levels

High-performance liquid chromatography (HPLC) analysis was performed on the perchloric acid extract of total liver for determining SAM, SAH, creatine and GAA levels as detailed previously[7,12]. We also calculated the hepatic methylation index which is defined as the ratio of SAM to SAH.

The triglyceride, cholesterol and non-essential fatty acid (NEFA) content in the liver lipid extract was quantified using the diagnostics kits (Thermo Electron Clinical Chemistry, Louisville, CO and Wako Diagnostics, Richmond, VA) as detailed previously following the manufacturer's instructions[7].

Serum homocysteine, aspartate transaminase, alanine transaminase, GAA, insulin, NEFA and ethanol levels

HPLC analysis was conducted to determine serum homocysteine and GAA levels as detailed in our previous publications[8,12]. Serum alanine transaminase (ALT)/aspartate transaminase (AST) levels were determined using the VITROS 5.1 FS Chemistry System (Ortho Clinical Diagnostics, Raritan, NJ). Commercially available ELISA kits from EMD Millipore (Billerica, MA) and Wako Diagnostics (Richmond, VA) were used to determine serum Insulin (and NEFA levels, respectively. Ethanol levels were quantified by gas chromatography using a Perkin-Elmer system[25].

GAMT and L-arginine:glycine amidinotransferase activity measurements

Liver cytosols were used for determining hepatic GAMT activity as detailed in our publication[12]. L-arginine: glycine amidinotransferase (AGAT) activity was assayed in kidney homogenates as detailed[12].

Proteasome activity

Trypsin-like (Suc-LSTR-AMC hydrolysis) and Chymotrypsin-like (Suc-LLVY-AMC hydrolysis) activity was determined as previously described[13,14] using liver cytosol fractions. Protein concentration were measured by the Bradford dye-binding procedure[26] and the specific enzyme activities were expressed as nanomoles of 4-amino, 7-methyl coumarin formed per mg protein per hour.

Statistical analysis

Data were analyzed by ANOVA followed by Tukey test for specific comparisons between means. A P value < 0.05 was regarded as statistically significant.

RESULTS

The body weights of the GAA-treated and their pair-fed controls were comparable. However, an approximately 10% to 20% lower body weight was noted for the ethanol-alone and the GAA-supplemented ethanol-fed rats despite the fact that all rats had identical caloric intake (Table 1). The liver weight and the percent liver-to-body weight ratio of GAA-treated and their pair-fed controls were similar at the end of 6-wk of the feeding regimen. Ethanol treatment for 6 wk increased the liver weight and percent liver-to-body ratio which was

Table 1 Effect of dietary ethanol or/and guanidinoacetate ingestion on pathology

	Control	Control + GAA	Ethanol	Ethanol + GAA
Body weight (gm)	366.71 ± 20.26^{a}	345.93 ± 12.30^{a}	301.00 ± 12.49^{b}	323.90 ± 12.89^{a}
Liver weight (gm)	9.82 ± 0.44^{a}	9.47 ± 0.44^{a}	11.70 ± 0.36^{b}	15.05 ± 0.71^{c}
Liver-to body ratio (%)	2.67 ± 0.07^{a}	2.74 ± 0.10^{a}	3.88 ± 0.25^{b}	4.64 ± 0.06^{c}
Serum ALT (U/L)	54.43 ± 1.65^{a}	56.57 ± 3.96^{a}	120.00 ± 15.59^{b}	222.00 ± 50.52^{b}
Serum AST (U/L)	63.00 ± 3.95^{a}	68.40 ± 10.79^{a}	135.23 ± 13.86^{b}	252.60 ± 54.19^{b}
Serum GAA (μmol/L)	1.92 ± 0.31^{a}	60.60 ± 6.73^{b}	2.97 ± 1.05^{a}	23.63 ± 1.32^{c}
Serum ethanol levels (mmol/L)	0 ± 0.00^{a}	0 ± 0.00^{a}	51.10 ± 4.346^{b}	44.94 ± 6.28^{b}
Hepatic GAMT activity (pmol Creatine synthesized/min/mg protein)	247.58 ± 22.31^{a}	412.13 ± 24.92^{c}	207.19 ± 22.32^{b}	445.198 ± 23.19^{c}
Kidney AGAT activity (nmol GAA synthesized/min/mg protein)	10.03 ± 1.17^{a}	2.30 ± 0.177^{b}	10.39 ± 1.07^{a}	2.882 ± 0.44^{b}

Data represent mean ± SEM. of n = 5 animals/group. Values not sharing a common subscript letter are statistically different, $P < 0.05$. AGAT: L-arginine: glycine amidinotransferase; GAA: Guanidinoacetate; AST: Aspartate transaminase; ALT: Alanine transaminase.

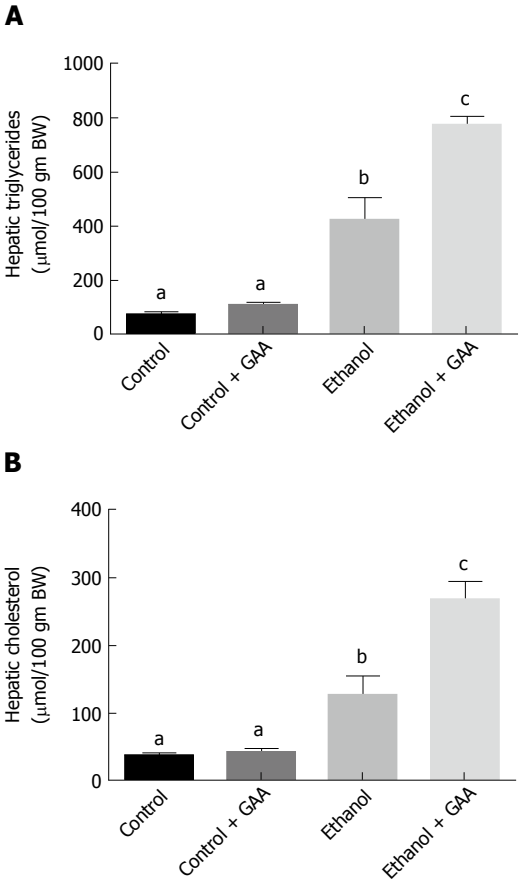

Figure 1 Effect of dietary ethanol or/and guanidinoacetate ingestion on hepatic triglycerides and cholesterol levels. Male Wistar rats were fed the control or ethanol Lieber DeCarli diet with or without 0.36% GAA. After 6 wk of feeding, triglyceride (A) and cholesterol (B) content in the liver lipid extract was determined using the diagnostics kit (Thermo Electron Clinical Chemistry, Louisville, CO). The data shown are mean ± SEM of 5 determinations. Values not sharing a common subscript letter are statistically different, $P < 0.05$ *vs* control. GAA: Guanidinoacetate.

further augmented in the group of rats fed the GAA-supplemented ethanol diet (Table 1).

GAA enhances ethanol-induced steatosis

We observed that 6 wk of ethanol administration increased hepatic triglyceride and cholesterol accumulation by approximately 6- and 3-fold, respectively compared with the pair-fed controls (Figure 1A and B). There was no change in the triglyceride or cholesterol content in liver of GAA-fed rats compared to controls. However, a approximately 11- and 7-fold increase in triglyceride and cholesterol accumulation, respectively, was observed in livers of rats fed the GAA-supplemented ethanol diet compared with the pair-fed controls (Figure 1A and B).

Histological evaluation

The livers of the rats fed the control diet showed no macro- or microvesicular steatosis and the total percent of hepatocytes possessing lipid vesicles was less than 10% in all animals. There was no fibrosis present or inflammation (small foci of inflammatory cells, lipogranulomas, or portal triad inflammation). Furthermore no hepatocyte cell injury, sinusoidal dilatation or congestion was observed in any animal in this group (Figure 2A).

The histology of the GAA treated group (n = 5) was similar to the control group. None of the animals presented with either macro- or microsteatosis. There also was no evidence of fibrosis, inflammatory changes or hepatocyte cell injury in this group of animals (Figure 2B).

In the ethanol-treated group (n = 5), all rats exhibited a panlobular microvesicular pattern of steatosis and low levels of macrosteatosis in zones 2 and 3. No fibrosis, inflammatory changes or hepatocyte cell injury was seen in this group of animals (Figure 2C and 3A).

In the ethanol plus GAA- treated group (n = 5) there appeared to be some synergy between the treatments. All 5 animals had macrosteatosis in 10%-66% of their hepatocytes which was present in a panlobular pattern, although there was more macrosteatosis in zones 2 and 3. Microsteatosis was also present in all 5 animals and it too was found in a panlobular pattern (Figure 2D).

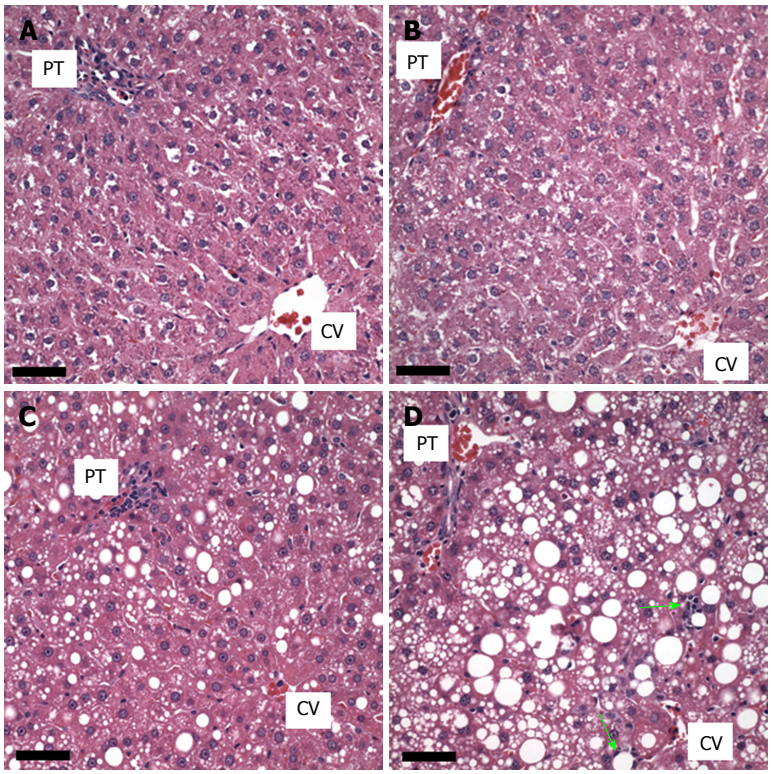

Figure 2 Effect of dietary ethanol or/and guanidinoacetate ingestion on liver histology. Hematoxylin-eosin stained images of livers from animals fed the following diets are shown: Pair-fed control (A); 0.36% guanidinoacetate (GAA) (B); ethanol (C); ethanol + 0.36% GAA (D). Scale bars = 50 microns. Limited macrosteatosis was observed following the ethanol diet while much more extensive macrosteatosis with a limited number of cells exhibiting microsteatosis and a couple of small lipogranulomas (arrows) are seen in the livers of animals on the ethanol + 0.36% GAA diet. The livers of the animals on the 0.36% GAA diet are similar to the control livers. PT: Portal triad; CV: Central vein.

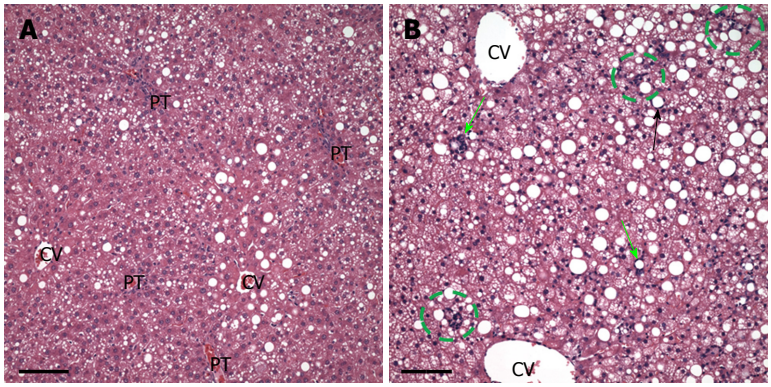

Figure 3 Hematoxylin-eosin staining of liver sections from rats fed ethanol (A) or ethanol + 0.36% guanidinoacetate (B) for 6-wk. There is a diffuse inflammatory infiltrate in the liver from the rat fed ethanol plus guanidinoacetate. Green arrows indicate lipogranulomas, green circle indicates an inflammatory cell foci, Scale bar = 100 microns. PT: Portal triad, CV: Central vein.

Further, all rats in the ethanol + GAA-treated group exhibited small foci of inflammatory cells per 200 × field and the presence of lipogranulomas (Figure 3B). Analysis of the Mallory trichrome stained slides revealed a very small amount of fibrosis in 2 of the 5 animals in this treatment group and only one of these two exhibited small amount of sinusoidal fibrosis (Figure 4). One animal showed a few hepatocytes with cell injury while another exhibited slightly dilated sinusoids in the centrilobular region.

No ballooning of hepatocytes, significant numbers of acidophil bodies, pigmented macrophages, mega-mitochondria, Mallory Bodies, glycogenated nuclei, significant numbers of mitotic figures, or hyalinized and thickened portal veins or hepatic arteries were present in any of the groups in this experiment.

Hepatocellular levels of SAM, SAH, SAM:SAH Ratio, GAA and creatine

Hepatic SAM levels were similar between the control

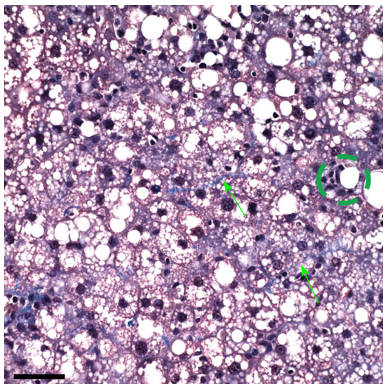

Figure 4 Higher magnified image from a Mallory trichrome stained liver section from a rat fed the ethanol + 0.36% guanidinoacetate for 6 wk. Green arrows point to delicate trichrome collagen fiber and green circle surrounds small inflammatory cell foci. Note the presence of many macro- and micro-steatotic hepatocytes Scale bar = 50 microns.

and ethanol-fed rats. This was in accordance with our previously published results[7,12]. However, SAM level was decreased in the GAA-treated rats, which was more pronounced in the rats fed the GAA-supplemented ethanol diet (Figure 5A).

In regards to hepatic SAH levels, comparable increases in the level of this metabolite compared to controls were observed in GAA or ethanol treated rats. However, markedly increased hepatic SAH levels was noted in the group of rats fed the GAA-supplemented ethanol diet compared to other treatment groups (Figure 5B).

Since the SAM:SAH ratio is an important factor regulating cellular methylation reactions, calculation of this ratio revealed a lower SAM:SAH ratio in the ethanol-fed and the GAA-alone treated groups compared with the controls. A dramatic decrease in hepatocellular SAM:SAH ratio was seen in rats fed the GAA-supplemented ethanol diet in comparison with the other treatment groups (Figure 5C).

Regarding hepatic creatine and GAA levels after 6 wk of the dietary regimens, we observed a 60% decrease in hepatic creatine and a approximately 2-fold increase in GAA accumulation in rats exposed to ethanol as compared with the pair-fed controls (Figure 6A and B). This was in accordance with our published data obtained on feeding rats a control or ethanol diet for 4-5 wk[12]. However, GAA ingestion either alone or in combination with ethanol treatment elevated hepatic creatine and GAA levels, as expected. While the combined GAA + ethanol treatment had a significantly higher hepatic creatine content than the GAA-alone treatment, a statistically similar increase in hepatic GAA level was noted in these two groups of animals, showing almost 20-fold increased levels as compared with the control diet-fed rats (Figure 6B).

Serum levels of homocysteine, GAA, insulin, NEFA, AST and ALT

Similar serum homocysteine levels were observed in the

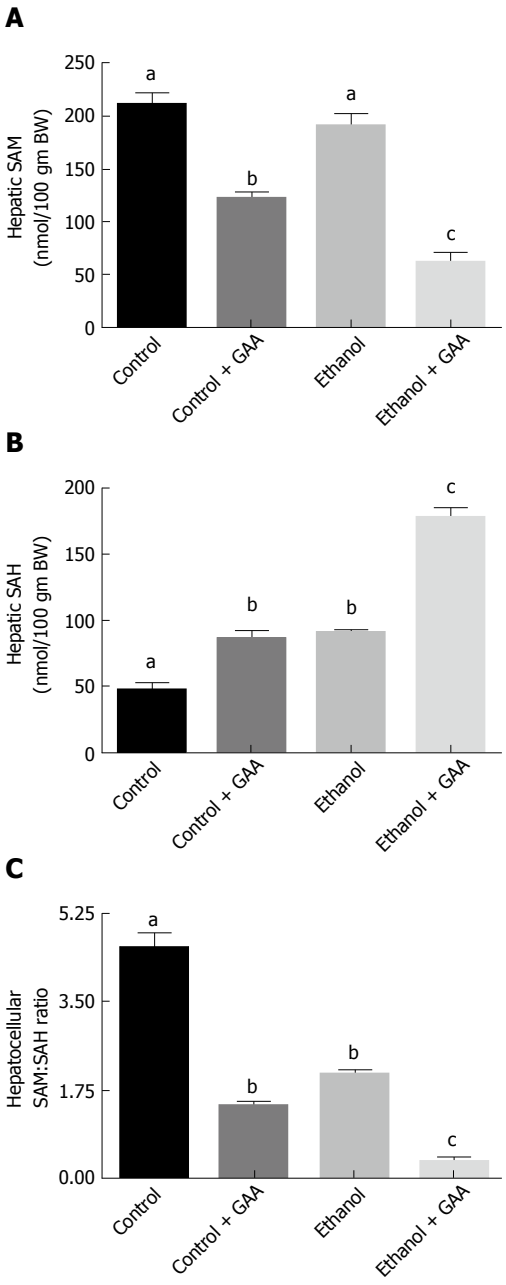

Figure 5 Effect of dietary ethanol and/or guanidinoacetate ingestion on hepatic S-adenosylmethionine, S-adenosylhomocysteine and S-adenosylmethionine: S-adenosylhomocysteine ratio. Rats were fed the Lieber DeCarli control or ethanol diet with or without 0.36% GAA supplementation. After 6 wk of feeding, Hepatocellular SAM (A) and SAH (B) levels were determined by HPLC analysis as detailed in the "MATERIALS AND METHODS" section and (C) SAM:SAH ratio calculated. The data shown are mean ± SEM of 5 determinations. Values not sharing a common subscript letter are statistically different, $P < 0.05$ *vs* control. GAA: Guanidinoacetate; SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine.

rats fed the control or ethanol diets for 6 wk. However feeding rats a GAA-supplemented diet increased circulating homocysteine level which was further dramatically increased in in the GAA-supplemented ethanol treated group (Figure 7A).

GAA alone had no effect on circulating insulin or NEFA level (Figure 7B and C). On the other hand, ethanol administration decreased serum insulin levels

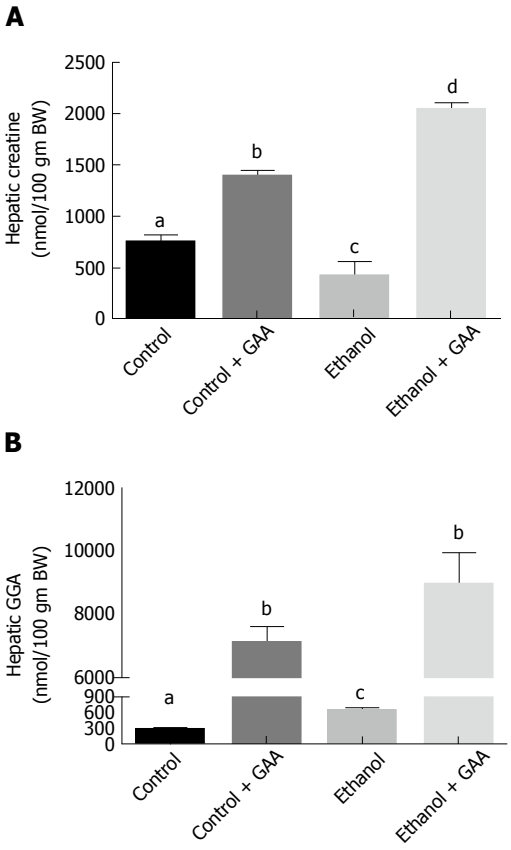

Figure 6 Effect of dietary ethanol and/or guanidinoacetate ingestion on hepatic creatine and guanidinoacetate levels. Rats were fed the Lieber DeCarli control or ethanol diet with or without 0.36% GAA supplementation. After 6 wk of feeding, liver Creatine (A) and GAA (B) levels were determined by HPLC analysis as detailed in the "MATERIALS AND METHODS" section. The data shown are mean ± SEM of 5 determinations. Values not sharing a common subscript letter are statistically different, $P < 0.05$ *vs* control. GAA: Guanidinoacetate.

and increased circulating NEFA levels, while GAA co-treatment had no further effect on these parameters (Figure 7B and C). All treatments produced a much higher hepatic NEFA compared with controls, although maximum elevation was seen in the combined treatment group (Figure 7D).

As expected, both the GAA-alone and combined ethanol and GAA treatment groups exhibited elevated serum GAA levels in comparison with the controls. However, the circulating GAA level in the combined treatment group was significantly lower than the level observed in the GAA-alone treatment group (Table 1).

Regarding liver toxicity, the serum levels of AST and ALT in the GAA-treated rats were similar to controls. Ethanol treatment significantly elevated serum AST and ALT levels, which were further enhanced approximately 2-fold in the combined GAA and ethanol treatment group (Table 1).

The blood ethanol levels were comparable in the ethanol-alone and combined ethanol and GAA treatment group despite dramatic differences in many metabolite levels and indices of liver toxicity between the two treatment groups (Table 1).

Hepatic GAMT and kidney AGAT activity

Ethanol administration for 6 wk resulted in a small (20%), but significant, decrease in hepatic GAMT activity compared to controls (Table 1). A approximately 1.7-fold increase in hepatic GAMT activity was observed in both GAA treatment groups (either alone or supplemented in the ethanol diet) (Table 1).

Kidney AGAT activity was unaffected after 6 wk of ethanol treatment (Table 1). A substantially suppressed kidney AGAT activity was observed after GAA administration for 6 wk which was comparable in the combined GAA-ethanol treatment group (Table 1).

Liver proteasome activity

A significant decline in trypsin- and chymotrypsin-like specific activities of the proteasome was noted in the rats fed ethanol for 6 wk which further decreased in the combined GAA and ethanol treatment group (Figure 8). Liver proteasome activity was unaffected by GAA-alone treatment (Figure 8).

DISCUSSION

Our findings indicate that the longer period of 6 wk of feeding GAA (a methyl group consumer) along with ethanol (a metabolic stressor) imposes a substantial burden on the cellular methylation index, which results in increased liver toxicity in comparison to our recently published 2-wk study[15]. However, some unexpected results were obtained on analyzing liver damage upon feeding GAA alone. While 2 wk of GAA treatment generated hepatic steatosis[15], this pathology was surprisingly absent at 6 wk of administration of this agent.

Our laboratory has been examining the consequences of the alcohol-induced changes in the methionine metabolism and have made a seminal contribution in identifying the causal role of the reduction in the hepatocellular SAM:SAH ratio in the pathogenesis of alcoholic liver injury[3,4,7-12,15,27-34]. In a recent study, we reported that 2 wk of ethanol feeding promoted hepatic SAH accumulation and caused a significant reduction in hepatocellular SAM:SAH ratio[15]. The consequent reduction in the hepatic methylation capacity resulted in hepatic steatosis and proteasome inhibition, the two examined functional consequences of low SAM:SAH ratio[15]. We also showed that feeding a methylation stressor, GAA, for 2 wk also perturbed the methionine metabolic pathway that caused a reduction in the hepatic SAM:SAH ratio. This ultimately resulted in similar histopathology as seen after ethanol exposure[15]. More importantly, we showed that the combined treatment with GAA and ethanol treatment further lowered the SAM:SAH causing even more profound pathological changes

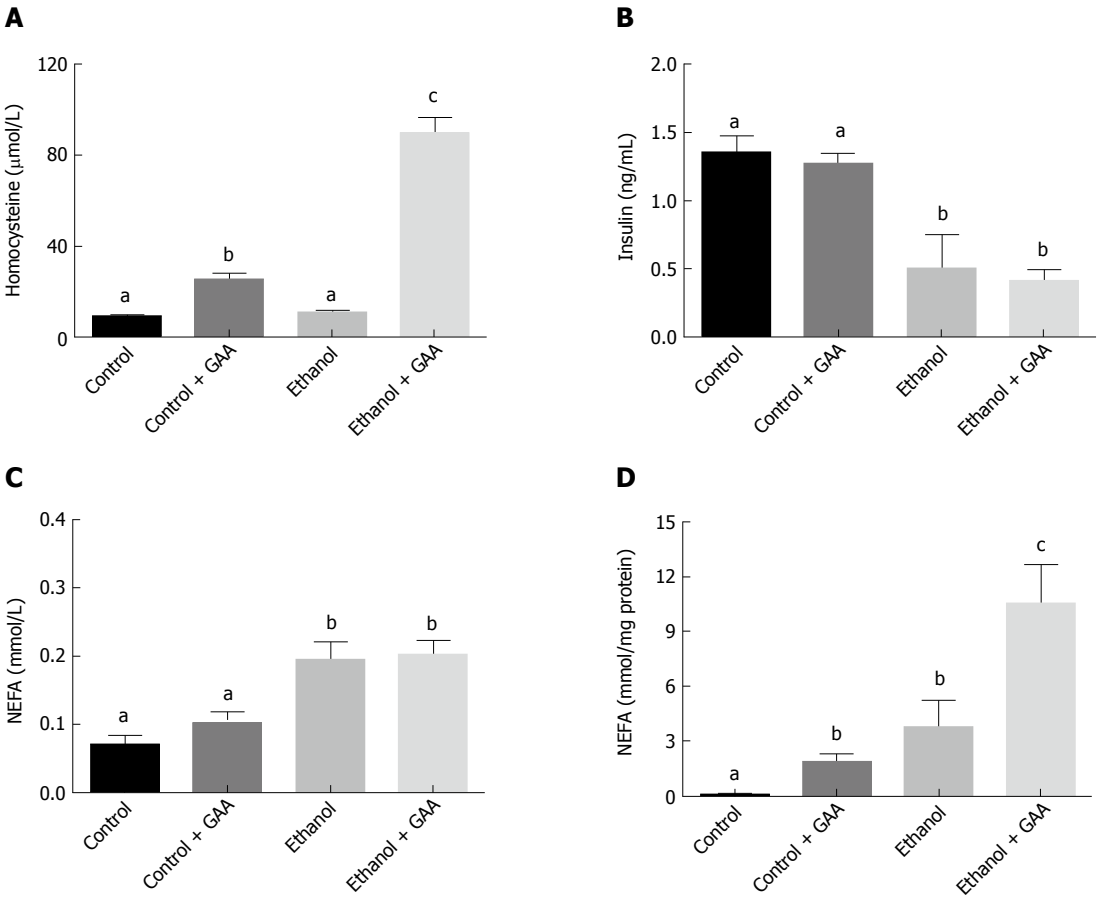

Figure 7 Effect of dietary ethanol and/or guanidinoacetate ingestion on serum homocysteine, insulin, non-essential fatty acid and hepatic non-essential fatty acid levels. Rats were fed the Lieber DeCarli control or ethanol diet with or without 0.36% GAA supplementation. After 6 wk of feeding, serum homocysteine (A) insulin (B) NEFA (C) and hepatic NEFA (Dlevels were determined by HPLC or biochemical analysis as detailed in the Materials and Methods section. The data shown are means ± SEM of 5 determinations. Values not sharing a common subscript letter are statistically different, $P < 0.05$ *vs* control. GAA: Guanidinoacetate; NEFA: Non-essential fatty acid.

than either treatment alone[15].

To further investigate whether prolonged treatment with the combined exposure could worsen liver pathology, we observed that indeed 6 wk of treatment led to persistent and more pronounced liver toxicity. Several biochemical changes identified at 2 wk of combined exposure were also noted at 6 wk of treatment[15]. At both 2 and 6 wk of the combined treatment, we observed hyperhomocysteinemia, significantly lower hepatocellular SAM:SAH ratio, panlobular macro- and microvesicular hepatic steatosis, accumulation of cholesterol, triglyceride and NEFA in the liver and decreased hepatic proteasome activity as compared to the rest of the groups. The combined treatment group exhibited the lowest SAM:SAH ratio and the highest homocysteine serum levels that have been causally linked with hepatic lipid accumulation, reduced proteasome function and increased adipose tissue lipolysis[3-5,7,12-14,35,36]. The only significant differences between the 2 and 6 wk of combined exposure was an approximately 8-fold increased GAA accumulation, much higher homocysteinemia and approximately 2-fold increased serum AST and ALT levels at the latter time-point[15]. Overall, all these biochemical and pathological changes seen after 6 wk of combined exposure were as we had predicted.

However, some novel observations were made when the various parameters were analyzed in rats fed only GAA for 6 wk. Increases in serum homocysteine level and lowering of the hepatocellular SAM:SAH ratio noted at 6 wk were similar to our previous observations at the end of 2 wk of GAA treatment[15]. The only biochemical difference identified was a approximately 8-fold higher GAA accumulation at week 6 compared to the earlier time period[15]. However, despite the increased GAA and persistence of biochemical abnormalities that are known to reduce methylation capacity, there was no evidence of hepatic steatosis after 6 wk of GAA treatment. This was in contrast to our previous 2-wk study where we noted the development of hepatic steatosis with GAA treatment[15]. We believe that the significant elevation in the liver creatine level seen after prolonged GAA administration may be hepato-protective since such effects of creatine have been reported in both *in*

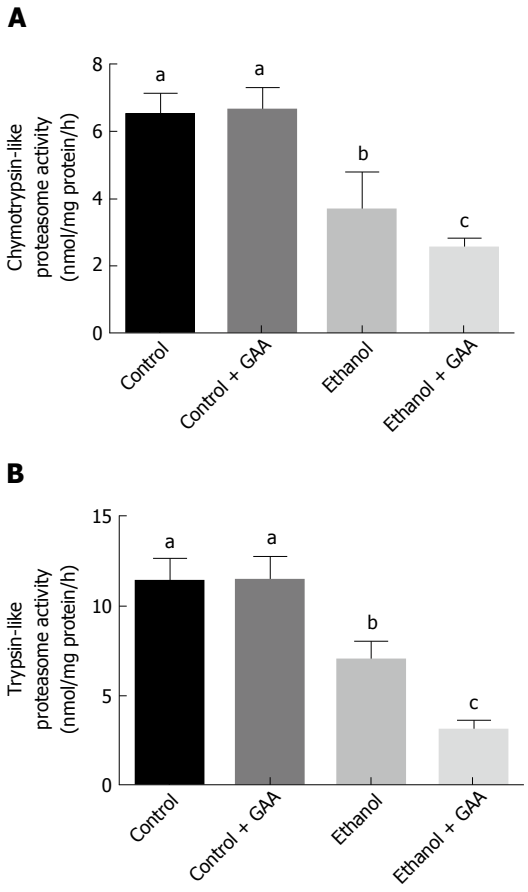

Figure 8 Effect of dietary ethanol and/or guanidinoacetate on peptidase specific activities of the proteasome. Rats were fed the Lieber DeCarli control or ethanol diet with or without 0.36% GAA supplementation. After 6 wk of feeding, liver cytosols were prepared and assayed for the chymotrypsin- (A) and trypsin-like (B) specific activities of the proteasome as detailed in the "MATERIALS AND METHODS" section. The results are mean ± SEM of 8-10 determinations from each group and activities are expressed as nmol AFC generated/mg protein/h. Values not sharing a common subscript letter are statistically different, $P < 0.05$ *vs* control. GAA: Guanidinoacetate.

vivo and *in vitro* injury models unrelated to ethanol exposure[12,37-43]. Mechanistic studies have indeed revealed that creatine supplementation prevents hepatic triglyceride accumulation by promoting hepatocellular β-oxidation *via* upregulating PPARα and its targets[38,42,43], inhibiting triglyceride synthesis and increasing its efflux[43]. These effects of creatine on lipid metabolism are considered to be independent of methylation and phosphatidylcholine synthesis *via* phosphatidylethanolamine methyltransferase-catalyzed pathway[43].

We were also surprised to find that hepatic creatine level was higher in rats administered the combined treatment compared with the GAA treatment group. Yet, this accumulated creatine was unable to exert its known protective effect when alcohol was present suggesting that creatine cannot prevent the well-documented ethanol-induced aberrations in several metabolic pathways of fat metabolism involving triglyceride efflux and fatty acid synthesis and oxidation. These findings are intriguing and mechanistic studies are underway to explain our findings. However, similar results that support our data were also obtained by us in a recent study that revealed an inability of oral creatine supplementation to prevent alcoholic steatosis[44].

We also examined two enzymes that are involved in GAA synthesis (AGAT) and utilization (GAMT). We observed that 6 wk of exposure to ethanol lowered the GAMT enzyme activity by approximately 20%, while GAA-alone treatment increased GAMT activity possibly to accelerate the utilization and removal of its substrate, GAA. Similar increases in GAMT activity were also noted in the combined GAA + ethanol treatment group. With regards to AGAT, in accordance with reports showing that GAA feeding represses the enzyme activity[45], we observed that 6 wk of GAA treatment either alone or combined with ethanol decreased AGAT activity by approximately 75%. It appears that a longer period of GAA administration represses GAA activity even more since approximately 50% repression was noted in the GAA-treated groups in our previous 2-wk study[15]. Overall, these two enzymes showed comparable changes as those reported after 2 wk of the different treatment regimens[15] and therefore, could not account for the differences in the hepatic steatosis seen in the GAA-fed rats at 6 wk *vs* 2 wk of treatment.

Another parameter to discuss is the loss of body weight in the rats fed ethanol either alone or in combination with GAA. Such decreases in body weight may be attributed to the ethanol-induced lipolysis of the adipose tissue triglyceride store with a concomitant increase in circulating and hepatic NEFA[46,47]. Clinical studies have also demonstrated that alcoholics have significantly lower body weight and lower fat mass than controls[48,49].

Further, a decrease in serum insulin level observed in ethanol-fed rats was also not surprising given that such a decrease has been reported before[46,50,51]. Furthermore, a recent preliminary study revealed that indeed it is the alcohol-induced increase in the serum ghrelin level and decrease in the pancreatic Rab3D content that both contribute to impaired insulin secretion from pancreatic β-cells, thereby causing a low serum insulin level in alcoholic rats[52].

To summarize, increased methylation demand by GAA treatment superimposed on the ethanol-induced stress on the methionine metabolic pathway results in steatohepatitis, proteasome inhibition and more pronounced indices of liver injury. These biochemical and pathological changes could be attributed to profoundly reduced hepatic SAM:SAH ratio, hyperhomocysteinemia, increased accumulation of GAA in the liver and the inability of creatine to protect the liver in the setting of ethanol abuse. Another important point that should be stressed is that while the prolonged administration of GAA alone

did not produce any evidence of liver injury in the present study, sustained use of a different methyl group consumer that does not generate a protective agent such as creatine could have an entirely different (adverse) outcome. Thus, ingesting large amounts of methyl group consuming compounds either alone or in combination with ethanol is not recommended.

COMMENTS

Background

The authors have recently reported that rats exposed to guanidinoacetate (GAA, a potent methyl-group consumer) for 2 wk results in hepatic steatosis. They further showed that a combined exposure to GAA and ethanol (a cellular methylation stressor) for 2 wk caused more hepatosteatosis and inflammatory changes as compared to either treatment alone. Therefore, they investigated the hypothesis that exposure to GAA either alone or combined with ethanol intake for a prolonged period of time would cause more advanced liver pathology thus identifying methylation defects as the initiator and stimulator for progressive liver damage.

Research frontier

There are many endogenous and exogenous compounds that consume labile methyl groups during metabolism. The consumption of such compounds has increased in the last decade which puts a substantial burden on the cellular methylation index. The prevalence of binge and heavy alcohol drinking is growing. Thus, this area of research is extremely important to understand the effects of prolonged exposure to two different methylation stressors.

Innovations and breakthroughs

Prolonged treatment with both ethanol and a methyl consumer such as GAA causes worsening of liver pathology compared to either treatment alone. These detrimental consequences were related to the profound reduction in the hepatic methylation index, an increased accumulation of GAA and the inability of creatine generated to exert its hepato-protective effects in the setting of ethanol. Thus, ingesting large amounts of methyl group consuming compounds either alone or in combination with ethanol is not recommended.

Applications

This model of the combined treatment with a methyl consumer and ethanol for a prolonged duration could be used for better understanding of the role of severe impairments in critical methylation reaction to promote progressive liver injury including fibrosis, cirrhosis and hepatocellular carcinoma.

Terminology

Hepatic methylation index is defined as the ratio of the methyl donor, S-adenosylmethionine to its product S-adenosylhomocysteine. This ratio controls the biochemical transmethylation reactions.

Peer-review

An important point is to know whether prolonged administration of both GAA and ethanol would lead to hepatocellular carcinoma development and whether the observations are species-specific (rats, mice). Thus, this novel synergy could replace the current experimental models of ASH lacking the NASH component as observed after Lieber DeCarli feeding and represent an alternative to this diet alone. Moreover, if HCC development exists in the chronic situation this would a very valuable experimental tool for the study of ASH.

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ORIGINAL ARTICLE

Basic Study

TM6SF2 E167K variant predicts severe liver fibrosis for human immunodeficiency/hepatitis C virus co-infected patients, and severe steatosis only for a non-3 hepatitis C virus genotype

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Conflict-of-interest statement: All the authors of the manuscript declare that they have no conflict of interest in connection with this paper.

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Abstract

AIM

To evaluate the impact of the Glu167Lys (E167K) transmembrane 6 superfamily member 2 (TM6SF2) variant on the biochemical and morphologic expression of liver lesions in human immunodeficiency virus (HIV)/hepatitis C virus (HCV) co-infected patients.

METHODS

The study comprised 167 consecutive patients with HIV/HCV coinfection and biopsy-proven chronic hepatitis. A pathologist graded liver fibrosis and necroinflammation

using the Ishak scoring system, and steatosis using Kleiner's scoring system. Patients were genotyped for TM6SF2 E167K (rs58542926) by real-time Polymerase chain reaction. The 167 patients, 35 therapy-naive and 132 receiving ART, were prevalently males (73.6%), the median age was 40.7 years and the immunological condition good (median $CD4^+$ cells/mm^3 = 505.5).

RESULTS

The 17 patients with the TM6SF2 E167K variant, compared with the 150 with TM6SF2-E/E, showed higher AST (P = 0.02) and alanine aminotransferase (P = 0.02) and higher fibrosis score (3.1 ± 2.0 *vs* 2.3 ± 1.5, P = 0.05). In a multivariate analysis, TM6SF2 E167K was independently associated with severe fibrosis. The same analysis showed that HCV-genotype 3, present in 42.2% of patients was an independent predictor of severe steatosis. The association of TM6SF2 E167K with severe steatosis, absent for the whole group of 167 patients, was re-evaluated separately for HCV-genotype 3 and non-3 patients: No factor was independently associated with severe steatosis in the HCV-genotype-3 subgroup, whereas an independent association was observed between severe steatosis and TM6SF2 E167K in non-3 HCV genotypes. No association between the TM6SF2 E167K variant and severe liver necroinflammation was observed.

CONCLUSION

In HIV/HCV coinfection the TM6SF2 E167K variant is an independent predictor of severe fibrosis, but appears to be independently associated with severe steatosis only for patients with a non-3 HCV genotype.

Key words: Human immunodeficiency virus/hepatitis C virus co-infection; TM6SF2; Liver histology; Liver steatosis; Liver biopsy

Core tip: The transmembrane 6 superfamily member 2 (TM6SF2) E167K variant has been identified as an independent predictor of severe liver steatosis in patients with hepatitis C virus (HCV)-related chronic hepatitis (CH) lacking human immunodeficiency virus (HIV) infection. We analyzed the impact of the TM6SF2 E167K variant on the liver histology of 167 HIV/HCV co-infected patients with CH. The TM6SF2 E167K variant was found to be an independent predictor of severe fibrosis, while an independent association with severe steatosis was demonstrated only for patients with a non-3 HCV genotype.

Sagnelli C, Merli M, Uberti-Foppa C, Hasson H, Grandone A, Cirillo G, Salpietro S, Minichini C, Starace M, Messina E, Morelli P, Miraglia Del Giudice E, Lazzarin A, Coppola N, Sagnelli E. TM6SF2 E167K variant predicts severe liver fibrosis for human immunodeficiency/hepatitis C virus co-infected patients, and severe steatosis only for a non-3 hepatitis C virus genotype. *World J Gastroenterol* 2016; 22(38): 8509-8518 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i38/8509.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i38.8509

INTRODUCTION

The non-synonymous sequence variation (rs58542926 C to T, NP_001001524.2: p. E167K) in the transmembrane 6 superfamily member 2 (TM6SF2) gene encodes for an adenine-to-guanine substitution at nucleotide 499 that replaces glutamate at residue 167 with lysine[1].

The *TM6SF2* gene is considered a regulator of liver fat metabolism since it influences the secretion of triglyceride-rich lipoproteins and the hepatic triglyceride content[2]. In patients with non-alcoholic fatty liver disease (NAFLD), the TM6SF2 E167K variant induces a reduction in very-low-density-lipoprotein secretion and a predisposition to retain triglycerides in lipid droplets in liver cells[1-3], conditions associated with higher alanine aminotransferase (ALT) serum levels, lower lipoprotein plasma concentrations and advanced hepatic fibrosis[1,3-5].

In addition, the TM6SF2 E167K variant has been identified as an independent predictor of severe liver steatosis in patients with hepatitis C virus (HCV)-related chronic hepatitis (CH) lacking human immunodeficiency virus (HIV) infection[6,7], whereas controversial results have been published on the impact of this polymorphism on liver fibrosis[8-10].

As no data were available on this topic for HIV/HCV coinfection, we studied the impact of the TM6SF2 E167K variant on the biochemical and morphologic expression of liver lesions in 167 HIV/HCV coinfected patients with CH.

MATERIALS AND METHODS

Patients

This investigation comprised 167 consecutive anti-HIV-positive patients with CH (defined by positive HCV RNA) first observed from 1996 to 2008 who underwent a diagnostic liver biopsy at one of the two participating Units of Infectious Diseases after an observation period of nearly 18 mo. The two participating Units have been cooperating for years in clinical investigations and apply the same clinical and laboratory approach[11-14].

At the time the liver biopsy was performed, all patients were naive for anti-HCV treatment, were HBsAg-negative and had no evidence of autoimmune or genetic disorders inducing liver disease. No patient declared alcohol abuse (a daily intake of > 40 g/d for males and > 30 g/d for females for at least 5 years). No patient had serological or ultrasound evidence of HCC.

The patients received ART or were left untreated

according to the current DHHS guidelines at the time of liver biopsy[15]. For each patient, a serum sample obtained at liver biopsy was stored at -80 ℃ and thawed for this investigation.

The standards of human experimentation of the local Ethics Committees and with the Helsinki Declaration of 1975, revised in 1983. The present study was designed as retrospective and was approved by the Azienza Ospedaliera Universitaria - Second University of Naples in 2013 (protocol number 112/15 March 2013). Each patient signed their informed consent to undergo liver biopsy, for the collection and storage of biological material and for the use in clinical research of the data obtained.

Liver biopsy

Percutaneous liver biopsy was performed under US guidance using a modified disposable Menghini needle (16 gauge - external diameter 1.65 mm). A liver specimen of more than 1.5 cm in length was always obtained and at least 11 portal tracts were examined in each sample. Specimens were fixed in formalin, embedded in paraffin and stained with hematoxylin-eosin and Masson's trichrome stain[16]. A skilled pathologist unaware of the clinical and laboratory data examined the liver biopsies and used the Ishak scoring system to grade liver necroinflammation and fibrosis[17]. To assess liver steatosis, a home-made scoring system obtained with a partial modification of Kleiner's scoring system for NAFLD was used[6,18]: score 1 identifies the presence of fatty deposition in 1%-10% of hepatocytes, score 2 in 11%-31%, score 3 in 31%-60% and score 4 in > 60%[12-14,16,19].

Serological determinations

Serum HBsAg was sought using a commercial immunoenzymatic assay (Abbott Laboratories, North Chicago, IL, United States). The anti-HCV antibody was sought using a 3rd generation commercial immunoenzymatic assay (Ortho Diagnostic Systems, Neckargemund, Germany). Antibodies to HIV 1 and 2 were sought using a commercial ELISA (Abbott Lab., North Chicago, IL, United States) and positive results were confirmed by Western blot (Genelabs Diagnostics, Science Park Drive, Singapore).

HCV RNA was quantified by performing a real-time polymerase chain reaction (PCR) in a Light cycler 1.5 (Roche Diagnostics, Branchburg, NJ, United States), with the detection limit in plasma samples estimated at around 40 IU/mL. HCV genotyping was performed by a Line-Probe assay (INNO-LIPA HCV-Ⅱ; Innogenetics, Zwigndrecht, Belgium). The HIV viral load was assessed using the Amplicor HIV Monitor 1 test (Roche Molecular Systems Inc., Branchburg, New Jersey) with the lowest detection limit of 400 copies/mL.

Lymphocyte subsets ($CD4^+$, $CD8^+$) were evaluated by flow cytofluorimetry using monoclonal antibodies and a fluorescence-activated cell sorter scan (Becton Dickinson, Mountain View, CA, United States). Liver function tests, serum triglycerides, cholesterol and routine analyses were performed applying standard procedures.

Detection of TM6SF2 and PNPLA3 polymorphisms

A blood sample obtained from each patient was frozen (-80 ℃) and never thawed until used for this investigation.

Genomic DNA was extracted from peripheral whole blood with a DNA extraction kit (Promega, Madison WI, United States). Patients were genotyped for TM6SF2 E167K (rs58542926) and PNPLA3 I148M (rs738409) polymorphisms by Taqman allelic discrimination assay on an ABI 7900HT real-time PCR system, as previously described[6,14,19].

The genotype distributions of TM6SF2 and PNPLA3 polymorphisms were in the Hardy-Weinberg equilibrium, $P = 0.4$ and $P = 0.3$, respectively.

Statistical analysis

Continuous variables not normally distributed were summarized as median and interquartile range (IQR), and categorical variables as absolute and relative frequencies. Differences in the continuous data were evaluated by a non-parametric test (Kruskal-Wallis), and the χ^2 test was applied to categorical variables. A generalized linear model analysis was applied to perform multivariate analysis. All independent variables with a significant effect on liver steatosis, fibrosis or necroinflammation at univariate analysis or with biological plausibility were included in the multivariate analysis. A P value < 0.05 was considered to be statistically significant. Statistical analysis was performed using StatGraph, version 3.0 (Adalta, Arezzo, Italy).

RESULTS

The 167 patients enrolled were prevalently males (72.5%), with a median age of 40.7 years (IQR: 37.6-44.1) and a good immunological condition shown by the $CD4^+$ cell count (median 505.5 cells/mm^3, IQR: 397.3-665.5) at the time of liver biopsy and by the nadir of $CD4^+$count (median 258.0 cells/mm^3, IQR: 166.5-407.3). At the time of the liver biopsy, 35 (21.0%) patients were left untreated because they showed a high $CD4^+$cell count (median 507.5 cells/mm^3, IQR: 398.0-662.0), whereas 132 (79.0%) had been receiving ART for a median duration of 8.0 years (IQR: 6.0-11.0). HCV-genotype 3 was detected in 42.2% of the cases, HCV-genotype 1 in 38.5%, HCV-genotype 4 in 14.3% and HCV-genotype 2 in 5.0%.

Of the 167 patients investigated, 89.8% were TM6SF2 167E homozygous and 11.2% heterozygous for the TM6SF2 E167K variant. The PNPLA3 I/I, I/M and M/M variants at codon 148 were observed respectively in 52.7%, 40.1% and 7.2% of patients.

The 167 patients showed a mean fibrosis score of 2.3 ± 1.46, a mean histological activity index (HAI) score of 5.9 ± 3.0 and a mean steatosis score of 1.7 ± 1.3. Thirty-seven (22.2%) patients showed a HAI of 9-18, 58 (34.7%) a fibrosis score 4-6 and 56 (33.5%) a steatosis score 3-4.

The demographics and initial biochemical, virological and histological data according to the TM6SF2 variants are shown in Table 1 (Table 1). Compared with the 150 patients with the TM6SF2 E167E variant, the 17 with the TM6SF2 E167K variant were older (P = 0.04), showed higher AST (P = 0.02) and ALT (P = 0.02) serum values and a higher fibrosis score (P = 0.05), whereas no difference was observed in the degree of steatosis or liver necroinflammation.

The 31 patients with a liver fibrosis score 4-6, compared with the 136 with a lower score (0-3), were older (P = 0.05) and showed higher bilirubin (P = 0.01), AST (P = 0.0008), ALT (P = 0.01) and ALP (P = 0.01) serum values (Table 2). They had a lower $CD4^+$/mL cell count (P = 0.01), a higher HAI score (P = 0.0001), a higher percentage of cases with an HAI score > 9 (P = 0.0001), a higher steatosis score (P = 0.02), a higher percentage of patients with a steatosis score 3-4 (P = 0.0001) and a greater frequency of cases with the TM6SF2 E167K variant (P = 0.03) (Table 2). Other differences shown in Table 2 were not significant to the statistical analysis or of low or no clinical impact.

To identify factors independently associated with a severe fibrosis, a multivariate analysis was performed, including the TM6SF2 variants (p. 167 E/K *vs* p. 167 E/E), the PNPLA3 variants (p. 148 I/I *vs* p. 148 I/M + M/M), HCV-genotype 3 *vs* other genotypes, BMI and other potential confounding factors (sex, nadir of $CD4^+$ cell count, HIV viral load, ART, AST, ALT, GGT, total bilirubin, triglyceride and cholesterol values. The TM6SF2 E167K variant (P = 0.0272) and the total cholesterol serum value (P = 0.0203) were the only factors independently associated with the severity of liver fibrosis.

Compared with the 111 with a lower score, the 56 patients with severe steatosis (score 3-4) showed a higher BMI (P = 0.03), higher bilirubin (P = 0.01), AST (P = 0.0004), ALT (P < 0.0001), GGT (P = 0.04) and glucose serum values (P = 0.004), and lower cholesterol serum levels (P < 0.01). In addition, they had a lower $CD4^+$/mL cell count (P = 0.002), a higher mean HAI score (P = 0.002), a higher percentage of cases with an HAI score > 9 (P = 0.01), a higher mean fibrosis score (P = 0.003), a higher percentage of patients with a fibrosis score 4-6 (P = 0.001) and a higher percentage of patients with HCV-genotype 3 (P = 0.04) (Table 3). Other differences shown in Table 3 were not significant to the statistical analysis or of low or no clinical impact.

To identify factors independently associated with severe steatosis, a multivariate analysis was performed, including the TM6SF2 variants (p. 167 E/K *vs* p. 167 E/E), the PNPLA3 variants (p. 148 I/I *vs* p. 148 I/M + M/M), HCV genotype (3 *vs* other genotypes), BMI and other potential confounding factors (sex, nadir of $CD4^+$ cell count, HIV viral load, duration of HIV infection, ART, GGT, AST, ALT, total bilirubin, glucose). The only factors independently associated with severe steatosis were HCV-genotype 3 (P = 0.0227), the PNPLA3 p. 148 I/M plus M/M variants (P = 0.0321) and the glucose serum values (P = 0.0441).

Due to the high percentage of patients with HCV-genotype 3 and the association of this genotype with severe steatosis in this and other investigations[6,8-10,20], the initial characteristics of 161 of the HIV/HCV coinfected patients (HCV genotype was not available in 6 cases) were analyzed according to the presence or absence of HCV-genotype 3. Compared to the 93 patients with a non-3 HCV genotype, the 68 with HCV-genotype 3 showed higher AST (P = 0.004) and ALT (P = 0.0002) serum values, a higher HAI score (P = 0.03), higher steatosis scores (P = 0.002), a higher rate of patients with severe steatosis (P = 0.03) and lower serum levels of GGT (P = 0.004), triglycerides (P = 0.003) and cholesterol (P = 0.0006) (Table 4). The association between the TM6SF2 variants and severe steatosis was investigated separately for patients with HCV-genotype 3 and for those with a non-3 HCV genotype in multivariate analyses, including the TM6SF2 variants (p. 167 E/K *vs* E/E), the PNPLA3 variants (p. 148 I/I *vs* p. 148 I/M + M/M), BMI, sex, the nadir of $CD4^+$ cell count, HIV viral load, triglyceride, cholesterol, GGT and ART at the time of the liver biopsy. None of the factors considered was identified as an independent predictor of severe steatosis in the HCV-genotype-3 subgroup, whereas the TM6SF2 E167K variant (P = 0.0339), the PNPLA3 I/I variant (P = 0.0263), BMI (P = 0.0348) and GGT (P = 0.0049) were independently associated with severe steatosis in patients with a non-3 HCV genotype.

We admit that the present study has some limitations considering the relatively small number of patients, barely sufficient for a genetic association study. These limitations are offset by the gold-standard method used to detect liver lesions (liver biopsy examination by a skilled pathologist) and by the new information regarding HIV/HCV coinfection.

DISCUSSION

This is the first investigation, to our best knowledge, to demonstrate that the TM6SF2 E167K variant is independently associated with severe liver fibrosis in HIV/HCV coinfected patients with CH.

This association was previously identified in HCV-mono-infected patients with CH or cirrhosis[10,20] and in patients with NAFLD[5,10].

In a cross-sectional cohort of 815 Italian therapy-naïve HCV-mono-infected patients, the TM6SF2 E167K variant was independently associated with histological cirrhosis[10], and in a subset of 645 Swiss/German CHC patients in the same study, it was associated with a fibrosis Metavir stage F2-F4[10]. In a cross-sectional

Table 1 Initial characteristics of 167 patients with human immunodeficiency virus infection and chronic hepatitis C, according to the *TM6SF* variants

	TM6SF2 p. 167 E/K	TM6SF2 p. 167 E/E	*P* value
Patients, *n*	17	150	
Age (yr)	42.6 (40.4-47.8)	40.3 (37.4-44.0)	0.04
Males	12 (70.6)	109 (72.7)	0.8
IVDU	8 (61.5)	95 (74.8)	0.2
BMI (kg/m^2)	24.0 (22.0-25.0)	23.0 (21.3-25.0)	0.3
Glucose (mg/dL)	81.0 (75.0-87.0)	88.0 (81.0-98.0)	0.002
Bilirubin (mg/dL)	0.9 (0.7-1.2)	0.7 (0.5-1.0)	0.06
AST (IU/L)	108.0 (52.0-134.0)	59.0 (40.0-94.0)	0.02
ALT (IU/L)	141.0 (122.0-172)	81.5 (46.0-131.0)	0.02
Cholesterol (mg/dL)	144.0 (123.0-194.0)	164.0 (135.5-191.0)	0.4
Triglycerides (mg/dL)	136.0 (118.0-154.0)	125.5 (83.5-182.3)	0.6
GGT (IU/mL)	65.0 (46.0-95.0)	79.0 (39.0-165.0)	0.6
ALP (IU/mL)	192.0 (156.3-238.3)	186.5 (139.3-252.0)	0.9
HCV RNA (IU/mL), median (IQR)	598679.0 (140698.5-997750.0)	601550.0 (202000.0-1432735.0)	0.7
Nadir of CD4$^+$ cells/mm^3	214.0 (153.0-380.0)	266.0 (176.3-413.3)	0.5
HIV RNA (cps/mL)	7638.0 (4112.5-19215.5)	10870.0 (3100.5-35374.4)	0.7
HIV RNA < 50 cps/mL	6 (35.3)	66 (44.0)	
CD4$^+$ cell/mmc	435.0 (380.0-650.0)	508.0 (399.3-670.0)	0.2
ART, treated	14 (82.3)	118 (78.7)	0.6
PI/r-NRTI-NNRTI	0	6 (5.3)	
PI/r-NRTI	6 (46.1)	40 (35.1)	
NRTI-NNRTI	2 (15.4)	30 (26.3)	
PI-NRTI	0	7 (6.1)	
NRTI	5 (38.5)	31 (27.2)	
Therapy missing, *n*	1	4	
Therapy-naïve	3 (17.6)	32 (21.3)	
Duration of ART (yr)	7.7 (6.4-12.8)	8.0 (5.7-10.8)	0.3
Duration of HIV infection (yr)	17.4 (12.5-21.4)	14.0 (7.7-17.9)	0.05
HCV Genotype			
1	5 (29.4)	57 (39.6)	0.3
2	1 (5.9)	7 (4.9)	
3	6 (35.3)	62 (43.1)	
4	5 (29.4)	18 (12.5)	
Missing	0	6	
HAI score	6.8 ± 2.9	5.8 ± 3.0	0.2
HAI: score 0-8	13 (76.5)	117 (78.0)	0.2
score 9-18	4 (23.5)	33 (22.0)	
Fibrosis score	3.1 ± 2.0	2.3 ± 1.5	0.05
Degree of fibrosis			
0	2 (11.8)	11 (7.3)	0.1
1	2 (11.8)	48 (32.0)	
2	2 (11.8)	33 (22.0)	
3	6 (35.3)	32 (21.3)	
4	0	11 (7.3)	
5	2 (11.8)	6 (4.0)	
6	3 (17.6)	9 (6.0)	
Steatosis score, mean ± SD	1.9 ± 1.2	1.7 ± 1.3	0.4
Degree of steatosis			0.8
0	3 (17.6)	45 (30.0)	
1	3 (17.6)	19 (12.7)	
2	4 (23.5)	37 (24.7)	
3	6 (35.3)	40 (26.7)	
4	1 (5.8)	9 (6.0)	
PNPLA3			
p. 148 I/I	9 (52.9)	79 (52.7)	0.7
p. 148 I/M	6 (35.3)	61 (40.7)	
p. 148 M/M	2 (11.8)	10 (6.7)	

Data represent as *n* (%), mean ± SD, or median (IQR). HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; BMI: Body mass index; HAI: Histological activity index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: γ glutamyl transferase; ART: Antiretroviral therapy; NRTIs: Nucleoside/nucleotide analogue reverse transcriptase inhibitors; NNRTIs: Non-nucleoside reverse transcriptase inhibitors; PIs: Protease inhibitors; TM6SF2: Transmembrane 6 superfamily member 2; PNPLA3: Patatin-like phospholipase domain-containing 3 gene; IQR: Interquartile range.

Table 2 Initial characteristics of 167 patients with human immunodeficiency virus infection and chronic hepatitis C, according to the fibrosis score

	Fibrosis score 0-3	Fibrosis score 4-6	*P* value
Patients, *n*	136	31	
Age (yr)	40.3 (37-44)	42.0 (39.5-46.7)	< 0.05
Males	96 (70.6)	25 (80.6)	0.1
BMI (kg/m^2)	23 (21.2-24.9)	22.8 (21.7-26.5)	0.5
Glucose (mg/dL)	87.0 (80.0-95.0)	91.0 (81-102)	0.6
Bilirubin (mg/dL)	0.68 (0.4-1.0)	0.97 (0.7-1.5)	< 0.01
AST (IU/L)	55.0 (39-91)	96.0 (63.0-143.0)	0.0008
ALT (IU/L)	79.0 (44-131)	120.0 (67-180)	< 0.01
Cholesterol (mg/dL)	162 (137-194.0)	158 (116.8-177)	0.3
Triglycerides (mg/dL)	122.0 (84.0-164)	147.5 (55-227)	0.1
GGT (IU/mL)	70.0 (36.0-150)	108 (60.0-227.0)	0.2
ALP (IU/mL)	178.0 (136.0-239)	221 (182-269)	< 0.01
HCV RNA (IU/mL)	5.2e5 (1.8e5-1.4e6)	7e5 (2.3e5-1.7e6)	0.4
Nadir of CD4$^+$ cells/mm^3	271 (175.5-429.5)	238.0 (145.0-347.5)	0.1
HIV RNA (copies/mL)	10914.5 (4402.3-31052.0)	4500.0 (784.0-36959.0)	0.1
CD4$^+$ cells/mm^3	510.0 (403-708)	454.0 (338-586)	< 0.01
ART, Treated	105 (77.2)	27 (87.1)	0.4
PI/r-NRTI-NNRTI	6 (5.9)	0	
PI/r-NRTI	35 (33.6)	11 (42.3)	
PI-NRTI	6 (5.9)	1 (3.8)	
NRTI-NNRTI	25 (24.8)	7 (26.9)	
NRTI	29 (28.7)	7 (26.9)	
Therapy missing, *n*	4	1	
Therapy-naïve	31 (22.8)	4 (12.9)	
Duration of ART (yr)	7.8 (5.4-10.9)	9.6 (6.3-12)	0.2
Duration of HIV infection (yr)	14.4 (7.5-18.1)	13.9 (8.4-17.4)	0.6
HCV Genotype			
1	51 (38.9)	11 (3.7)	0.4
2	7 (5.3)	1 (0.4)	
3	54 (41.2)	14 (46.7)	
4	19 (14.5)	4 (13.4)	
Missing, *n*	5	1	
HAI score	5.0 ± 2.7	8.6 ± 2.3	< 0.0001
HAI			
score 0-8	115 (84.6)	15 (48.4)	< 0.0001
score 9-18	21 (15.4)	16 (51.6)	
Steatosis score	1.4 ± 1.2	1.9 ± 1.3	< 0.02
Degree of steatosis			
0	44 (32.4)	4 (12.9)	0.0001
1	18 (13.2)	4 (12.9)	
2	34 (25.0)	7 (22.6)	
3	32 (23.55)	14 (45.2)	
4	8 (5.9)	2 (6.5)	
TM6SF			
p. 167 E/K	12 (8.8)	5 (16.1)	< 0.03
p. 167 E/E	124 (91.2)	26 (83.9)	
PNPLA3,			
p. 148 I/I	75 (55.1)	13 (41.9)	0.2
p. 148 I/M	49 (36.1)	18 (58.6)	
p. 148 M/M	12 (8.8)	0	

Data represent as *n* (%), mean ± SD, or median (IQR). e: Elevated; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; BMI: Body mass index; HAI: Histological activity index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: γ glutamyl transferase; ART: Antiretroviral therapy; NRTIs: Nucleoside/nucleotide analogue reverse transcriptase inhibitors; NNRTIs: Non-nucleoside reverse transcriptase inhibitors; PIs: Protease inhibitors; TM6SF2: Transmembrane 6 superfamily member 2; PNPLA3: Patatin-like phospholipase domain-containing 3 gene; IQR: Interquartile range.

analysis of 2023 HCV-mono-infected patients with a chronic liver disease recently performed by Eslam *et al*[8], TM6SF2-E rs58542926 was only marginally associated with the severity of liver fibrosis in a univariate analysis, but not in a multivariate regression analysis. In the same study, the Authors evaluated the association between the rs58542926 variants and fibrosis progression in 1174 of the 2023 patients with a chronic liver disease: Despite a marginal association in the univariate analysis, after adjustment for other variables, the T allele was not independently associated with fibrosis. Instead, no association with the severity

Table 3 Initial characteristics of 167 patients with human immunodeficiency virus infection and chronic hepatitis C, according to the steatosis score

	Steatosis score 0-2	Steatosis score 3-4	*P* value
Patients, *n*	111	56	
Age (yr)	40.3 (37.4-44.2)	41.8 (38.4-44.0)	0.3
Males	79 (71.2)	42 (75)	0.6
BMI (kg/m^2)	22.8 (21.3-24.5)	23.9 (21.9-25.6)	0.03
Glucose (mg/dL)	85.5 (79.3-94.0)	92.5 (82.0-102.0)	0.004
Bilirubin (mg/dL)	0.6 (0.4-1.0)	0.8 (0.6-1.1)	0.01
AST (IU/L)	54.0 (38.3-79.0)	92.0 (55.8-142.3)	0.0004
ALT (IU/L)	67.0 (43.0-114.5)	129.0 (78.8-201.5)	0.00006
Cholesterol (mg/dL)	168.0 (141.0-196.5)	147.0 (120.0-174.0)	0.01
Triglycerides (mg/dL)	130.5 (84.0-188.5)	123.0 (87.0-156.0)	0.5
GGT (IU/mL)	70.0 (34.5-142.5)	97.0 (46.5-229.5)	0.04
ALP (IU/mL)	185.0 (138-240.3)	196.0 (147.5-265.0)	0.5
HCV RNA (IU/mL)	5.2e5 (1.5e5-1.4e6)	7.4e5 (2.4e5-1.4e6)	0.7
Nadir of CD4$^+$ cells/mm^3	272.0 (166.5-425.8)	257.5 (172.8-360.5)	0.2
HIV RNA copies/mL	7484.0 (2925.3-18994.0)	18918.0 (3991.0-37219.0)	0.1
HIV RNA < 50 copies/mL	61 (54.9)	25 (44.6)	
CD4$^+$ cell/ mm^3	527.0 (425.3-720.5)	463.0 (373.0-542.8)	0.002
ART, Treated	88 (79.3)	44 (78.6)	0.91
PI/r-NRTI-NNRTI	5 (5.9)	1 (2.4)	
PI/r-NRTI	33 (38.8)	13 (30.2)	
PI-NRTI	3 (3.5)	4 (9.5)	
NRTI-NNRTI	21 (24.7)	11 (26.2)	
NRTI	23 (27.1)	13 (30.9)	
Therapy missing, *n*	3	2	
Therapy-naïve	23 (41.1)	12 (21.4)	
Duration of HAART (yr)	8.2 (6.2-11.5)	7.6 (4.5-10.8)	0.3
Duration of HIV infection (yr)	14.1 (7.8-17.8)	14.2 (8.1-18.1)	0.8
HCV-genotype			
1	44 (41.1)	18 (33.3)	0.04
2	5 (4.7)	3 (5.6)	
3	38 (35.5)	30 (55.6)	
4	20 (18.7)	3 (5.6)	
Missing, *n*	4	2	
HAI score	5.4 ± 2.9	6.9 ± 3.0	0.002
Degree of HAI			
score 0-8	98 (88.3)	38 (67.8)	0.01
score 9-18	19 (17.1)	18 (32.1)	
Fibrosis score	2.1 ± 1.6	2.9 ± 1.6	0.003
Degree of fibrosis			
0	12 (10.8)	1 (1.8)	0.001
1	40 (36.0)	10 (17.9)	
2	21 (18.9)	14 (25)	
3	23 (20.7)	15 (26.3)	
4	5 (4.5)	6 (10.7)	
5	3 (2.7)	5 (8.9)	
6	7 (6.3)	5 (8.9)	
TM6SF			
p. 167 E/K	10 (0.9)	7 (12.5)	0.48
p. 167 E/E	101 (90.9)	49 (87.5)	
PNPLA3			
p. 148 I/I	66 (59.4)	22 (39.3)	0.06
p. 148 I/M	38 (38.3)	29 (51.8)	
p. 148 M/M	7 (6.3)	5 (8.9)	

Data represent as *n* (%), mean ± SD, or median (IQR). e: Elevated; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; BMI: Body mass index; HAI: Histological activity index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: γ glutamyl transferase; ART: Antiretroviral therapy; NRTIs: Nucleoside/nucleotide analogue reverse transcriptase inhibitors; NNRTIs: Non-nucleoside reverse transcriptase inhibitors; PIs: Protease inhibitors; TM6SF2: Transmembrane 6 superfamily member 2; PNPLA3: Patatin-like phospholipase domain-containing 3 gene; IQR: Interquartile range.

of fibrosis was observed for 694 Caucasian patients with CHC due to HCV-genotype 1[9].

Some studies on the association between the TM6SF2 rs58542926 variants and the severity of liver fibrosis in patients with NAFLD have provided interesting information. Using two histologically characterized cohorts including steatosis and steatohepatitis, and fibrosis and cirrhosis, Liu *et al*[5]

Table 4 Initial characteristics of 161 patients with human immunodeficiency virus infection and chronic hepatitis C, according to the hepatitis C virus genotype

	HCV genotype 3	non- 3 HCV genotype	*P* value
Patients, *n*	68	93	
Age (yr)	40.6 (36.7-44.0)	40.8 (37.8-44.1)	0.3
Males	43 (63.2)	69 (74.2)	0.07
IVDU	44 (64.7)	57 (61.3)	0.8
BMI (kg/m^2)	22.7 (21.2-25.2)	23.1 (21.7-24.9)	0.5
Glucose (mg/dL)	87.0 (79.0-94.0)	89.0 (82.0-98.0)	0.1
Bilirubin (mg/dL)	0.7 (0.6-1.0)	0.7 (0.5-1.0)	0.5
AST (IU/L)	77.5 (53.3-124.0)	55.0 (38.0-85.0)	0.004
ALT (IU/L)	120.5 (62.5-187.5)	66.0 (40.0-117.0)	0.0002
Cholesterol (mg/dL)	143.5 (115.3-173.0)	171.0 (147.5-197.0)	0.0006
Triglycerides (mg/dL)	99.0 (69.5-158.0)	139.0 (103.5-216.8)	0.003
GGT (IU/mL)	61.0 (32.8-106.0)	96.0 (49.5-226.5)	0.004
ALP (IU/mL)	196.0 (143.0-260.5)	192.0 (150.0-246.0)	0.9
HCV RNA (IU/mL)	477624.0 (124539.0-1050000.0)	730500.0 (233250.0-1941008.0)	0.07
Nadir of CD4$^+$ cells/mm^3	247.7 (155.3-380.0)	252.0 (175.0-404.0)	0.6
HIV RNA copies/mL	5607.5 (1972.3-18918.0)	12130.0 (1806.8-37226.5)	0.7
HIV RNA < 50 copies/mL	30 (44.0)	42 (45.2)	
CD4$^+$ cell/mm^3	506.5 (397.8-638.0)	494.0 (398.0-697.5)	0.7
ART, Treated	50 (73.5)	79 (84.9)	0.4
PI/r-NRTI-NNRTI	1 (2.0)	5 (6.6)	
PI/r-NRTI	22 (44.9)	24 (31.6)	
PI-NRTI	3 (6.1)	4 (5.3)	
NRTI-NNRTI	13 (26.5)	18 (23.7)	
NRTI	10 (20.4)	25 (32.9)	
Therapy missing, *n*	1	3	
Therapy-naïve	18 (26.47)	14 (15.0)	
Duration of ART (yr)	9.0 (4.3-12.6)	7.7 (6.1-10.4)	0.4
Duration of HIV infection (yr)	14.0 (7.7-18.5)	14.1 (8.1-17.1)	0.8
HAI score	6.5 ± 3.1	5.5 ± 2.9	0.03
HAI			
score 0-8	48 (70.6)	76 (81.7)	0.09
score 9-18	20 (14.7)	17 (18.3)	
Fibrosis score	2.5 ± 1.6	2.2 ± 1.6	0.2
Degree of fibrosis			
0	4 (5.9)	9 (9.7)	0.7
1	16 (23.5)	32 (34.4)	
2	16 (23.5)	17 (18.3)	
3	18 (26.5)	19 (20.4)	
4	4 (5.9)	5 (5.3)	
5	5 (7.3)	4 (4.3)	
6	4 (5.9)	7 (7.5)	
Steatosis score	2.0 ± 1.3	1.4 ± 1.2	0.002
Degree of steatosis			
0	14 (20.6)	31 (33.3)	0.03
1	7 (10.3)	14 (15.0)	
2	16 (23.5)	20 (21.5)	
3	23 (33.8)	22 (23.7)	
4	7 (10.1)	2 (2.1)	
TM6SF			
p. 167 E/K	6 (8.8)	11 (11.8)	0.5
p. 167 E/E	62 (91.2)	82 (88.2)	
PNPLA3			
p. 148 I/I	38 (55.9)	48 (51.6)	0.5
p. 148 I/M	24 (35.3)	40 (43.0)	
p. 148 M/M	6 (8.8)	5 (5.4)	

HCV genotype missing in 6 cases. Data represent as *n* (%), mean ± SD, or median (IQR). e: Elevated; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; BMI: Body mass index; HAI: Histological activity index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: γ glutamyl transferase; ART: Antiretroviral therapy; NRTIs: Nucleoside/nucleotide analogue reverse transcriptase inhibitors; NNRTIs: Non-nucleoside reverse transcriptase inhibitors; PIs: Protease inhibitors; TM6SF2: Transmembrane 6 superfamily member 2; PNPLA3: Patatin-like phospholipase domain-containing 3 gene; IQR: Interquartile range.

demonstrated that the TM6SF2 E167K rs58542926 variant enhances hepatic fibrosis progression in patients with NAFLD. Eslam *et al*[8] investigated 502 Caucasian patients with NAFLD and demonstrated that the TM6SF2 E167K rs58542926 variant is independently associated with the risk of a > F2 Metavir score.

Concluding on this point, an independent association between the TM6SF2 E167K variant and severe liver fibrosis has been demonstrated in HIV/HCV co-infected patients with CH and in NALFD patients, whereas contrasting results have been observed for HCV-monoinfected CH patients.

In the present study, the TM6SF2 E167K variant has been identified as an independent predictor of severe steatosis in HIV/HCV coinfected patients with HCV-genotype other than 3, an association not detected in those with HCV-genotype 3 most probably because this genotype favors lipid droplet deposition and storage in hepatocytes, possibly obscuring other associations.

In accordance with this, in HCV-mono-infected CH patients, Milano *et al*[10] observed a marginal association between the E167K variant and severe steatosis, but more interestingly, an independent association was found for patients with a non-3 HCV genotype and no association for those with HCV-genotype 3[10]. These data were confirmed by Eslam *et al*[8], who observed a marginal but independent association between the TM6SF2 rs58542926 T allele and the severity of steatosis, but stratifying this cohort by HCV-genotype (3 *vs* non-3), they demonstrated that the association persisted only for the subgroup of patients with a non-3 HCV genotype. They concluded that the TM6SF2 E167K substitution may promote lipid abnormalities and steatosis by altering TM6SF2 and the microsomal triglyceride transfer protein expression[8]. Petta *et al*[9], however, found no association between the *TM6SF2* gene and the severity of steatosis in HCV-genotype-1 mono-infected CH patients.

The TM6SF2 E167K variant was not identified as an independent predictor of severe necroinflammation in the HIV/HCV co-infected CH patients enrolled in the present study. In accordance with this, in HCV-mono-infected CH patients the association between the TM6SF2 E167K variant and severe liver necroinflammation was marginal in the study by Milano *et al*[10] and absent in the study by Eslam *et al*[8].

In conclusion, the data from the present study show that in HIV/HCV coinfected CH patients the TM6SF2 E167K variant plays an important role in steatosis severity, as demonstrated in patients infected with a non-3 HCV genotype and not found in those harboring HCV-genotype 3, most probably due to the strong action of this viral genotype on fat deposition in liver cells. This phenomenon seems to be independent of HIV infection, since it has been observed both in anti-HIV-positive (the present study) and anti-HIV-negative patients[10,20].

The data from the present study also demonstrate that the TM6SF2 E167K variant is an independent predictor of severe fibrosis in HIV/HCV coinfected CH patients, an association identified in anti-HIV-negative subjects by some Authors[10,20] and not found by others[9].

The role of the TM6SF2 E167K variant as a predictor of severe necroinflammation remains uncertain, since it was not observed in the HIV/HCV CH patients in the present study and not or only marginally observed in HCV-monoinfected CH patients[9,10,20].

COMMENTS

Background

The transmembrane 6 superfamily member 2 (*TM6SF2*) gene is a regulator of liver fat metabolism since it influences the secretion of triglyceride-rich lipoproteins and the hepatic triglyceride content. In addition, it has been demonstrated that in patients with non-alcoholic fatty liver disease (NAFLD), the TM6SF2 E167K variant induces a reduction in very-low-density-lipoprotein secretion and a predisposition to retain triglycerides in lipid droplets in liver cells, conditions associated with increased alanine aminotransferase serum levels, lower lipoprotein plasma concentrations and advanced hepatic fibrosis. Previous studies identified the TM6SF2 E167K variant as an independent predictor of severe liver steatosis in patients with hepatitis C virus (HCV)-related chronic hepatitis (CH) lacking human immunodeficiency virus (HIV) infection, whereas controversial results have been published on the impact of this polymorphism on liver fibrosis.

Research frontiers

As no data were available on this topic for HIV/HCV coinfection, the authors studied the impact of the TM6SF2 E167K variant on the biochemical and morphologic expression of liver lesions of 167 HIV/HCV coinfected patients with CH.

Innovations and breakthroughs

This is the first study investigating the association of TM6SF2 E167K variant with the entity of liver lesions in patients with HIV/HCV coinfection and biopsy-proven CH. Considering the whole group of patients, the TM6SF2 E167K variant was not associated with severe steatosis. However, nearly 40% of patients in this study had HCV-genotype 3, which resulted to be independently associated with severe steatosis. An independent association was found between severe steatosis and TM6SF2 E167K in the non-3 HCV-genotype subgroup, whereas no association was found in the HCV-genotype-3 subgroup, most probably because this viral genotype favors fat deposition in liver cells. This phenomenon seems to be independent of HIV infection, since it was observed both in anti-HIV-positive patients in the present study and in anti-HIV-negative patients in other investigations. The data from the present study also demonstrate that the TM6SF2 E167K variant is an independent predictor of severe fibrosis in HIV/HCV coinfected CH patients, an association identified in anti-HIV-negative subjects by some Author but denied by others. No association was found between the TM6SF2 E167K variant and severe liver necroinflammation.

Applications

As the TM6SF2 E167K variant is an independent predictor of severe liver steatosis and severe liver fibrosis in HIV/HCV coinfected CH patients, its detection has a marked diagnostic and clinical value. The authors believe that their article will be a stimulus for other clinicians to start testing patients with CH C and in particular those with HIV infection for the TM6SF2 E167 variants.

Peer-review

This is a well-conducted research paper that illustrates the potential specialty for using HIV/HCV patients in NAFLD study. The authors results showed that 167 consecutive patients with HIV/HCV coinfection and biopsy-proven CH and showed A pathologist graded liver fibrosis and necroinflammation. The

authors reported that the TM6SF2 E167K variant on the liver histology of HIV/HCV co-infected patients with CH and independent predictor of severe fibrosis with severe steatosis was demonstrated only for patients with a non-3 HCV genotype. There are no major criticisms of the work and I would support acceptance of the manuscript. The paper is able to publish to World Journal of Gastroenterology.

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ORIGINAL ARTICLE

Basic Study

Embryonic liver fordin is involved in glucose glycolysis of hepatic stellate cell by regulating PI3K/Akt signaling

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Author contributions: Tu W performed the majority of experiments and analyzed the data; Wang ZJ participated equally in treatment of animals; Ye J designed and coordinated the research; Wang ZJ wrote the paper.

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Abstract

AIM

To investigate the role of embryonic liver fordin (ELF) in liver fibrosis by regulating hepatic stellate cells (HSCs) glucose glycolysis.

METHODS

The expression of ELF and the glucose glycolysis-related proteins were evaluated in activated HSCs. siRNA was used to silence ELF expression in activated HSCs *in vitro* and the subsequent changes in PI3K/Akt signaling and glucose glycolysis-related proteins were observed.

RESULTS

The expression of ELF increased remarkably in HSCs of the fibrosis mouse model and HSCs that were cultured for 3 wk *in vitro*. Glucose glycolysis-related proteins showed an obvious increase in the activated HSCs, such as phosphofructokinase, platelet and glucose transporter 1. ELF-siRNA, which perfectly silenced

the expression of ELF in activated HSCs, led to the induction of glucose glycolysis-related proteins and extracellular matrix (ECM) components. Moreover, pAkt, which is an important downstream factor in PI3K/Akt signaling, showed a significant change in response to the ELF silencing. The expression of glucose glycolysis-related proteins and ECM components decreased remarkably when the PI3K/Akt signaling was blocked by Ly294002 in the activated HSCs.

CONCLUSION

ELF is involved in HSC glucose glycolysis by regulating PI3K/Akt signaling.

Key words: Liver fibrosis; Embryonic liver fordin; PI3K/Akt signaling; Hepatic stellate cells; Glucose glycolysis

Core tip: The metabolism of activated hepatic stellate cells (HSCs) was reprogrammed. Silence of embryonic liver fordin (ELF) led to the inhibition of PI3K/Akt signaling and decrease of glycolysis in activated HSCs. Glucose glycolysis of activated HSCs was regulated by ELF through PI3K/Akt signaling The present study indicated that metabolism of HSCs may be a novel target for the diagnosis and treatment of liver cirrhosis in clinical practice.

Tu W, Ye J, Wang ZJ. Embryonic liver fordin is involved in glucose glycolysis of hepatic stellate cell by regulating PI3K/Akt signaling. *World J Gastroenterol* 2016; 22(38): 8519-8527 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i38/8519.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i38.8519

INTRODUCTION

Liver fibrosis occurs in most chronic liver diseases and is characterized by the accumulation of extracellular matrix following sustained inflammation[1]. The main causes of liver fibrosis include chronic HBV and HCV infection, parasitic infections, alcohol abuse, and NASH. Liver fibrosis can lead to the dysfunction of hepatocytes and obstruction of the intrahepatic blood flow, which resulting in the subsequent hepatic insufficiency and hypertension of the portal vein[2].

HSCs, which are the main source of ECM in injured livers, play a crucial role in the progress of liver fibrosis. In normal livers, HSCs are located in the Disse space, between the sinusoidal endothelium and hepatocytes. HSCs become activated and acquire contractile, proinflammatory, and fibrogenic properties when subjected to diverse chronic injuries. Researchers have identified many factors that promote the transdifferentiation of quiescent HSCs into activated HSCs[3].

The TGF-β signaling plays a very important role in various cellular physiological processes through the regulation of downstream proteins[4]. Frequent inactivation of the TGF-β pathway components in liver fibrosis demonstrates a powerful promoting role of the TGF-β pathway. The multifunctional effects of TGF-β in cellular actions occur through the binding of its receptors, TGF-β receptor II and receptor I; the activation of intrinsic kinase activity; and the phosphorylation and translocation of mediators, Smads, followed by TGF-β target gene activation[5]. ELF, also known as β2-spectrin, is important for maintaining function of cellular membranes and polarization of epithelial cells. Meanwhile, ELF also plays an important role in TGF-β pathway.

Smad 3 and Smad 4 are the main proteins of Smad family which is crucial for the activation of TGF-β pathway. The interaction of ELF and Smad proteins was able to facilitate the transport of Smad 3-Smad4 complex into nucleus, which lead to the subsequent activation of TGF-β pathway[6-8].

Our previous study demonstrated that ELF is involved in the activation of HSCs. First, Western blot and RT-qPCR evaluation indicated that ELF expression was increased remarkably in fibrotic mouse model. Moreover, the silence of ELF in activated HSCs significantly reduced the ECM components such as collagen and α-SMA. Clarification of the mechanism is needed[9]. In addition, our study showed that the PI3K/Akt signaling is regulated by ELF in the process of hepatocyte proliferation[10]. The PI3K/Akt signaling is essential in the regulation of glucose metabolism in many types of cells[11,12]. Researchers have identified a novel mechanism for reprogramming quiescent HSCs into activated HSCs that depends on the induction of glycolysis, similar to the Warburg state that has been described in cancer cells[13]. We hypothesized that ELF is involved in glucose metabolism of HSCs through PI3K/Akt signaling.

MATERIALS AND METHODS

Ethics statement

All the processes were approved by the Animal Care and Use Committee of Huazhong University of Science and Technology, Wuhan, China.

Mouse model of liver fibrosis

Eight-week-old male mice(C57/Black/6) that purchased from Animal Center of HUST (Wuhan, China) were challenged by classic subcutaneous injection with carbon tetrachloride (CCl_4) diluted at a 3:7 (v/v) ratio in 5 mL/kg olive oil twice weekly for 12 wk as described previously.

HSC isolation

Primary hepatic stellate cells used in this study were isolated from C57/Black/6 mice (including control and fibrosis model) by *in situ* perfusion and centrifugation.

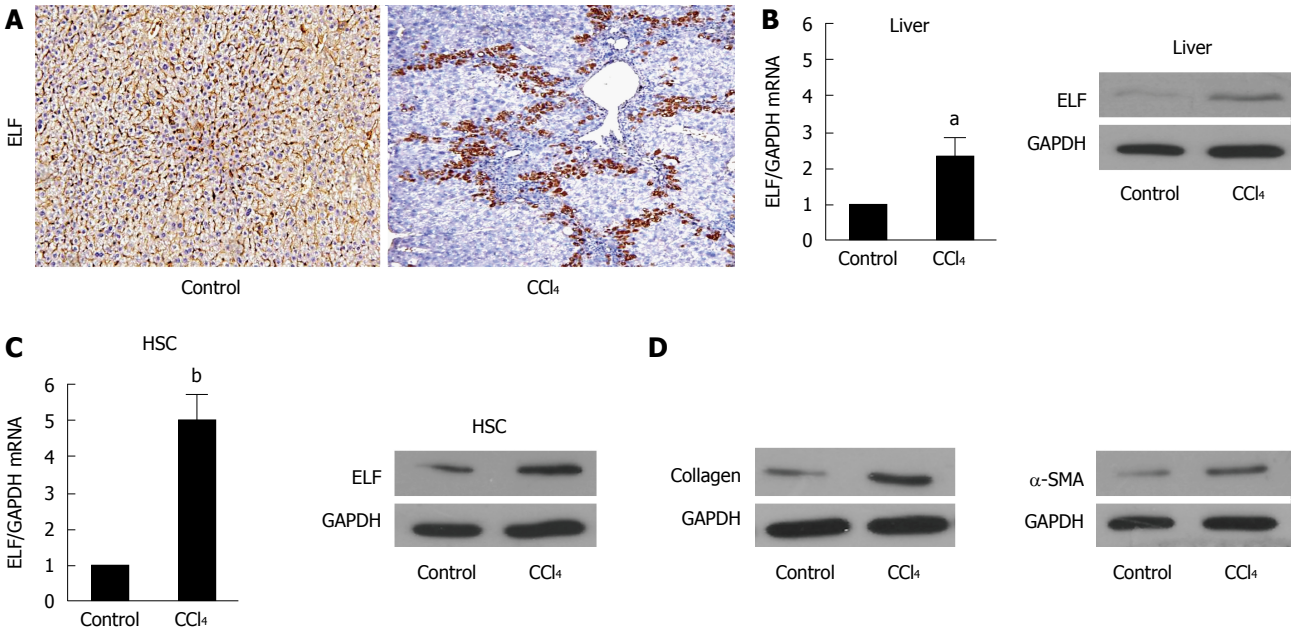

Figure 1 Embryonic liver fordin expression is upregulated in fibrotic livers and hepatic stellate cells. A: The Embryonic liver fordin (ELF) expression in cirrhotic livers was determined by the immunohistochemical analysis. Magnification × 200; B: Real-time RT-PCR and Western blot analysis were used to evaluate the ELF expression in the liver homogenates from the control and CCl_4-treated mice. $^aP < 0.05$, the CCl_4-treated mice *vs* the control mice. GAPDH was used as the control; C: Real-time RT-PCR and Western blot analysis were used to evaluate the ELF expression in the primary hepatic stellate cells (HSCs) isolated from the control and CCl -treated mice. $^bP < 0.01$, the CCl_4-treated mice *vs* the control mice; D: The α-SMA and collagen I expression at the protein level were upregulated in the whole liver homogenates from the CCl_4-treated mice compared with the controls.

The details were described in our previous study[9].

Preparation and transfection of siRNAs

siRNA used to silence ELF expression was synthesized by Applied Biosystems. The concentration of siRNAs (and scrambled siRNAs) was 50 pmol/L. Lipo2000 (Invitrogen) was used to transduce the siRNA into mice hepatic stellate cells on six wells when the confluence was about 30%-50%. RNA extraction was performed 72 h later. According to previous identification, ELF siRNA s74307 was chosen because of its best efficacy.

Western blot

The total protein was extracted from cell and tissue using RIPA buffer with protease inhibitors. Concentrations of proteins were evaluated by BCA Assay Kit. Total proteins (50 μg) were separated on 10% SDS-PAGE. The immunoblotting was performed. The immune complex was visualized by ECL detection

Immunohistochemistry

Liver specimens for histology and immunohistochemistry were fixed in 10% buffered formalin for 48 h, and then sliced into sections. Staining was performed using ABC kit. Sections were incubated at 4 ℃ with antibody for 12 h. DAB was used to visualize immunocomplexes.

Measurement of lactate

Whole cell lysates of liver sample and HSCs were prepared with pyruvate assay buffer and then filtered through a 10-kilodalton molecular weight spin filter for deproteinization. Levels of lactate were measured using a lactate assay kit or pyruvate assay kit from BioVision according to the manufacturer's instructions, and normalized to the control group.

Statistical analysis

The results were presented as the mean ± SD. The differences between groups were tested by Student two-tailed *t* test, and $P < 0.05$ was considered statistically significant.

RESULTS

ELF expression is upregulated in fibrotic liver and HSCs

To evaluate whether ELF is involved in liver fibrosis, we generated a fibrotic mouse model. The immunohistochemical (IHC) examination found that ELF expression was increased in the fibrotic livers compared with controls (Figure 1A). Moreover, the ELF expression was observed near the bridging fibrotic areas. In addition, ELF mRNA (with RT-qPCR, approximately 2.5 times) and protein expression by Western blot analysis was increased in the CCl_4-treated animals (Figure 1B). HSCs are predominantly located in the areas of bridging fibrosis, and we isolated HSCs from the fibrotic and normal livers. The RT-qPCR and Western blot test of the ELF expression found that there was a more significant increase in the HSCs isolated from the fibrotic livers than from the normal livers (Figure 1C). This finding indicated that the upregulated expression of ELF in

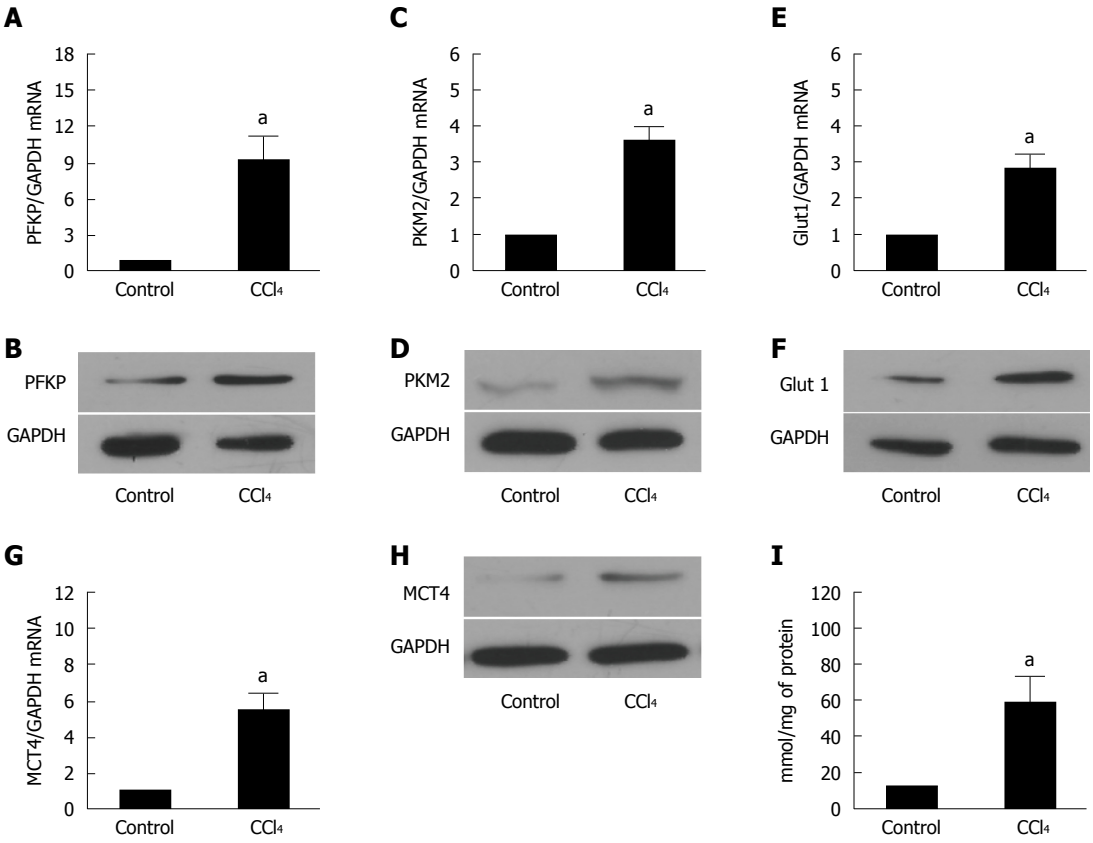

Figure 2 Glycolysis related-genes are upregulated in liver fibrosis. A, C: The real-time RT-PCR analysis evaluated the expression of the hepatic glycolytic enzymes, PFKP and PFKM2 in fibrotic and control mice. The PFKP and PFKM2 expression of the mRNA level was increased compared with that of the control ($^aP < 0.05$ *vs* control). GAPDH was used as the control; B, D: Western blot analysis indicated that the PFKP and PFKM2 expression of the protein level was upregulated in the fibrotic livers; E: The real-time RT-PCR analysis evaluated the hepatic glucose transporter, Glut 1, expression in the fibrotic livers and the control mice. The expression of Glut 1 mRNA was increased compared with that of the controls ($^aP < 0.05$ *vs* control); F: Western blot analysis indicated that the expression of the Glut 1 at protein level was upregulated in the fibrotic livers; G: Real-time RT-PCR analysis evaluated the MCT4 hepatic lactate transporter expression in the fibrotic livers and the control mice. The expression of the Glut 1 mRNA was increased compared with that of the controls ($^aP < 0.05$ *vs* control); H: Western blot analysis indicated that the expression of MCT4 at protein level was upregulated in the fibrotic liver; I: The intracellular lactate was evaluated by a lactate assay kit ($^aP < 0.05$ *vs* control).

HSCs might play an important role in liver fibrosis.

Glycolysis-related genes are upregulated in fibrotic livers

Previous study had demonstrated that glucose metabolism was reprogrammed in fibrotic livers[13]. To confirm whether glycolysis-related genes were changed in fibrotic livers, we selected some key proteins which are involved in glucose glycolysis, such as phosphofructokinase (PFKP), Glut1, PKM2, and MCT4.

PFKP, a gene which encodes the platelet isoform of phosphofructokinase (PFK), plays an important role in glucose glycolysis of various cell types[14,15]. As shown in Figure 2A and 2B, the PFKP expression was increased at both the mRNA and protein levels. Pyruvate kinase isozymes M1/M2 (PKM1/M2) is an enzyme encoded by the PKM2 gene. The function of PKM2 is to catalyze the last step within glycolysis, and leads to the dephosphorylation of phosphoenolpyruvate[16]. Real-time quantitative PCR and Western blot (Figure 2C and D) demonstrated that PKM expression was increased significantly in fibrotic liver at both mRNA and protein levels. Glucose transporter 1 (Glut 1) is a uniporter protein that in humans is encoded by the *SLC2A1* gene. Glut 1 is the first glucose transporter which facilitates the transport of glucose from blood into membrane in various kinds of cells[17,18]. We found that Glut1 expression also showed a remarkable increase in fibrotic liver than control mice (Figure 2E and F).

Monocarboxylate transporter 4 (MCT4), the lactate export pump, plays an crucial role in the accumulation of intracellular lactate in various cells[19]. The upregulation of MCT4 in fibrotic livers indicates that lactate accumulation was increased in fibrotic livers compared with the controls (Figure 2G and H). These findings indicated that glycolysis related genes were overexpressed in fibrotic livers. However, the expression of these genes in HSCs remains unknown.

The intracellular lactate was evaluated by a lactate assay kit. As shown in Figure 2I, intracellular lactate level increased significantly in fibrotic liver.

Glycolysis related genes are upregulated significantly in HSCs isolated from fibrotic livers

The liver is composed of many cell types, and further

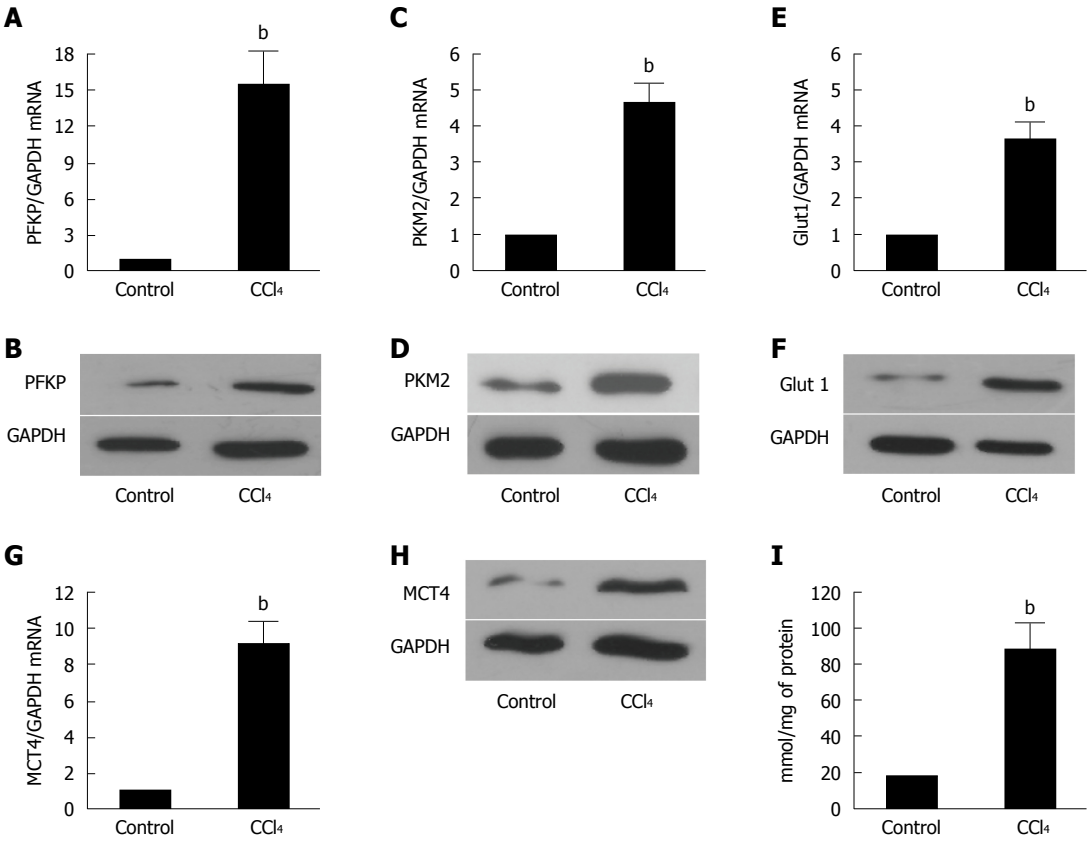

Figure 3 Glycolysis related-genes are upregulated significantly in the HSCs isolated from the fibrotic livers. A, C: The real-time RT-PCR analysis was used to evaluate the expression of the PFKP and PFKM2 hepatic glycolytic enzymes in the fibrotic livers and control mice. The PFKP, pAKT, and PFKM2 expressions of the mRNA level were increased compared with those of the controls ($^bP < 0.01$ *vs* control). GAPDH was used as the control; B, D: The Western blot analysis indicated that the PFKP and PFKM2 expression at the protein level was upregulated in the fibrotic livers; E: The real-time RT-PCR analysis evaluated the hepatic glucose transporter Glut 1 expression in the fibrotic livers and control mice. The expression of Glut 1 mRNA was increased compare with that of the controls ($^bP < 0.01$ *vs* control); F: The Western blot analysis indicated that the Glut 1 expression at the protein level was upregulated in the fibrotic livers; G: The real-time RT-PCR analysis evaluated the hepatic lactate transporter MCT4 expression in the fibrotic livers and the control mice. The Glut 1 expression at the mRNA level was increased compared with that of the controls ($^bP < 0.01$ *vs* control); H: The western blot analysis indicated that the MCT4 expression at the protein level was upregulated in the fibrotic livers; I: The intracellular lactate was evaluated by a lactate assay kit ($^bP < 0.01$ *vs* control).

studies should be performed if a certain protein is overexpressed in the liver. Therefore, we first isolated the HSCs from a fibrotic liver by *in situ* perfusion. Then, the total protein and mRNA were extracted. As demonstrated in Figure 3, the expression of PFKP, GLUT 1, PKM2, and MCT4 increased remarkably ($P < 0.01$) shown by real time-quantitative PCR (Figure 3A, C, E and G) and Western blot (Figure 3B, D, F and H). Moreover, lactate (Figure 3I) showed a significant increase compared with the controls ($P < 0.01$). This finding indicates that the glucose metabolism of activated HSCs was reprogrammed. The reprogrammed metabolism might supply energy to HSCs for their proliferation, ECM production, and migration.

Silence of ELF in activated HSCs leads to the inhibition of glycolysis

The efficiency of the knockdown of ELF in activated HSCs by siRNA was tested by Western blot (Figure 4A) and real-time quantitative PCR (Figure 4B). The ELF-siRNA treatment led to a remarkable decrease in the ELF expression. A significant reduction in SMA and collagen 1 expression was observed in the ELF-siRNA treated HSCs (Figure 4H and I). In addition, PFKP, GLUT 1, PKM2, and MCT4 showed a significant decrease in response to the ELF-siRNA (Figure 4C-F). The intracellular lactate also decreased significantly in response to the silence of ELF (Figure 4G). This finding indicates that the silence of ELF leads to the inhibition of glucose glycolysis-related genes. These results indicated that silence of ELF could block HSC activation, reduce ECM production, and inhibit glycolysis.

ELF-PI3K/Akt-glycolysis axis in HSC

Although previous data indicated that ELF might play an important role in HSC activation and glycolysis in the pathogenesis of liver fibrosis, the mechanism needs to be clarified. Since PI3K/Akt signaling is important in glucose glycolysis of many cell types, and based on our previous study of the interaction between ELF and PI3K/Akt pathway, we hypothesized that the cross talk of ELF and PI3K/Akt signaling was involved in the activation of HSCs.

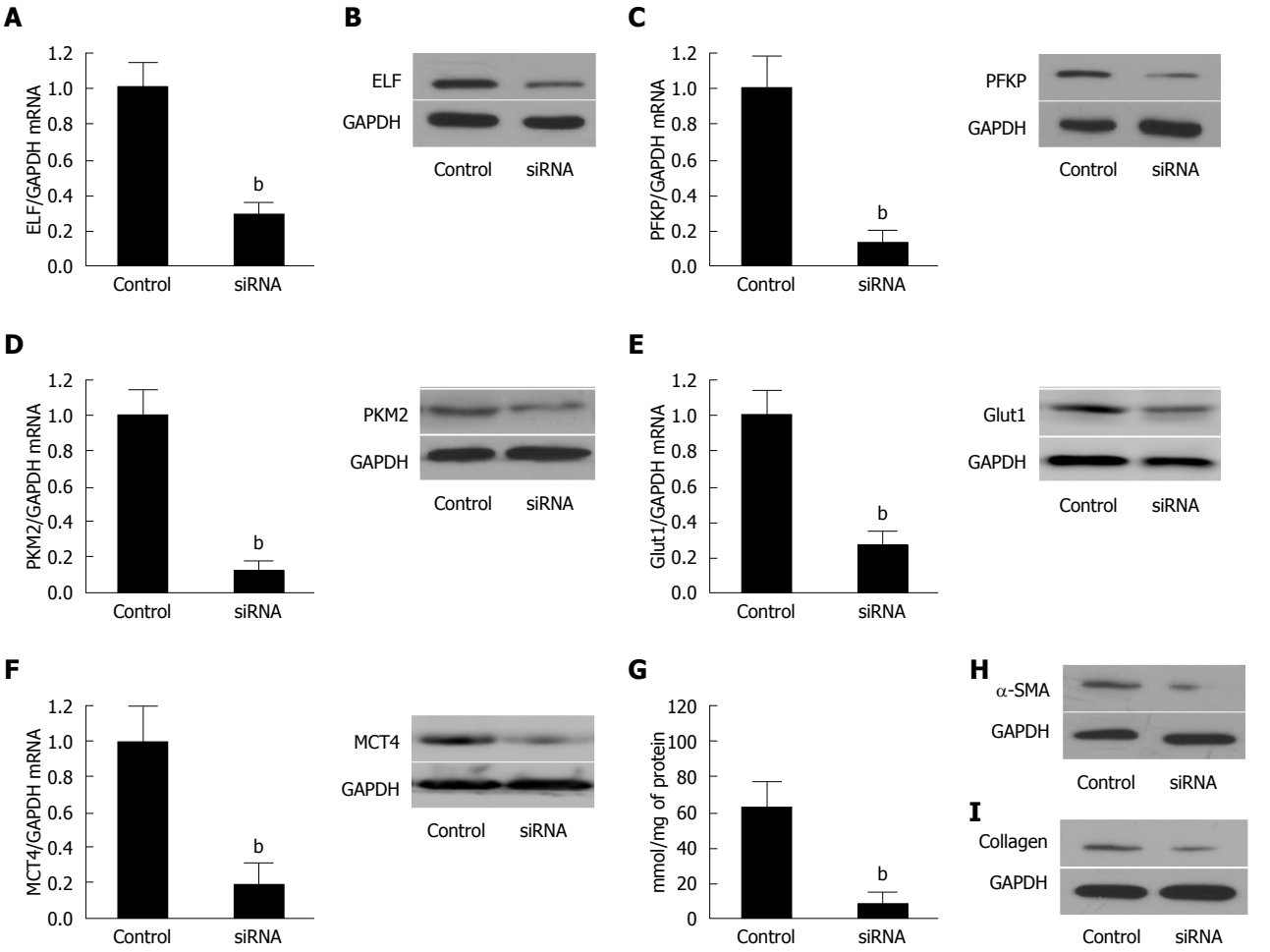

Figure 4 Silencing of Embryonic liver fordin in the activated hepatic stellate cells led to the inhibition of glycolysis. A: The ELF mRNA was reduced in the activated hepatic stellate cells (HSCs) transfected synthetic siRNA against ELF was assessed by real-time RT-PCR. $P < 0.01$ for the ELF siRNA versus the siRNA controls. GAPDH was used as the control; B: Western blot analysis confirmed that synthetic siRNA inhibited the ELF expression in the activated HSCs; C, D: The hepatic glycolytic enzymes, PFKP and PFKM2, expression of the mRNA and protein level decreased significantly after the ELF siRNA treatment ($^aP < 0.05$ *vs* control). E, F: The hepatic glycolytic enzymes, Glut 1 and MCT 4, expression of the mRNA at the protein level decreased significantly after the ELF siRNA treatment ($^aP < 0.05$ *vs* control). G: The intracellular lactate level decreased significantly after the ELF siRNA treatment ($^aP < 0.05$ *vs* control); H, I: The main components of the extracellular matrix, α-SMA and collagen I, expression showed a remarkable decrease in the activated HSCs, and the HSC transfected synthetic siRNA against ELF.

To confirm this finding, we first evaluated Akt, the key protein of PI3K signaling, in ELF-siRNA treated HSCs. As shown in Figure 5A, the expression of pAkt decreased significantly after the silencing of ELF in activated HSCs, however the total AKT was not influenced by ELF-siRNA treatment. Then, we administered activated HSCs with LY294002, a PI3K/Akt signaling inhibitor, and made the following evaluation. The remarkable downregulation of the pAkt expression confirmed that the PI3K/Akt signaling was well blockaded by LY294002 (Figure 5D). According to the Western blot and RT-qPCR testing, we found that inhibition of the PI3K/akt signaling does not affect the ELF expression (Figure 5B and C). Therefore, the key proteins of glycolysis, PFKP and PKM2, showed a significant decrease in the activated HSCs treated with LY294002 at the mRNA (Figure 5E and G) and protein levels (Figure 5F and H). In addition, SMA and collagen, the main components of the extracellular matrix, were effectively repressed by the LY294002 treatment (Figure 5I and J). Based on these data, we first demonstrated that ELF could regulate pAkt and glycolysis. Moreover, the blockade of PI3K/akt led to the repression of glycolysis and ECM production. The ELF was involved in the HSC glycolysis through the regulation of PI3K/Akt signaling.

DISCUSSION

The destruction of the sinusoidal architecture in hepatic fibrosis always leads to hypoxia of the HSCs around the sinusoids. Simultaneously, the HSCs show a strong ability to activate and proliferate, which requires sufficient energy. The mechanism by which the HSCs ensure sufficient energy for activation and proliferation in the anoxic microenvironment is unknown. As shown in recent research, glycolysis was increased significantly in activated HSCs. Cellular metabolism might be a novel aspect in the pathogenesis study of liver fibrosis. To date, most studies of liver fibrosis

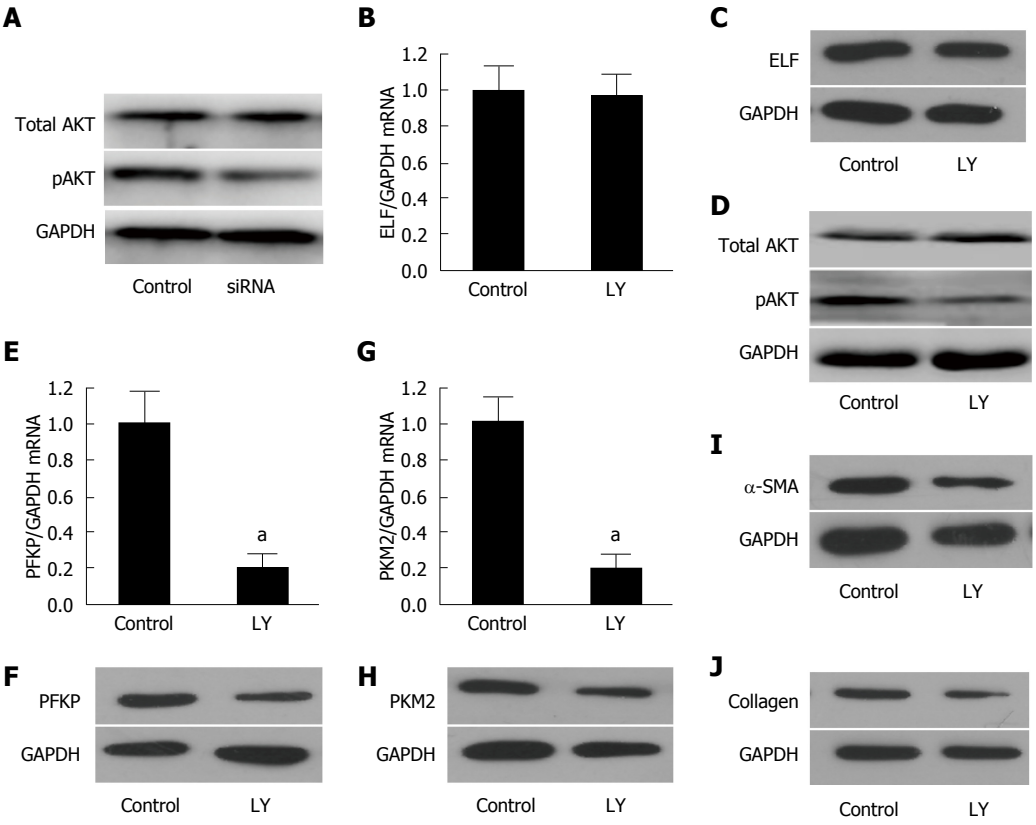

Figure 5 Embryonic liver fordin was involved in the regulation of HSC glycolysis through PI3K/AKT signaling. A: The key protein of PI3K/akt signaling, pAKT, decreased significantly in the embryonic liver fordin (ELF)-siRNA treated hepatic stellate cells (HSCs), but the expression of total AKT was not affected by ELF-siRNA treatment. GAPDH was used as the control; B, C: The Western blot and real-time RT-PCR analysis indicated that the ELF expression was not affected by Ly294002, an inhibitor of the PI3K/akt signaling; D: The pAKT expression decreased obviously after the inhibition of the PI3K/akt signaling in the HSCs; E, F: The hepatic glycolytic enzymes PFKP expression of mRNA and the protein level decreased significantly after the Ly294002 treatment in the activated HSCs ($^aP < 0.05$ *vs* control); G, H: The hepatic glycolytic enzyme PKM2 expression at mRNA and the protein levels decreased significantly after the Ly294002 treatment in the activated HSCs ($^aP < 0.05$ *vs* control). I, J: The main components of the extracellular matrix, α-SMA and collagen I expression showed a remarkable decrease in the activated HSCs treated by the PI3K/akt signaling inhibitor, Ly294002.

focused on HSC activation and ECM production. We showed that energy metabolism, particularly glycolysis, represents an innovative role in HSC activation.

In this study, we found a novel role for ELF in the metabolic process of HSC s activation; the PI3K/Akt pathway was shown to regulate the glycolysis of HSCs. We first found that ELF expression was increased in the HSCs of the fibrosis mouse model and the HSCs cultured for 3 wk *in vitro*. PFKP is the key enzyme in glycolysis and GLU, which showed a significant increase in the activated HSCs compared with the controls. Next, we tested whether increased ELF expression affects glycolysis related-proteins. The silencing of ELF in the activated HSCs leads to decreased expression of glycolysis-related enzymes, such as PFKP, GLUT1, PKM2, and MCT4. Further studies indicated that ELF was involved in HSC glucose metabolism through regulation of pAkt, an important downstream factor in PI3K/Akt signaling.

PI3K signaling, one of the most important pathways that involved various human diseases, provides intense growth and survival signals to many cell types and has profound effects on cellular metabolism[20]. As demonstrated in previous studies, the integration of growth and proliferation signals with alterations to the central metabolism is important for the oncogenic effects of the PI3K pathway[21,22]. Protein kinase B (PKB), also known as Akt, is a serine/threonine-specific protein kinase that plays a key role in multiple cellular processes such as glucose metabolism, apoptosis, cell proliferation, transcription and cell migration. Akt is the best-studied downstream factor of PI3K signaling, and an important driver of the glycolytic phenotype tumor, and stimulates ATP generation, ensuring that cells have the bioenergetic capacity required to respond to growth signals[23]. Akt was able to phosphorylate key glycolytic enzymes such as hexokinase, phosphofructokinase 2, and Glu 1. This function leads to increased expression and membrane translocation of the glucose transporters[24-26].

Abundant evidence has demonstrated that PI3K/Akt pathway is crucial for the proliferation and metabolism of many kinds of cells. Therefore, many studies have suggested that TGF-β is able to activate PI3K/Akt signaling and leads to the subsequent phosphorylation of the downstream genes without an interaction with Smad proteins[27-29]. TGF-β was shown to downregulate PI3K/Akt signaling activity

through Smad proteins[30,31]. Therefore, the PI3K/Akt signaling can also antagonize the Smad-mediated effects in other TGF-β related conditions[32,33]. Our previous studies showed that the activation of PI3K/Akt signaling through insulin stimulation induced the activation of TGF-β/Smad signaling, as indicated by the nuclear translocation of Smad3/4. Moreover, the inhibition of PI3K/Akt signaling by the use of the LY294002 inhibitor led to the blockage of TGF-β/Smad signaling[9]. These findings indicated that the activated PI3k/Akt pathway led to the activation of TGF-β/Smad signaling in the hepatocytes. ELF was shown to play an important role in this process by regulating the localization of Smad3/4 in the nucleus[10]. However, the details underlying the interaction between TGF-β/Smad and PI3K/Akt signaling, particularly in the field of cell energy metabolism, should be investigated in the future.

Multiple intrinsic and extrinsic molecular mechanisms contribute to cellular metabolism and provide support for the three basic needs of dividing cells, as follows: rapid ATP generation to maintain the energy status; increased biosynthesis of macromolecules; and tightened maintenance of appropriate cellular redox status[34,35]. Metabolic changes represent a common feature of all cell types and various diseases including liver fibrosis. This effect is regulated by diverse pathways and factors, such as PI3K signaling, the hypoxia-inducible factor, p53, MYC and AMP-activated protein kinase, liver kinase B1, and the hedgehog pathways[36,37]. Liver fibrosis represents a very complicated process. In recent years, scientists have revealed various pathways that contribute to the pathogenesis of liver fibrosis. Although we showed that PI3K/Akt, which was regulated by ELF, could affect glycolysis in HSCs, other signaling could perform this function. Additional signals that were involved in the regulation of energy metabolism should be investigated in liver fibrosis. Our study could increase the knowledge of the pathogenesis of hepatic fibrosis and might be applied to the diagnosis and treatment of patients.

COMMENTS

Background

The activation of hepatic stellate cells is a key event in pathogenesis of liver fibrosis.

Research frontiers

In recent studies, various factors which were involved in the proliferation, apoptosis, ageing and extracellular matrix secretion were found to be contributive to the activation process of hepatic stellate cells.

Innovations and breakthroughs

This study found that embryonic liver fordin (ELF) was involved in hepatic stellate cells activation by regulating glucose metabolism, which was a novel aspect in the area of liver fibrosis.

Applications

The glucose metabolism of activated hepatic stellate cells provides us a new vision in the diagnosis and therapy of liver fibrosis.

Peer-review

In this study, the authors found that the ELF was involved in the couples of signaling pathways to activate the glucose glycolysis of hepatic stellate cells during the liver fibrosis. The PI3K/Akt signaling and glucose glycolysis-related proteins were evaluated in this study.

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ORIGINAL ARTICLE

Basic Study

Special AT-rich sequence-binding protein 2 acts as a negative regulator of stemness in colorectal cancer cells

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Abstract

AIM

To find the mechanisms by which special AT-rich sequence-binding protein 2 (SATB2) influences colorectal cancer (CRC) metastasis.

METHODS

Cell growth assay, colony-forming assay, cell adhesion assay and cell migration assay were used to evaluate the biological characteristics of CRC cells with gain or loss of SATB2. Sphere formation assay was used to detect the self-renewal ability of CRC cells. The mRNA expression of stem cell markers in CRC cells with upregulated or downregulated SATB2 expression was detected by quantitative real-time polymerase chain reaction. Chromatin immunoprecipitation (ChIP) was used to verify the binding loci of SATB2 on genomic sequences of stem cell markers. The Cancer Genome Atlas (TCGA) database and our clinical samples were

analyzed to find the correlation between SATB2 and some key stem cell markers.

RESULTS

Downregulation of SATB2 led to an aggressive phenotype in SW480 and DLD-1 cells, which was characterized by increased migration and invasion abilities. Overexpression of SATB2 suppressed the migration and invasion abilities in SW480 and SW620 cells. Using sequential sphere formation assay to detect the self-renewal abilities of CRC cells, we found more secondary sphere formation but not primary sphere formation in SW480 and DLD-1 cells after SATB2 expression was knocked down. Moreover, most markers for stem cells such as CD133, CD44, AXIN2, MEIS2 and NANOG were increased in cells with SATB2 knockdown and decreased in cells with SATB2 overexpression. ChIP assay showed that SATB2 bound to regulatory elements of CD133, CD44, MEIS2 and AXIN2 genes. Using TCGA database and our clinical samples, we found that SATB2 was correlated with some key stem cell markers including CD44 and CD24 in clinical tissues of CRC patients.

CONCLUSION

SATB2 can directly bind to the regulatory elements in the genetic loci of several stem cell markers and consequently inhibit the progression of CRC by negatively regulating stemness of CRC cells.

Key words: Special AT-rich sequence-binding protein 2; Colorectal cancer; Stemness; Metastasis

Core tip: We found that special AT-rich sequence-binding protein 2 (SATB2) had a suppressive effect on the tumor growth, adhesion and migration *in vitro*. Moreover, SATB2 could negatively regulate the stemness of colorectal cancer (CRC) cells by directly binding to the regulatory elements in the genetic loci of several stem cell markers. Our study provides a new mechanism for the involvement of SATB2 in CRC progression and helps us to better understand the metastasis traits of cancer stem cells.

Li Y, Liu YH, Hu YY, Chen L, Li JM. Special AT-rich sequence-binding protein 2 acts as a negative regulator of stemness in colorectal cancer cells. *World J Gastroenterol* 2016; 22(38): 8528-8539 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i38/8528.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i38.8528

INTRODUCTION

Special AT-rich sequence-binding protein 2 (SATB2) is an important DNA-binding protein involved in transcriptional regulation and chromatin remodeling. SATB2 plays key roles in osteoblastic differentiation, cortical neuron differentiation and skeletal development[1-4]. However, the role of SATB2 in cancer initiation and progression is still not well-understood. We previously found that SATB2 was a potential marker for metastasis of colorectal cancer (CRC) and low expression of SATB2 was correlated with tumor progression and poor prognosis in CRC patients[5]. Interestingly, more evidence shows that SATB2 is involved in progression of breast cancer, head and neck squamous cell carcinomas and osteosarcoma[6-8]. Importantly, SATB2 is strongly expressed in normal colorectal and appendiceal epithelium[9], demonstrating SATB2 as a diagnostic marker for CRC.

Recently, SATB2 and its analogue protein SATB1 are found to regulate embryonic stem cell differentiation by directly binding with NANOG genomic locus[10]. Renew and differentiation of trophoblast stem cells are also considered to be related with SATB proteins[11]. In the process of cancer development and progression, very few cancer cells with stem cell-like properties have greatly enhanced tumor-initiating potential within a tumor and these cells are termed as cancer stem cells (CSCs)[12,13]. However, the regulatory mechanisms of CSCs and relations between CSCs and cancer metastasis are still needed to be elucidated.

In this study, we found that SATB2 had a suppressive effect on the tumor growth, adhesion, and migration *in vitro*. Moreover, SATB2 could negatively regulate the stemness of CRC cells by directly binding to the regulatory elements in the genetic loci of several stem cell markers. Our study provides a new mechanism for the involvement of SATB2 in CRC progression and helps better understand the metastasis traits of CSCs.

MATERIALS AND METHODS

Cell lines

The CRC cell lines used in our experiments were bought from the Cell Bank at the Chinese Academy of Sciences. The cells were cultured in RPMI-1640 medium in which fetal bovine serum (Hyclone, United States) was added to a final concentration of 10%. The cells were sustained in an incubator with 5% CO_2 at 37 ℃.

Plasmid and lentivirus preparation

The information of pCAG-SATB2 vector and its control vector had been mentioned in our previous paper[5]. The pLKO.1-TRC vectors with different interference fragments targeting SATB2 were purchased from Thermo Scientific (United States, Item No. TRCN0000020684 to TRCN0000020688).

Table 1 Primer sequences used in quantitative real-time polymerase chain reaction for detection of stem cell marker mRNA expression

Gene	Sequence
CD133-F	5′ TTTGTCTTCTATTCTTGGCTTC 3′
CD133-R	5′ ACCTTGTCATAATCAATTTTGG 3′
CD44-F	5′ GGTTCATAGAAGGGCACGT 3′
CD44-R	5′ TGTCTTCGTCTGGGATGG 3′
CD24-F	5′ TCAAGTATTTGGGAAGTG 3′
CD24-R	5′ GTGTTCTAAATGTGGCTAT 3′
OCT3/4-F	5′ CGACCATCTGCCGCTTTGAG 3′
OCT3/4-R	5′ CCCCCTGTCCCCCATTCCTA 3′
KLF4-F	5′ TGGGTCTTGAGGAAGTGCTG 3′
KLF4-R	5′ TGTTTACGGTAGTGCCTGGTC 3′
SOX2-F	5′ CACCTACAGCATGTCCTACTC 3′
SOX2-R	5′ CATGCTGTTTCTTACTCTCCTC 3′
PRL-1-F	5′ GGCAAACTTCGAGTCTCCT 3′
PRL-1-R	5′ CCGGTTGATGAATGGCTAA 3′
MEIS2-F	5′ GTCCACGAACTGTGCGATAA 3′
MEIS2-R	5′ TTCGGAAGGGTACGGATG 3′
AXIN2-F	5′ AGCATTTTAATCAACAGCATCTA 3′
AXIN2-R	5′ TAACTAAGAATGTGATCCAAGAA 3′
NANOG-F	5′ CAACTGGCCGAAGAATAGCA 3′
NANOG-R	5′ GCAGGAGAATTTGGCTGGAA 3′
GAPDH-F	5′ GGAGCGAGATCCCTCCAAAAT 3′
GAPDH-R	5′ GGCTGTTGTCATACTTCTCATGG 3′

Transfection and lentiviral transduction

The pCAG-SATB2 was transfected into CRC cell lines to establish cells in which SATB2 expression was upregulated. The packaged virus with pLKO.1-TRC vectors were used to establish cells with stably downregulated expression of SATB2.

Quantitative real-time polymerase chain reaction

The mRNA expression levels of SATB2 and stem cell markers in CRC cell lines were measured by quantitative real-time polymerase chain reaction (qRT-PCR) using SYBR Green (Takara, China) run in a 7500 real-time PCR system (ABI, United States). Expression levels of SATB2 and stem cell markers were evaluated using the ΔΔCt method and normalized according to the mRNA level of GAPDH. Primer sequences for qRT-PCR are listed in Table 1.

Western blot analysis

Protein extracts were obtained using the lysis buffer (KeyGen Biotech). After being quantified, equivalent amounts of protein extracts were separated using SDS-PAGE and transferred to the PVDF membrane (Roche Applied Sciences). The primary antibody was added onto each membrane and incubated at 4 ℃ overnight. The appropriate second antibody was used on the following day. Mouse monoclonal anti-SATB2 antibody (1:100, Abcam, United Kingdom), rabbit polyclonal anti-CD133 antibody (1:500, Abnova, China) and mouse monoclonal anti-α-tubulin antibody (1:1000, proteintech, United States) were used. The targeted bands were visualized and photographed with the FluorChem system (Alpha Innotech).

Cell growth assay

Cell aliquots (100 μL) were transferred into each well of 96-well microtiter plates at a concentration of 1 × 10^4 cells/mL. CCK-8 (Dojindo Laboratories, Japan) was used to test the cell proliferation ability every 24 h and would last 6 d. Each day we added 10 μL of CCK-8 reagents into each well and then incubated the plates at 37 ℃ for 2 h. After the incubation, the absorbance of each well was measured at 450 nm using the Vmax microplate spectrophotometer (Molecular Devices, CA).

Colony-forming assay

Cells (1 × 10^2) were seeded into each well of 6-well culture plates and incubated at 37 ℃ for 14 d. Then the cells were stained with crystal violet solution and the pictures of stained cells were taken with a digital camera. Under a microscope, the colonies containing more than 50 cells were counted. The colony formation efficiency of each group was calculated as the colony number divided by inoculated cell number and then multiplied by 100%.

Cell adhesion assay

Fibronectin (Invitrogen, United States) was added into each well of 96-well plates at a concentration of 10 μg/mL and plates were incubated overnight at 4 ℃. After that, 1% BSA was added and incubated at 37 ℃ for 1 h. Then diluted cells (1 × 10^5 cells/100 μL) were added to the coated wells and incubated at 37 ℃ for 1 h. After the non-adherent cells were washed out, we added CCK-8 reagent into each well and the plates were incubated at 37 ℃ for 2 h. The absorbance of each well was measured at 450 nm.

Cell migration assay

Transwells (BD Biosciences, United States) inserted with 8 μm pores were put into wells of 24-well plates. Cells were suspended with serum free medium and 2 × 10^5 cells were added inside the chamber. Below the matched chamber, 600 μL of RMPI-1640 medium containing 10% FBS was added. After incubation for 24 h, noninvasive cells on the membrane inside the transwell were removed. Invaded cells were fixed with methanol, stained with Giemsa or crystal violet and photographed.

Immunofluorescence analysis

CRC cells were stained with CD133 antibody as mentioned previously. Then the goat anti-rabbit secondary antibody conjugated with Alexa Fluor 594 (ZSGB-Bio, China) was used. DAPI was used to counterstain nuclei. The fluorescence was scanned and photographed with a confocal laser scanning microscope (Olympus, Japan). The average fluorescence

Table 2 Primer sequences used in polymerase chain reaction for detecting genomic binding sites for stem cell markers

Gene	Sequence
CD133-F	5′ TTTGTCTTCTATTCTTGGCTTC 3′
CD133-R	5′ ACCTTGTCATAATCAATTTTGG 3′
CD44(1)-F	5′ CTCATGGCTCAGTCGCCCAATCA 3′
CD44(1)-R	5′ TTTGCTCCTGAGCTGTTGCGTGG 3′
CD44(2)-F	5′ AGATTAAGGAGCTAGGACTC 3′
CD44(2)-R	5′ AAGATCACTTGGCAAGAAAG 3′
CD44(3)-F	5′ GGCACGTGTGAAACCTTTCCATTC 3′
CD44(3)-R	5′ GCTGAGCTGGACGCCAAGCA 3′
CD44(4)-F	5′ GCCTTTCATCCCTCGGGTGTGC 3′
CD44(4)-R	5′ TTCCTCCCAGGGACCAGGCC 3′
MEIS2(1)-F	5′ GGATTCCTGGCCAAAGGACGC 3′
MEIS2(1)-R	5′ CTCCCCCTAAGAGCGGCTCCA 3′
MEIS2(2)-F	5′ ACTGCCCGCAAGGATTCCACAA 3′
MEIS2(2)-R	5′ GGACTGTGGACCAAATCCAGCACAG 3′
AXIN2-F	5′ TATTCAAGGCATCTTTTACTGGAC 3′
AXIN2-R	5′ AGCAAAGAACTAGCCAATAAGGAG 3′

intensity was calculated with Image J software.

Sphere formation assay

The low attachment plates (Corning Incorporated, United States) were used to culture cells using serum-free medium according to a previous study[14]. We prepared the serum-free medium for sphere culturing by adding 10 μg of EGF, 5 μg of LIF and 10 μg of bFGF (Invitrogen, United States) into 500 mL of DMEM/F12 medium. Cells were cultured in the 24-well ULLA plates at a density of 5000 or 10000 cells/well for 1 wk. Spheres (> 50 μm) were counted using an immunofluorescent microscope (Olympus, Japan).

Chromatin immunoprecipitation

SW480 cells were cultured and harvested. Following procedures were provided by chromatin immunoprecipitation (CHIP-IT) Express Enzymatic and Enzymatic shearing Kit (Active Motif). Mouse monoclonal anti-SATB2 antibody (Abcam, Cambridge, United Kingdom) was used. The positive and negative control antibodies were provided in CHIP-IT control (Active Motif). The immunoprecipitated DNA was amplified by PCR. The primer sequences for PCR are listed in Table 2.

Correlation analysis using The Cancer Genome Atlas database and clinical samples of CRC patients

RNA-Seq expression data (combining level 3 data from IlluminaGA_RNASeqV2 platforms) from CRC patients were downloaded from The Cancer Genome Atlas (TCGA), which had been analyzed in Cancer Browser (https://genome-cancer.ucsc.edu/). Correlations between SATB2 and stem cell markers were analyzed according to data from these clinical samples.

We collected 68 fresh samples from CRC patients operated from March to April in 2010 at Nanfang Hospital. Among them, there were 45 males and 23 females. The average age was 63.77 ± 16.22 years. We collected the tumor tissue and its adjacent normal tissue. Then the tissues were preserved in liquid nitrogen and RNA was extracted and analyzed subsequently.

Statistical analysis

SPSS V.13.0 statistical software package was used to perform all statistical analyses. The Student's *t*-test was used to compare two groups of independent samples. One-way ANOVA was used to analyze differences among multiple groups and differences between groups were analyzed by LSD pairwise comparison. Pearson correlation analysis was used to calculate the correlation between SATB2 and stem cell markers. $P < 0.05$ was considered to be statistically significant for all the analyses.

RESULTS

Overexpression of SATB2 inhibits the proliferation and migration of CRC cells in vitro

SATB2 was successfully overexpressed in SW480 and SW620 cells both at mRNA (SW480, $P < 0.001$; SW620, $P < 0.001$; Figure 1A) and protein (SW480, $P < 0.05$; SW620, $P < 0.01$; Figure 1B) levels. CCK-8 cell proliferation assay showed that overexpression of SATB2 inhibited cell proliferation in SW480 ($P < 0.001$) and SW620 ($P < 0.001$) cells (Figure 1C). Moreover, the colony formation assay indicated that cells with SATB2 overexpression had a deceased formation of colonies compared with control cells (SW480, $P < 0.001$; SW620, $P < 0.01$; Figure 1D). A significant decrease in cell migration was showed in CRC cells after the exogenous expression of SATB2 (SW480, $P < 0.001$; SW620, $P < 0.001$; Figure 1E).

Knockdown of SATB2 promotes adhesion, colony-formation and migration of CRC cells in vitro

To further confirm the effect of SATB2 on the biological properties of CRC cells, we used the pLKO.1-TRC system with shRNA interference targeting SATB2 to produce virus to knock down SATB2 expression in CRC cells. The lentiviruses with different shRNAs targeting SATB2 were tested in SW480, SW620 and DLD-1 cells for optimal selection. The lentivirus with shRNA#1 targeting SATB2 had the optimal efficiency to knock down SATB2 expression in three tested CRC cell lines (SW480, $P < 0.001$; SW620, $P < 0.01$; DLD-1, $P < 0.01$) and was then used to establish the cell lines with SATB2 stable knockdown (Figure 2A). Single cells were isolated from the cells infected by the lentivirus with shRNA#1 targeting SATB2 and cultured for 2 wk to establish clones with SATB2 stable knockdown (Figure 2B). SW480/clone7 ($P < 0.001$) and DLD-1/clone5 ($P < 0.01$) were used in our next experiments. In

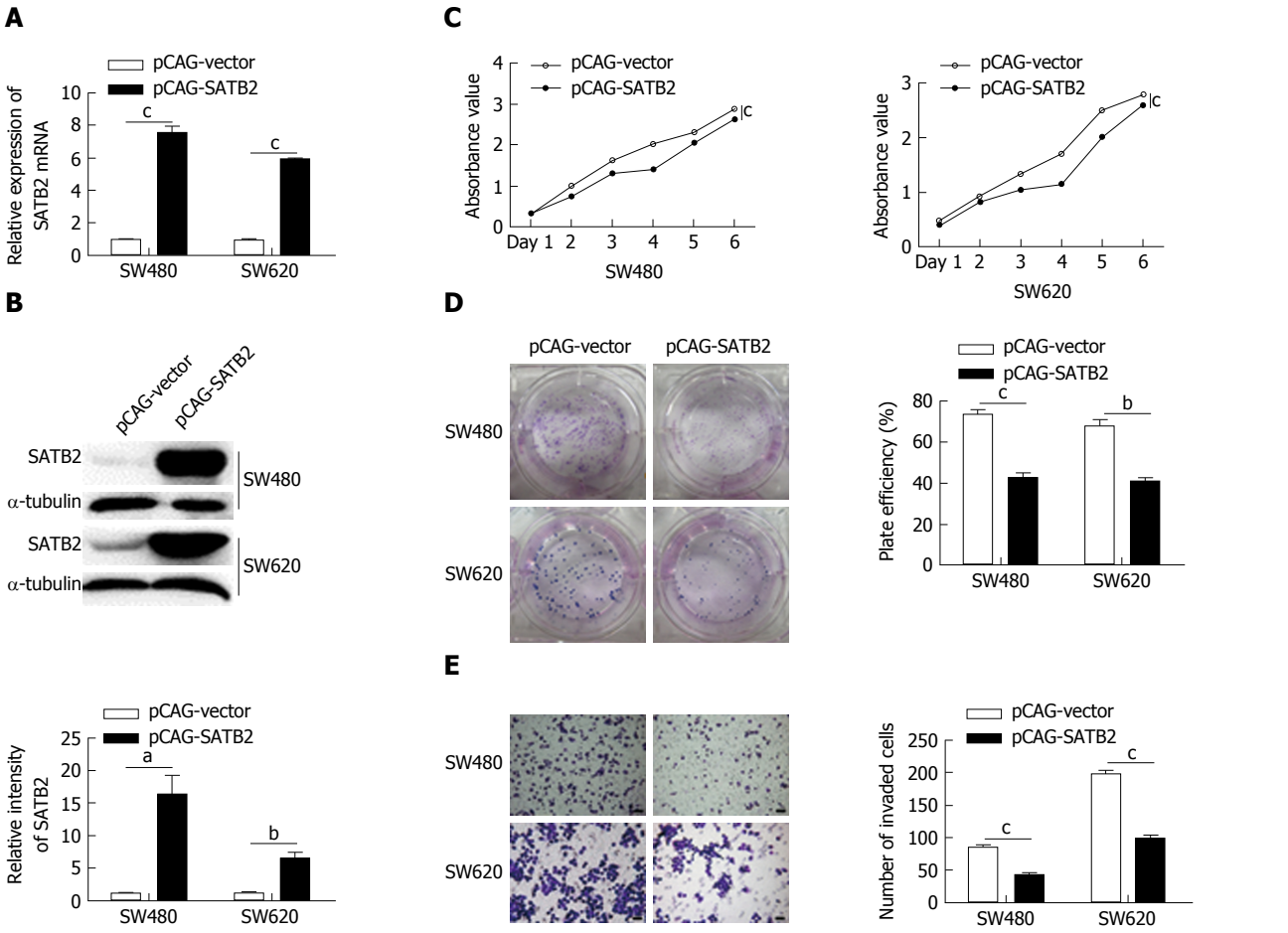


Figure 1 Overexpression of special AT-rich sequence-binding protein 2 inhibits the proliferation and migration of colorectal cancer cells *in vitro*. A, B: Expression levels of SATB2 in SW480 and SW620 cells transfected with pCAG-SATB2 were increased whenever detected by qRT-PCR (A) or Western blot (B); C: The proliferation abilities of cells with SATB2 overexpression were detected to have a decrease in CCK-8 cell proliferation assay. The *P*-values of time effect, group effect and their interaction effect were all below 0.001 in SW480 and DLD-1 cells; D: Colony formation assay was used to analyze ability of clone formation in SATB2 overexpressing cells. Cells with SATB2 overexpression formed less clones; E: Cell migration capacities of control cells and SATB2 overexpressing cells were compared by detecting the invaded cell numbers in transwell chambers. Fewer invaded cells were found in SATB2 overexpressing cells. Scale bar is 50 μm. Data shown are mean ± SEM. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* control.

contrast to our previous results, enhanced adhesion ability (SW480, $P < 0.001$; DLD-1, $P < 0.001$; Figure 2C), colony-forming capacity (SW480, $P < 0.05$; DLD-1, $P < 0.01$; Figure 2D) and migration ability (SW480/shRNA#1, $P < 0.05$; DLD-1/shRNA#1, $P < 0.001$; SW480/clone7, $P < 0.001$; DLD-1/clone5, $P < 0.001$; Figure 2E and F) were found in SW480 and DLD-1 cells after SATB2 was downregulated.

SATB2 knockdown enhances secondary sphere formation of CRC cells in vitro

In our previous studies, we found that SATB2 expression was closely correlated with tumor invasion, lymph node metastasis, distant metastasis and Dukes' classification in CRC patients[5]. Further, we found that SATB2 overexpression inhibited the proliferation and migration of CRC cells while knockdown of SATB2 promoted adhesion, colony-formation and migration of CRC cells *in vitro*. There is a subpopulation of CSCs that contributes to the biological traits of high-grade malignancy[15-17]. The CSCs were possibly the main cause of new tumor formation and tumor metastasis. We checked whether SATB2 could influence phenotype of stemness of CRC cells. As we know, self-renewal is one of the basic characteristics of stemness of CRC cells. So we observed the self-renewal of CRC cells using sequential sphere formation assay. Nevertheless, SATB2 knockdown had no effect on primary sphere formation in CRC cells (Figure 3A). Interestingly, more secondary sphere formation was found in SW480 and DLD-1 cells after SATB2 expression was knocked down (SW480/shRNA#1, $P < 0.05$; DLD-1/shRNA#1, $P > 0.05$; SW480/clone7, $P < 0.05$; DLD-1/clone5, $P < 0.05$; Figure 3B), indicating that SATB2 repressed the self-renewal ability of CRC cells.

SATB2 knockdown increases the expression of several markers for CSCs in CRC cells in vitro

We found that SATB2 knockdown enhanced secondary sphere formation of CRC cells *in vitro*. It is logically

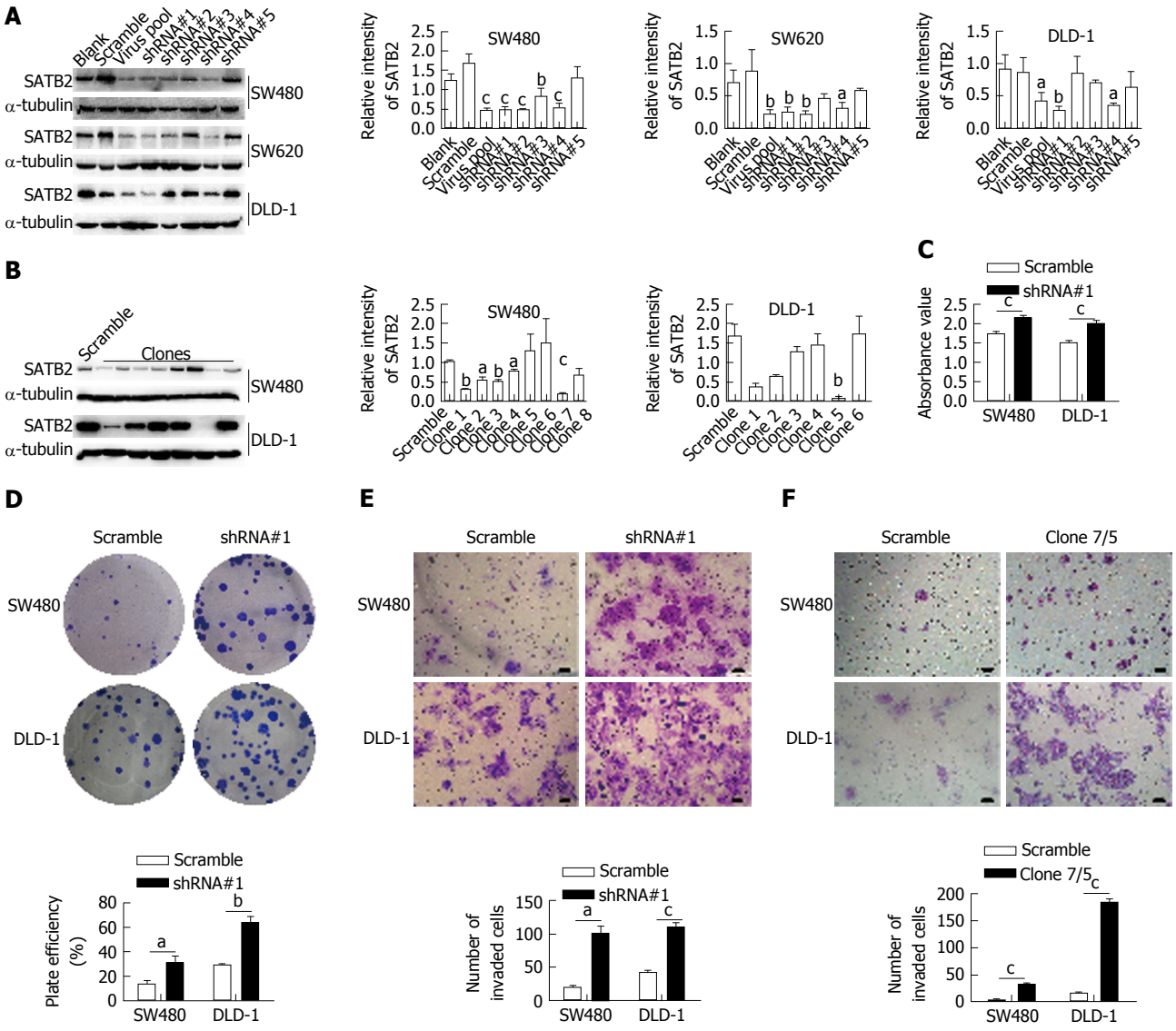


Figure 2 Knockdown of special AT-rich sequence-binding protein 2 promotes adhesion, colony-formation and migration of colorectal cancer cells *in vitro*. A: SATB2 expression levels in cells infected by virus with different shRNAs targeting SATB2 were detected by Western blot. shRNA named shRNA#1 had the best effect in silencing SATB2 expression in SW480, SW620 and DLD-1 cells; B: Cells in which SATB2 was stably knocked down were isolated into single cells and single cells were cultured for 2 wk to form clones. Then we detected SATB2 expression in different clones by Western blot. We chose SW480/clone7 and DLD-1/clone5 cells to be used in the following assays; C: Adhesion capabilities of cells infected by shRNA#1 virus and its control virus were compared by detecting the cells' absorbance values to reflect the numbers of adhered cells. Cells with low SATB2 expression had increased adhesion capabilities; D: Abilities of clone formation of CRC cells with stably reduced expression of SATB2 by an infection of shRNA#1 virus were detected to have an increase in colony formation assay; E and F: Transwell chambers were used to detect migration ability of cells with stably reduced expression of SATB2 by an infection of shRNA#1 virus (E) or by culturing the single cells isolated from shRNA#1 virus infected cells to clones (F) and both groups with low SATB2 expression had increased cell migration abilities. Scale bar is 50 μm. Data shown are mean ± SEM. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* control.

supposed that SATB2 may affect the expression of markers of CSCs as a key transcriptional factor which controls gene expression. Therefore, we detected the mRNA expression of several key markers of CSCs, such as CD133, CD44, AXIN2, MEIS2 and NANOG, by qRT-PCR in CRC cells with gain or loss of SATB2 expression. Accordingly, most markers for stem cells were increased in cells with SATB2 knockdown and decreased in cells with SATB2 overexpression (Figure 4A and B), especially CD133 (SW480/pCAG-SATB2, $P < 0.05$; DLD-1/pCAG-SATB2, $P < 0.001$; SW480/clone7, $P < 0.001$; DLD-1/shRNA#1, $P < 0.05$; DLD-1/clone5, $P < 0.001$), CD44 (SW480/pCAG-SATB2, $P < 0.05$; HCT-116/pCAG-SATB2, $P < 0.05$; DLD-1/pCAG-SATB2, $P < 0.05$; SW480/clone7, $P < 0.01$; DLD-1/clone5, $P < 0.001$) and PRL1 (HCT-116/pCAG-SATB2, $P < 0.01$; SW480/shRNA#1, $P < 0.01$; SW480/clone7, $P < 0.01$; DLD-1/shRNA#1, $P < 0.01$; DLD-1/clone5, $P < 0.01$). Specifically, CD133 expression was further analyzed by Western blot (SW480, $P < 0.05$; SW620, $P < 0.001$; DLD-1, $P < 0.05$) and immunofluorescent staining (SW480, $P < 0.001$; SW620, $P < 0.001$; DLD-1, $P < 0.001$). And CD133 expression was increased in CRC cells after SATB2 was knocked down

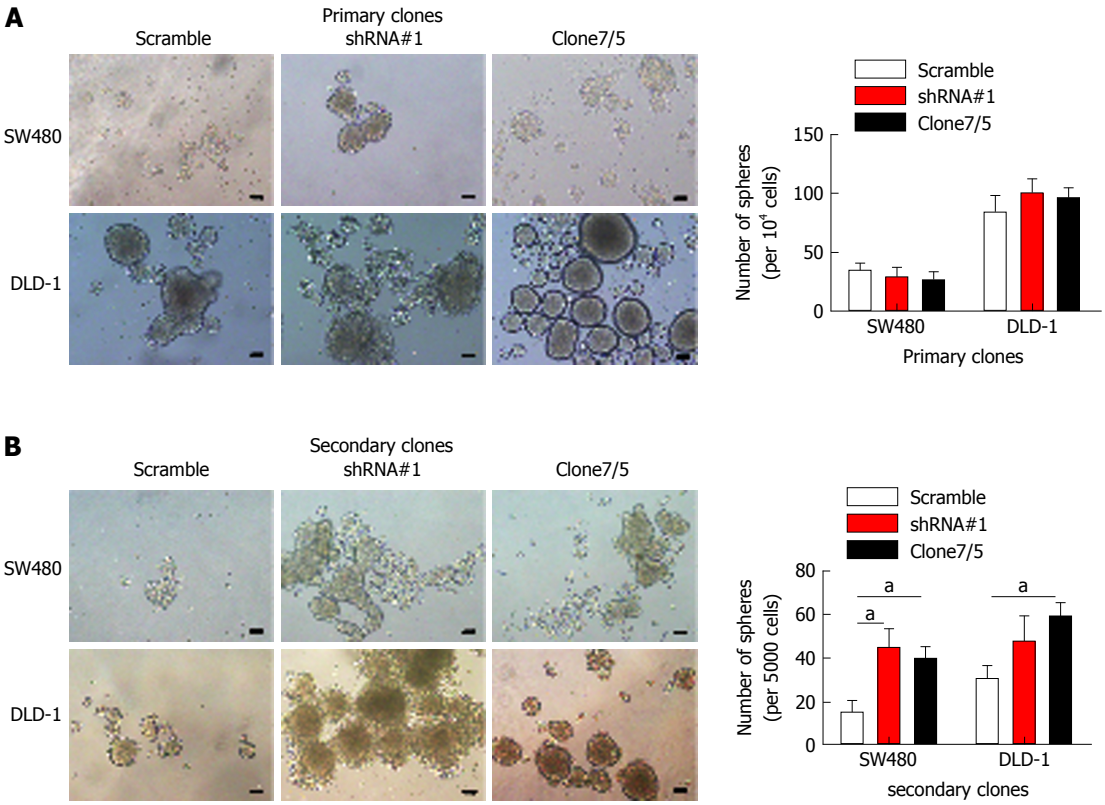


Figure 3 Special AT-rich sequence-binding protein 2 knockdown enhances secondary sphere formation of colorectal cancer cells *in vitro*. A: Self-renewal abilities of CRC cells in which SATB2 was stably knocked down were evaluated by sequential sphere formation assay; B: Spheres formed primarily were isolated into single cells and then equal amounts of cells were cultured to form secondary spheres. More secondary spheres were formed in SATB2 downregulated cells in SW480 and DLD-1. Scale bar is 50 μm. Data shown are mean ± SEM. $^aP < 0.05$ *vs* control.

(Figure 4C and D).

SATB2 binds to regulatory elements of CD133, CD44, MEIS2 and AXIN2 genes

As a transcriptional factor, SATB2 may affect gene expression of stem cell markers by directly binding to regulatory elements of those genes. We used the Genomatix online software to find the possible SATB2 binding loci of those stem cell marker genes. We found that SATB2 may bind to regulatory elements of CD133 (Figure 5A), CD44 (Figure 5B), MEIS2 (Figure 5C) and AXIN2 (Figure 5D), at single or multiple sites. Then, we employed ChIP, followed by PCR, to test whether SATB2 could bind to regulatory elements of these genes. Chromatin fragments were prepared from SW480 cells. Mouse monoclonal anti-SATB2 antibody was used to precipitate the needed chromatin. Anti-RNA pol II and anti-IgG antibodies were used as positive and negative controls separately. Our results indicated that regulatory elements of CD133 (Figure 5A), CD44 (Figure 5B), MEIS2 (Figure 5C) and AXIN2 (Figure 5D) contained SATB2-binding sequences.

SATB2 is correlated with some key stem cell markers including CD44 and CD24 in clinical tissues of CRC patients

To further analyze the correlation between SATB2 and some key stem cell markers, RNA-Seq expression data in clinical samples of CRC from TCGA (http://cancergenome.nih.gov/) were used. Using data from TCGA, we found that SATB2 was negatively correlated with expression of CD44 ($P < 0.001$), CD26 ($P < 0.001$), CD166 ($P < 0.01$), CD29 ($P < 0.001$) and KRT19 ($P < 0.01$) and positively correlated with expression of CD24 ($P < 0.001$) and LGR5 ($P < 0.01$) (Figure 6A). Significantly, in our clinical CRC tissues, we further confirmed that SATB2 was positively correlated with CD24 ($P < 0.001$) expression (Figure 6B). However, the correlation between SATB2 and CD133 was marginal for significance analysis both in TCGA data ($P = 0.072$, Figure 6A) and our own clinical samples of CRC ($P = 0.052$, Figure 6B), suggesting that limited samples were included in both studies.

DISCUSSION

In our previous studies, SATB2 has been found to be a potential novel prognostic factor for CRC because of its strong correlation with local invasion, lymph node metastasis and distant metastasis in CRC[5]. After then, more evidence has confirmed SATB2 as a useful marker for CRC metastasis[18-21]. Even so, the mechanisms by which SATB2 is involved in CRC metastasis are still largely unclear.

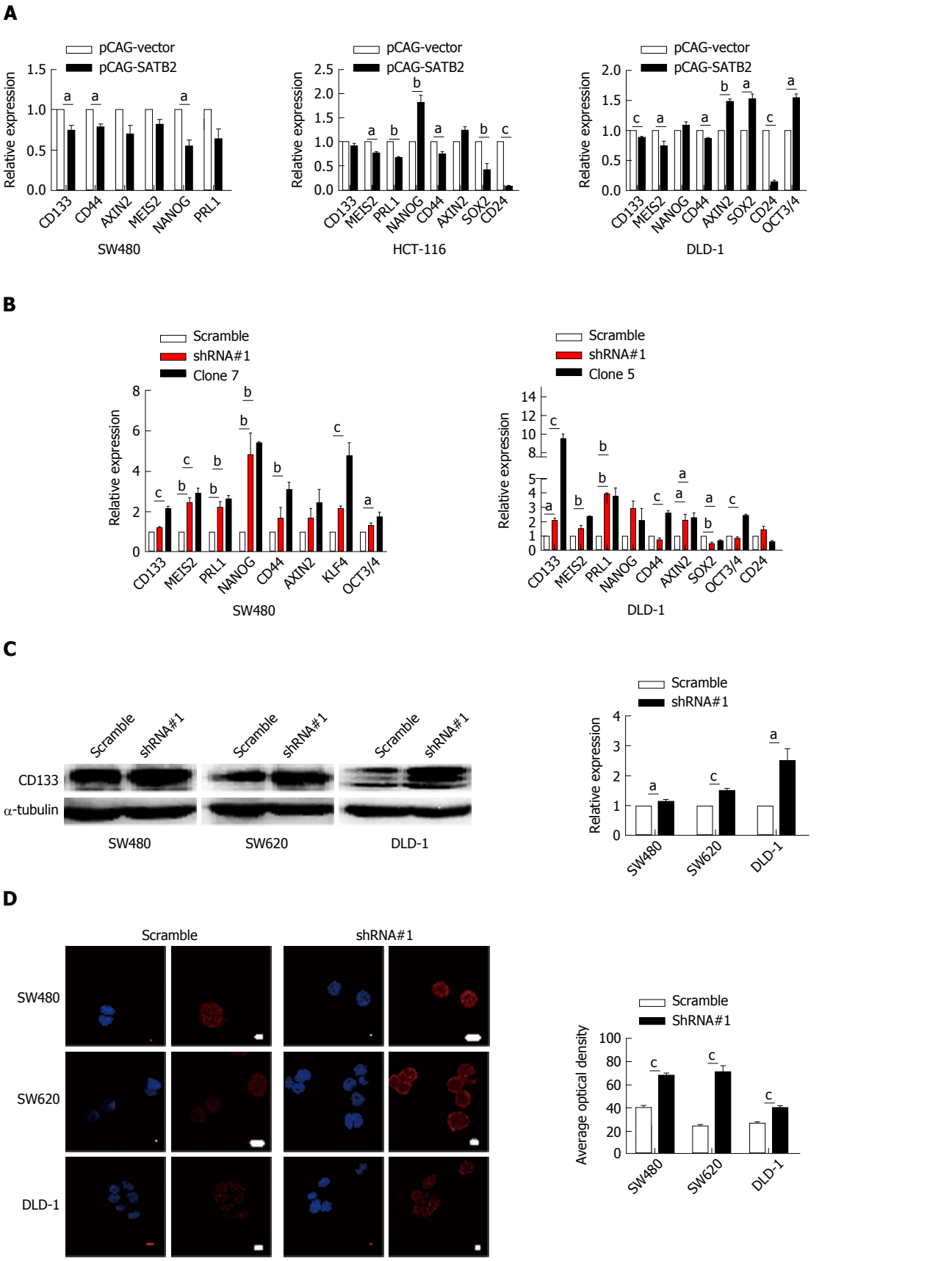


Figure 4 Special AT-rich sequence-binding protein 2 knockdown increases the expression of several markers for cancer stem cells in colorectal cancer cells *in vitro*. A-B: The mRNA expression of a series of stem cell markers was detected in SATB2 overexpressing cells (A) and SATB2 knockdown cells (B) by qRT-PCR; C-D: CD133 expression level in cells with stable SATB2 knockdown was increased when detected by Western blot (C) and immunofluorescence (D). Scale bar is 10 μm. Data shown are mean ± SEM. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, *vs* control.

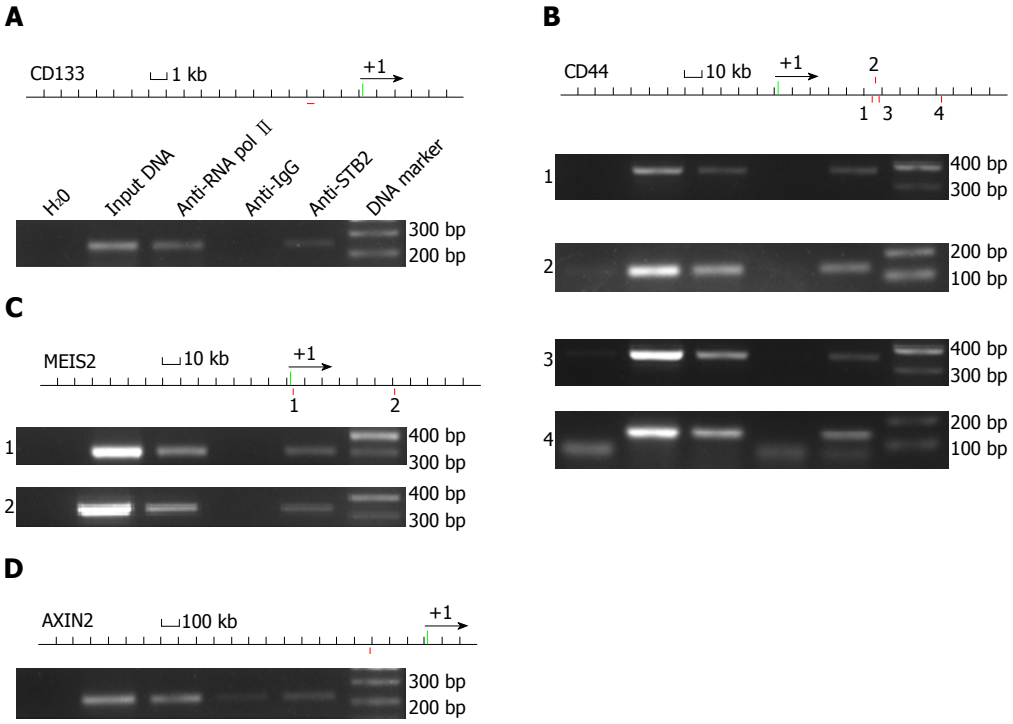


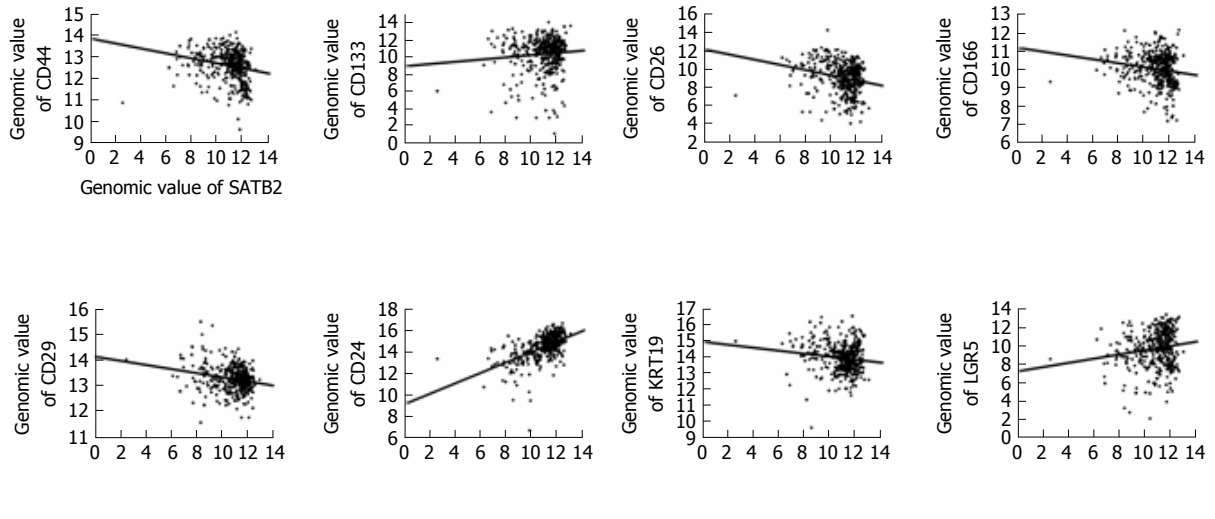
Figure 5 Special AT-rich sequence-binding protein 2 binds to regulatory elements of CD133, CD44, MEIS2 and *AXIN2* genes. A-D: ChIP was carried out with anti-SATB2 antibody, anti-RNA pol II antibody and anti-IgG antibody. Input DNA and immunoprecipitated DNA by anti-RNA pol II antibody were used as positive controls. Immunoprecipitated DNA by anti-IgG antibody and H_2O were used as negative controls. PCR was used to find out the genomic binding sites for each gene. Red squares under the genomic sequence mean the predicted Satb2-binding loci. Green boxes mean the exons for each genomic sequence. Black arrows mean the translation initiation sites. As expected, SATB2 bound CD133 (A), CD44 (B), MEIS2 (C) and AXIN2 (D) at their regulatory elements.

Here, we found that SATB2 was a tumor suppressor in CRC. Gain-of-function studies showed that overexpression of SATB2 inhibited the proliferation and migration of CRC cells *in vitro*. Meanwhile, loss-of-function studies indicated that knockdown of SATB2 promoted adhesion, colony-formation and migration of CRC cells *in vitro*. These results are consistent with our clinical data, supporting the importance of SATB2 in tumor metastasis in CRC.

We also discovered that SATB2 was a negative regulator of stemness in CRC cells. At present, many solid tumors, including brain, colon, lung, breast, liver, prostate and bladder cancers, have been identified to have CSCs[13,22-26]. CSCs, commonly identified by their cell-surface-marker expression, have self-renewal and tumor-initiating ability and account for cancer relapse and metastasis[27]. Self-renewal ability of CSCs, also called stemness, is one of the basic characteristic of CSCs. Using primary and secondary sphere formation assay to detect self-renew of CRC cells, we found that SATB2 knockdown enhanced secondary sphere formation of CRC cells *in vitro*. Consistently, expression of CD133, NANOG and CD44, the key markers for CRC, was significantly increased when SATB2 was stably knocked down in CRC cells. Meanwhile, CD133 and CD44 were downregulated when SATB2 was overexpressed in CRC cells. As a transcriptional factor, SATB2 may regulate gene expression by directly binding to the regulatory elements of these stemness genes. NANOG was found to be directly regulated by SATB2 because of the binding to its promoter region[10]. In our study, Genomatix, an online web-based bioinformatic system, was first used to predict the potential genetic locus which might be recognized and bound by SATB2. We found that SATB2 could bind to one or more regulatory elements of CD133, CD44, MEIS2 and AXIN2. Importantly, ChIP assay confirmed that SATB2 could bind directly to the regulatory elements of CD133, CD44, MEIS2 and AXIN2. Interestingly, in TCGA clinical database and our clinical data of CRC, SATB2 was correlated with expression of several stem cell markers such as CD44 and CD24. Much more evidence is needed to explore the precise mechanisms by which SATB2 regulates stemness of cancer cells.

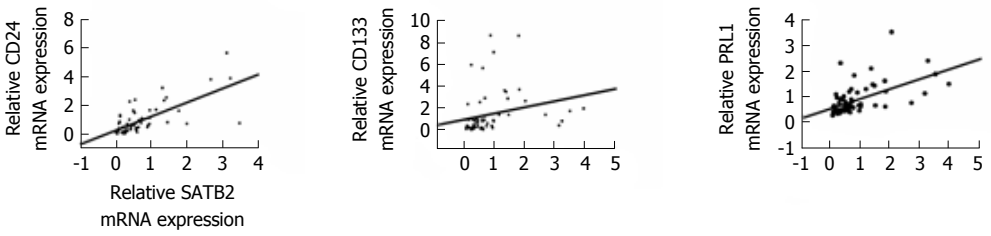
In conclusion, our studies found that SATB2 could directly bind to the regulatory elements in the genetic loci of several stem cell markers and consequently inhibit the progression of CRC by negatively regulating stemness of CRC cells.

A



Genes	Pearson correlation	Sig. (2-tailed)	Genes	Pearson correlation	Sig. (2-tailed)
CD44	-0.218	4.59×10^{-6}	CD29	-0.216	5.70×10^{-6}
CD133	0.087	0.072	CD24	0.561	3.05×10^{-37}
CD26	-0.224	2.38×10^{-6}	KRT19	-0.139	0.004
CD166	-0.163	0.001	LGR5	0.155	0.001

B



Genes	Pearson correlation	Sig. (2-tailed)
CD24	0.702	4.26×10^{-10}
CD133	0.248	0.052
PRL1	0.564	1.01×10^{-6}

Figure 6 Special AT-rich sequence-binding protein 2 is correlated with some key stem cell markers including CD44 and CD24 in clinical tissues of colorectal cancer patients. A: The correlations between SATB2 and stem cell markers were analyzed by Pearson correlation analysis according to RNA-Seq expression data of 434 primary colorectal tumors from TCGA. The matched scatter grams and the statistical *P*-values for different genes are showed separately; B: The correlations between SATB2 and stem cell markers were analyzed by Pearson correlation analysis according to the mRNA expression level of genes in 68 cases of colorectal cancer (CRC) tissues collected from NanFang hospital. The matched scatter grams and the statistical *P*-values for different genes are showed separately.

COMMENTS

Background

Special AT-rich sequence-binding protein 2 (SATB2) is a key factor for transcriptional regulation and chromatin remodeling. Previously, the authors found that decreased expression of SATB2 was correlated with metastasis in colorectal cancer (CRC). Unfortunately, how SATB2 influences CRC metastasis is still unclear.

Research frontiers

SATB2 and its analogue protein SATB1 are found to regulate embryonic stem cell differentiation by directly binding with NANOG genomic locus. Cancer stem cells (CSCs) with stem cell-like properties have been reported to enhance greatly tumor-initiating potential within a tumor. This implies the possible relationship between SATB2 and CSCs.

Innovations and breakthroughs

This is the first study to report the relationship between SATB2 and stemness of CRC cells. And our study provides a new mechanism for the involvement of SATB2 in CRC progression.

Applications

SATB2 inhibits the progression of CRC by negatively regulating stemness of CRC cells, which provides a new possible therapy for CRC patients.

Terminology

SATB2 is a protein that binds AT sequence on the targeted genes to regulate their transcription. Low expression of SATB2 has been reported in CRC tissues. SATB2 expression was closely correlated with tumor invasion, lymph node metastasis, distant metastasis and Dukes' classification in CRC patients. This predicts a possible role of SATB2 in regulating tumor metastasis.

Peer-review

This is a very interesting and may be a useful future technique. CRC is a leading cancerous disease affecting many people, therefore every method that could predict influencing factors on metastasis is very important for choosing the correct treatment or even follow-up schedule.

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ORIGINAL ARTICLE

Case Control Study

Association between gastrointestinal symptoms and affectivity in patients with bipolar disorder

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Author contributions: Karling P and Norrback KF constructed the study design, contributed in the acquisition of the data, analyzed the data, interpreted the data and wrote the manuscript; Maripuu M and Wikgren M constructed the study design, interpreted the data and wrote the manuscript; Adolfsson R constructed the study design, contributed in the acquisition of the data, interpreted the data and wrote the manuscript; all authors did a final approval of the version to be published.

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Clinical trial registration statement: The study was not registered at URL.

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Data sharing statement: Dataset available from the corresponding author at pontus.karling@umu.se.

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Abstract

AIM

To study if anxiety, depression and experience of stress are associated with gastrointestinal (GI) symptoms in patients with bipolar disorder.

METHODS

A total of 136 patients with bipolar disorder (mean age 49.9 years; 61% women) and 136 controls from the general population (mean age 51.0 years; 60% women) were included in the study. GI symptoms were assessed with The Gastrointestinal Symptom Rating Scale-irritable bowel syndrome (GSRS-IBS), level of anxiety and depression with The Hospital Anxiety and Depression Scale (HADS) and stress-proneness with Perceived Stress Questionnaire. Over a ten year period, all visits in primary care were retrospectively recorded in order to identify functional GI disorders.

RESULTS

In subjects with low total HADS-score, there were no significant differences in GI-symptoms between patients

and controls (GSRS-IBS 7.0 *vs* 6.5, *P* = 0.513). In the patients with bipolar disorder there were significant correlations between all GSRS and HADS subscores for all symptom clusters except for "constipation" and "reflux". Factors associated to GI symptoms in the patient group were female sex (adjusted OR = 2.37, 95%CI: 1.07-5.24) and high HADS-Depression score (adjusted OR = 3.64, 95%CI: 1.07-12.4). These patients had also significantly more visits for IBS than patients with low HADS-Depression scores (29% *vs* 8%, *P* = 0.008). However, there was no significant differences in consulting behaviour for functional GI disorders between patients and controls (25% *vs* 17%, *P* = 0.108).

CONCLUSION

Female patients and patients with high HADS depression score reported significantly more GI symptoms, whereas patients with low HADS scores did not differ from control subjects.

Key words: Anxiety; Bipolar disorder; Brain-Gut axis; Depression; Dyspepsia; Functional gastrointestinal disorder; Gastrointestinal Symptom Rating Scale-irritable bowel syndrome; Irritable bowel syndrome; Hospital Anxiety and Depression Scale; Stress

Core tip: Bipolar patients with depressive symptoms, but not with anxiety symptoms, reported more gastrointestinal (GI) symptoms than control subjects. Unexplained GI-symptoms in bipolar patients should be seriously considered to suffer from depression and receive adequate treatment.

Karling P, Maripuu M, Wikgren M, Adolfsson R, Norrback KF. Association between gastrointestinal symptoms and affectivity in patients with bipolar disorder. *World J Gastroenterol* 2016; 22(38): 8540-8548 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i38/8540.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i38.8540

INTRODUCTION

Affective disorders or affective symptoms such as depression and anxiety are common in those who seek help for functional gastrointestinal (GI) complaints[1]. Studies concerning the relationship between anxiety/depression and GI symptoms might be biased by a higher health care utilization that comes with psychological comorbidity[2,3]. To study the temporal relationship between the onset of gut symptoms and the onset of affective symptoms is difficult because of the insidious onset and fluctuating course of both affective and functional GI disorders[3,4]. Most studies that aim to characterize the relationship between bowel disorders and anxiety/depression are performed on patients from gastroenterology units. These patients often have longstanding and disabling gut symptoms with negative consequences on quality of life which in the long run may have an impact on mood. Therefore, a different approach for studying how affective syndromes influence the bowel and brain-gut interactions is to set the starting point at the psychiatric patients. Accordingly, there are relatively few studies using this approach[5]. In a large group of patients with unipolar depression, we have previously described that GI symptoms were common and related to symptoms of anxiety and depression[6]. Patients with unipolar depression have more pain, including abdominal pain, which in part correlates with the severity of the depressive mood, and patients with unipolar depression show a higher health care utilization for symptoms not denoted as "psychiatric"[5-9].

Bipolar disorder, including different subtypes such as bipolar disorder 1 and 2, is a common condition with reported life time prevalence in the population estimated at 2.4%[10]. Furthermore, in the last decade bipolar disorder is more described as a chronic, progressive disorder with significant residual symptoms between episodes of depression and mania/hypomania rather than classically cyclical illness[11]. It is estimated that bipolar disorder patients suffer from affective symptoms 50% of the time even if they are appropriately treated and are receiving mood stabilizing medication. The cost of total health care for patients with bipolar disorder is estimated at two to four times higher than for age- and sex matched controls[12]. In contrast to patients with unipolar depression, there are little published data concerning functional GI symptoms in patients with bipolar disorder.

The primary aim of this study was to compare the prevalence of GI symptoms in patients with bipolar disorder versus controls, and to determine the extent to which symptoms of anxiety/depression/stress and GI symptoms correlates in patients with an established bipolar disorder. A secondary aim was to determine if other factors than affectivity are associated to GI symptoms in patients with bipolar disorder.

MATERIALS AND METHODS

Study participants

Outpatients with a bipolar type 1 or type 2 diagnoses were considered for participation in the study, which is part of the multiple-outcome research project, the Umeå Bipolar project. The patients were treated at a specialized outpatient affective unit at Umeå University Hospital. The diagnoses were made according to DSM-IV criteria[13]. General exclusion criteria were dementia, mental retardation, relatedness as well as any other feature that would compromise the ability to fulfil the study protocol such as not having Swedish as a mother tongue, several visual or auditory handicaps. Pertaining to more specific exclusion criteria of the

present study, all subjects with abdominal surgery within three months before and after the survey, and all with established GI diseases, hepatic and renal diseases were excluded. Subjects on beta blockers, calcium antagonists, statines, antidepressants, pain medication including non-steroidal anti inflammatory medications were not excluded. Of 149 patients with bipolar disorder, 136 patients (88 bipolar type 1 and 48 bipolar type 2) between 20 and 84 years of age fulfilled the inclusion criteria and accepted participation. All patients were on stable medical treatment for three months prior to the study.

The control sample consisted of 136 age- and sex-matched subjects from a sub study of the Betula project (n = 299). The Betula project is a large multiple-outcome study focused at exploring memory, health and aging in the general population. All participants were randomly selected from the population registry of the same region as the patient sample (the Umeå region, northern Sweden) and have been shown to be representative of the general population[14]. The same exclusion criteria for the patients were applied to the control sample. The controls who took medications were likewise on stable treatment at least three month prior to the study.

Questionnaires

The Gastrointestinal Symptom Rating Scale-IBS (GSRS-IBS) is a validated self-assessment instrument to assess symptoms of irritable bowel syndrome (IBS)[15]. The GSRS-IBS questionnaire includes 13 items, each using a Likert scale (0-6 points). The items are grouped into symptom clusters: Abdominal pain (two items), bloating (three items), constipation (two items), diarrhoea (four items) and satiety (two items). There is currently not a defined cut-off level for having IBS in the GSRS-IBS questionnaire. Therefore, to explore the relationship between IBS-like symptoms with other factors in patients with bipolar disorder we used the median total GSRS-IBS score (the sum of all 13 items score) for the patients. In addition, but not included in the total GSRS-IBS score, we used five questions from the former Gastrointestinal Symptom Rating Scale (GSRS), that concern symptoms of gastroesophagal reflux (two items) and dyspepsia (three items)[16].

The Hospital Anxiety and Depression Scale (HADS), developed by Zigmond and Snaith in 1983[17], is a highly sensitive instrument to screen for symptoms of anxiety and depression among patients with somatic diseases. It consists of 7 items each for anxiety and depression, each using a 4-point Likert scale (0-3 points). We used the HADS scale because it has high sensitivity in detecting symptoms of anxiety and depression, it is well validated, and it is simple to fill in, which facilitates a higher response rate[18,19]. The accepted cut-off level of 8 points or more for the depression part of HADS (HADS-D) was used to define patients suffering from depression and the cut off level of 9 points or more was used to define patients suffering from anxiety (HADS-A).

The Perceived Stress Questionnaire (PSQ) was developed to measure general stress perceived during the past year and emphasizes cognitive perceptions more than emotional states or specific life events[20]. The PSQ consist of 30 items using a 4-point Likert scale (0-3 points). A PSQ index, varying from 0 (the lowest level) to 1 (the highest level) is calculated by dividing the total raw score with 90[20]. We used the estimated PSQ index of > 0.34 to define moderate level of perceived stress[21].

Medical records

After written consent from the subjects who responded to the questionnaires, records of primary care, surgery (including endoscopy unit) and infection clinics from 1999-2009, were searched twice for exclusion criteria (see study participants). The Swedish health care system includes a primary care health system with general physicians taking care of all initial referrals (except emergencies). Therefore, all patients who attend the gastroenterology out-patient clinic are initially referred by a general physician within the primary care. Blinded by the result of GSRS-IBS and HADS questionnaires, the records of primary care health centres were investigated twice to define consulters for IBS and any functional bowel disorders. Consulters for GI symptoms were defined by diagnosis of a functional bowel disorder as judged by their general physician or symptoms according to ROME Ⅲ criteria[22].

Statistical analysis

All analysis were carried out using IBM SPSS Statistics version 23. Non-parametric tests were used for comparing ordinal scales and continuous variables (Mann-Whitney test) and for correlations (Spearman's test). χ^2 test was used for crosstabs analyses and Fisher exact test if the number of cases was below 10. Student-t test was used for parametric comparison. A two-sided P value less than 0.05 were regarded significant. Means and standard deviations were used for continuous variables and medians and inter quartile range (IQR) for ordinal variables. No correction for multiple testing was applied. A logistic regression (SPSS/analyze/regression/binary logistic) was used for adjusting for possible confounders to the dependent variable GSRS-IBS score (dichotomous variable divided by median score). In the regression model age and body mass index were regarded as continues variables. HADS-D was categorized into two groups according to the accepted "cut-off" at ⩾ 8 points, HADS-A was categorized into two groups according to the accepted "cut-off" at ⩾ 9 points[18,19], PSQ index was categorized into two groups according the estimated moderate level of perceived stress (PSQ index > 0.34)[21] and the number of drugs was categorized into two groups

Table 1 Basal characteristic in patients with bipolar disorder and control subjects representative of a general population

	Bipolar disorder (n = 136)	Controls (n = 136)	P value
Mean age (SD) (yr)	49.9 (14.1)	51.0 (11.4)	0.505
Women	61% (n = 83)	60% (n = 81)	0.804
Mean body mass index (SD) (kg/m^2)	27.0 (5.50)	25.41 (3.19)	0.005[1]
Median HADS scores (IQR):			
HADS-anxiety	5 (7)	4 (5)	0.001[1]
HADS-depression	3 (5)	3 (3)	0.022[1]
PSQ index (IQR)	0.27 (0.27)	NA	
Median GSRS scores (IQR):			
Abdominal pain	1.00 (2.00)	0.50 (1.50)	0.076
Bloating	1.00 (1.67)	0.50 (1.34)	0.419
Diarrhoea	0.50 (1.50)	0.25 (0.75)	0.002[1]
Constipation	0 (2.00)	0 (1.00)	0.310
Satiety	0 (1.00)	0 (0.50)	0.019[1]
Dyspepsia	0 (1.00)	0.33 (0.67)	0.850
Reflux	0 (1.00)	0 (0.50)	0.376
Total GSRS-IBS	9.00 (17.00)	7.00 (13.00)	0.020[1]
Consulters for:			
Any functional GI disorder	25% (n = 34)	17% (n = 23)	0.108
IBS	12% (n = 17)	10% (n = 14)	0.582

[1]Statistical significance. For each separate symptom cluster in the Gastrointestinal Symptom Rating Scale the total score was divided by the amount of items. HADS: Hospital Anxiety and Depression Scale; GSRS: Gastrointestinal Symptom Rating Scale; IBS: Irritable Bowel Syndrome; PSQ: Perceived Stress Questionnaire; IQR: Intra quartile range; NA: Not available.

by the median value. Each single drug used by more than ten patients with bipolar disorder was separately analyzed with age, body mass index, sex HADS-A and HADS-D.

RESULTS

Patients with bipolar disorder in comparison to control subjects

Patients with bipolar disorder had significant higher body mass index and scored significant higher on HADS scales in comparison to control subjects. Forty-eight (35%) of the bipolar patients versus 18 (13%) of the controls had either HADS-D scores $\geqslant$ 8 or HADS-A scores $\geqslant$ 9 (P < 0.001). Thirty-four percent (n = 46) of the patients with bipolar disorder had a PSQ index > 0.34 (estimated moderate or high perceived stress level). Total GSRS-IBS score was significant higher for patients with bipolar disorder than for controls. There were two symptom clusters that were significant higher among the patients; "the diarrhoea cluster" and "the satiety cluster" (Table 1). The patients with bipolar disorder tended to more often consulted primary care for a functional GI disorder than control subjects. Drug intake (number of drugs) was significant higher in patients with bipolar depression disorder compared to controls (median number 3 *vs* 1, P < 0.001). Drugs for affective disorders, drugs for insomnia, levothyrexine, antacids therapy and IBS medications were significant more common among patients with bipolar disorder whereas analgesic were more seldom used in comparison to control subjects.

Association of affective symptoms and GI symptoms in bipolar disorder patients

Bipolar patients with low HADS-D and HADS-A scores reported GI symptoms to the same extent as control subjects with low HADS-D and HADS-A scores despite a higher use of medications (Table 2). Except for sub scores for "constipation" and "reflux", there were significant correlations between all GSRS and all HADS sub scores, and the bipolar patients with high HADS-A and/or HADS-D scored higher on GI symptom clusters (than patients with low HADS scores (Tables 2 and 3). There was a significant higher consulting rate in primary care for IBS in the patients with current high HADS-D in comparison to the patients with low HADS-D score (29% *vs* 8%, P = 0.008). Also control subjects with high HADS-A and/or HADS-D had higher GSRS scores than controls with low HADS scores [median scores of GSRS-IBS 12 (IQR 23) *vs* 6.5 (IQR 13), P = 0.021] as well as for sub scores for "bloating" (P = 0.005) and "diarrhoea" (P = 0.013).

Logistic regression of factors that may influence GI symptoms

Table 4 shows the characteristic of patients with high versus low GSRS-IBS score. Female sex, high scores on anxiety and depression, the use of benzodiazepines ("borderline significance") and the use of drugs for insomnia was significant more common in patients with high GSRS-IBS score. A logistic regression was preformed to analyze potential confounders that influence the presence of GI symptoms in patients with bipolar disorder. In the logistic regression model only

Table 2 Control subjects and patients with bipolar disorder with low scores on depression and low scores on anxiety score *vs* patients with high scores on depression and/or high scores on anxiety

	Bipolar patients with HADS-D ⩾ 8 and/or HADS-A ⩾ 9 (*n* = 48)	Bipolar patients with HADS-D < 8 and HADS-A < 9 (*n* = 88)	Controls with HADS-D < 8 and HADS-A < 9 (*n* = 118)
Mean age (SD) (yr)	46.7 (12.8) (P = 0.048)[1]	51.7 (14.4)	51.5 (11.8) (P = 0.903)
Proportions of women	58% (n = 28) (P = 0.634)	62% (n = 55)	59% (n = 70) (P = 0.644)
Mean Body mass index (kg/m^2) (SD)	27.2 (4.6) (P = 0.632)	26.8 (5.97)	25.6 (3.26) (P = 0.063)
Median HADS score (IQR)			
HADS-anxiety	10.5 (5.0)	3.0 (4.0)	3.0 (4.0) (P = 0.660)
HADS-depression	9.0 (9.0)	2.0 (3.0)	2.0 (3.0) (P = 0.505)
PSQ index (IQR)	0.47 (0.28) (P < 0.001)[1]	0.20 (0.17)	NA
Median GSRS score (IQR):			
Abdominal pain	1.50 (2.50) (P = 0.002)[1]	0.50 (1.50)	0 (1.50) (P = 0.558)
Bloating	1.67 (2.33) (P < 0.001)[1]	0.67 (1.33)	0.67 (1.67) (P = 0.415)
Diarrhoea	1.25 (2.25) (P < 0.001)[1]	0.50 (1.00)	0.25 (0.75) (P = 0.107)
Constipation	0 (2.00) (P = 0.380)	0 (1.50)	0 (1.00) (P = 0.453)
Satiety	0.50 (2.00) (P = 0.003)[1]	0 (0.50)	0 (0.50) (P = 0.513)
Dyspepsia	0.67 (1.33) (P = 0.002)[1]	0 (0.67)	0.33 (0.67) (P = 0.291)
Reflux	0 (2.13) (P = 0.098)	0 (0.88)	0 (0.50) (P = 0.796)
Total GSRS-IBS	15.0 (23.0) (P < 0.001)[1]	7.00 (12.0)	6.50 (13.0) (P = 0.513)
Consulters:			
For any functional GI disorder	29% (n = 14) (P = 0.407)	23% (n = 20)	14% (n = 17) (P = 0.131)
IBS	17% (n = 8) (P = 0.290)	10% (n = 9)	10% (n = 12) (P = 0.995)
Median number of drugs (IQR)	3.0 (3.0) (P = 0.557)	3.0 (3.0)	1.0 (2.0) (P < 0.001)[1]

[1]Statistical significance. For each separate symptom cluster in the Gastrointestinal Symptom Rating Scale the total score was divided by the amount of items. HADS: Hospital Anxiety and Depression Scale; GSRS: Gastrointestinal Symptom Rating Scale; GI: Gastrointestinal; IBS: Irritable bowel syndrome; IQR: Intra quartile range; NA: Not available.

Table 3 Hospital Anxiety Depression Scale scores and Perceived Stress Questionnaire index in correlation to different gastrointestinal symptom scores in patients with bipolar disorder (*n* = 136)

	HADS-Anxiety score. rs (*P* value)	HADS-Depression score. rs (*P* value)	PSQ index. rs (*P* value)
Abdominal pain	0.295 (0.001)[1]	0.248 (0.004)[1]	0.261 (0.002)[1]
Bloating	0.304 (< 0.001)[1]	0.365 (< 0.001)[1]	0.376 (< 0.001)[1]
Diarrhoea	0.334 (< 0.001)[1]	0.225 (0.009)[1]	0.268 (0.002)[1]
Constipation	0.099 (0.254)	0.028 (0.751)	0.063 (0.473)
Satiety	0.222 (0.010)[1]	0.253 (0.003)[1]	0.333 (< 0.001)[1]
Dyspepsia	0.293 (0.001)[1]	0.205 (0.017)[1]	0.287 (0.001)[1]
Reflux	0.245 (0.004)[1]	0.122 (0.160)	0.235 (0.007)[1]
Total GSRS-IBS score	0.336 (< 0.001)[1]	0.328 (< 0.001)[1]	0.348 (< 0.001)[1]

[1]Statistical significance. Statistics: Spearman's test. HADS: Hospital Anixiety and Depression Scale; GSRS: Gastrointestinal Symptoms Rating Scale; IBS: Irritable bowel syndrome; PSQ: Perceived Stress Questionnaire.

female sex and high HADS-D score was significantly associated to IBS symptoms (Table 5) in the patients with bipolar disorder.

Neither number of drugs ("cut off median number of drugs") (Table 5) or any single drug adjusted for age, sex, body mass index and HADS score significantly influenced GI symptoms.

Bipolar disorder type I vs type II

The patients with bipolar disorder type Ⅰ were older than the patients with bipolar disorder type Ⅱ (mean 51.9 years *vs* 46.2 years, P = 0.025). There were no significant differences in GSRS-IBS scores, HADS scores or GI visits between the subtypes of bipolar disorder.

DISCUSSION

This present study, for the first time, aims to determine the extent in which affectivity is related to GI symptoms in a patient sample with an established bipolar disorder, a disorder characterized with fluctuating periods of hypomania/mania and depression. Our study shows that there is a strong association between symptoms of affectivity and GI symptoms in patients with bipolar disorder but also shows that patients with bipolar disorder with low scores on affectivity do not have more GI symptoms than control subjects. The latter is despite a more frequent use of medications with GI side-effects (*i.e.*, neuroleptics, SSRIs) in patients with bipolar disorder. Therefore, unexplained GI-

Table 4 Comparison between patients with bipolar disorder who report high respective low scores on the Gastrointestinal Symptom Rating Scale for Irritable Bowel Syndrome

	HIGH GSRS-IBS SCORE (> 9) (*n* = 65)	LOW GSRS-IBS SCORE (≤ 9) (*n* = 71)	*P* value
Mean age (SD)(yr)	49.7 (13.7)	50.1 (14.5)	0.571
Women	71% (*n* = 46)	52% (*n* = 37)	0.026[1]
BMI (SD)	27.2 (4.46)	26.7 (6.32)	0.45
Median HADS scores (IQR)			
Anxiety score (median)	6.0 (9.0)	4.0 (6.0)	0.001[1]
Depression score (median)	4.0 (8.0)	2.0 (4.0)	0.002[1]
PSQ index (IQR)	0.32 (0.26)	0.21 (0.23)	< 0.001[1]
Consulters for:			
Any functional GI disorder	29% (*n* = 19)	21% (*n* = 15)	0.276
IBS	18% (*n* = 12)	7% (*n* = 5)	0.067
Bipolar type Ⅰ	46% (*n* = 40)	54% (*n* = 48)	0.460
Bipolar type Ⅱ	52% (*n* = 25)	37% (*n* = 23)	
Medications:			
Lithium	51% (*n* = 33)	42% (*n* = 31)	0.320
Neuroleptics	28% (*n* = 18)	20% (*n* = 14)	0.273
Anti-epileptics	31% (*n* = 20)	34% (*n* = 24)	0.706
SSRI	14% (*n* = 9)	11% (*n* = 8)	0.796
SNRI	9% (*n* = 6)	6% (*n* = 4)	0.519
Benzodiazepines	17% (*n* = 11)	6% (*n* = 4)	0.053
Drugs for insomnia	26% (*n* = 17)	10% (*n* = 7)	0.014[1]
Drugs for IBS	11% (*n* = 7)	1% (*n* = 1)	0.028[1]
Antacids	15% (*n* = 10)	7% (*n* = 5)	0.171
Statines	12% (*n* = 8)	8% (*n* = 6)	0.575
Levothyroxine	26% (*n* = 17)	13% (*n* = 9)	0.052
⩾ 3 drugs	64% (*n* = 41)	54% (*n* = 38)	0.214

[1]Statistical significance. HADS: Hospital anxiety and depression scale; GSRS-IBS: Gastrointestinal symptom rating scale- irritable bowel syndrome; PSQ: Perceived Stress Questionnaire; BMI: Body mass index; SSRI: Selective serotonin reuptake inhibitor; SNRI: Selective noradrenalin reuptake inhibitor; IQR: Intraquartile range.

symptoms in patients with bipolar disorder should be seriously considered to suffer from depression and receive adequate treatment. It is tempting to assume that this would reduce the number of unnecessary somatic examinations. We have previously shown that also patients with an established recurrent depression disorder report high scores on GI symptoms, but when in remission they do not differ from controls in reporting GI symptoms[6]. We believe that the present study and our previous study support that affectivity has an effect on the gut.

How the brain-gut axis is involved in the patho-physiology of anxiety/depression is not known. In the brain areas that process visceral afferents and areas involved in fear and anxiety are closely related. For example, functional imaging studies on patients with IBS have shown that balloon distension of the rectosigmoid colon increases activity in certain areas of the brain involved in the regulation of affective and sensory processes such as the amygdala, insula, cingulated and prefrontal cortex[23-26].

There is also evidence that gut symptoms and visceral hypersensitivity improve in patients with IBS treated with anti-depressants, hypnosis and cognitive-behavioural treatment[27-29]. One possible mechanism of these therapies could be an increase in prefrontal inhibition of the amygdale and anterior cingulated cortex[30].

Another factor that links affectivity and the gut is corticotrophin-releasing hormone (CRH). Anxiety, depression and stress are associated with increased activity of CRH[31]. CRH receptors are abundant in the amygdale as well in the gut and an exaggerated CRH response has been linked both to anxiety and depression[31-33] as well to gut physiology[34-37]. For example, injection of CRH results in an increased visceral hypersensitivity, exaggerated colonic motility and inhibition of upper gut motility[34-37]. In a clinical perspective a high CRH drive may result in simultaneous occurrence of increased affectivity, visceral pain, diarrhoea, urgency and dyspepsia. CRH also up regulates the hypothalamic-pituitary-adrenal axis leading to hypercortisolism and activates locus cereuleus[31] leading to a shift of the autonomic nervous system towards an increased sympathetic tone with possible complex downstream effects on the gut physiology (including motility, sensitivity, secretion and the gut immune system)[30,38,39].

The issue of the brain-gut axis is complex and many other possible factors may also be involved. For example, the gut microbiotica and/or subtle inflammation in the bowel may play a role in the regulation of mood[40], indicating that in addition to a brain-gut axis there is also a gut-brain axis involved in

Table 5 Logistic regression analysis studying factors which may influence gastrointestinal symptoms in patients with bipolar disorder

	Patients with bipolar disorder and GSRS-IBS > 9 *vs* patients with bipolar disorder and GSRS-IBS ≤ 9	
	Unadjusted OR	Adjusted OR
Age	1.00 (0.97-1.03)	1.01 (0.97-1.04)
Sex (male reference)	2.23 (1.09-4.52)[1]	2.37 (1.07-5.24)[1]
Body mass index	1.02 (0.95-1.09)	1.01 (0.93-1.08)
HADS-Depression ⩾ 8 (< 8 reference)	5.54 (2.07-14.8)[1]	3.64 (1.07-12.4)[1]
HADS-Anxiety ⩾ 9 (< 9 reference)	2.89 (1.34-6.22)[1]	1.82 (0.64-5.22)
PSQ index (⩽ 0.34 reference)	2.53 (1.21-5.30)[1]	1.30 (0.42-3.99)
Number of Drugs ⩾ 3 (< 3 reference)	1.55 (0.77-3.09)	1.29 (0.59-2.80)

[1]Statistical significance. The dependent variable is high versus low score on the Gastrointestinal Symptom Rating Scale. The studied covariates were: Age (continues variable), Sex (dichotomous variable), Body mass index (continues variable), Hospital Anxiety and Depression scale score (dichotomous variable), Perceived Stress Questionnaire score (dichotomous variable) and Number of drugs (dichotomous variable with "cut off" being the median value). OR is presented with 95%CI. GSRS-IBS: Gastrointestinal symptom rating scale-irritable bowel syndrome; HADS: Hospital anxiety depression scale; PSQ: Perceived Stress Questionnaire.

the "link between affectivity and bowel symptoms.

In the logistic regression analysis in our study depressive symptoms and not anxiety symptoms and not perceived stress were related to GI symptomatology. This is a novel and important finding, which is contrary to that seen in studies on patients with IBS[18,41] and control subjects[42] in where anxiety correlates to an higher extent to reported GI symptoms. Women and younger individuals score in general higher on HADS-anxiety[18] and perceived stress[21]. The majority of patients with IBS are women and younger, whereas the patients in the present study are older and involves relatively more men. These differences in age and gender distribution may partly explain the different impact of anxiety and depression on GI symptomatology in patients with IBS and patients with bipolar disorder. We suggest further studies that focus on the different aspects of affectivity and their impact on symptoms from the gut.

There are some limitations of our study. The GSRS and GSRS-IBS questionnaire is designed to be a sensitive tool for detecting symptoms typically of functional GI disorders[15,16]. However, the questions in the GSRS questionnaire only focus on symptoms the last week which increases the validity of the responses but at the same time disallows us from making a diagnosis according to the ROME criteria. GSRS is regarded by some authors to overestimate functional GI disorders in comparison to the ROME based questionnaires[43]. Two questions in the GSRS-IBS questionnaire issue the symptoms typically of postprandial dyspepsia (satiety and early satiety) and are inappropriate to be classed in the IBS-like symptom cluster. Because the questionnaire was valid with the "satiety" questions we have included the questions in the total GSRS-IBS[15].

The study design in the present study was mainly cross-sectional, which results in a lack of clear temporal relationship between depressive mood and GI symptoms. A prospective study design, analyzing GI symptoms in patients with affective disorder over time would better investigate this temporal relationship. Also, in studies comparing results from questionnaires there is some risk of reporting bias (reporting the same type of dignity on different scales). However, because of the fact that patients in remission (low HADS-D and HADS-A score) did not differ from controls in GI symptom score but the same patients tended to have more visits in primary care for functional GI complaints we argue that these data point toward a common pathophysiology between mood and gut symptoms in patients with bipolar disorder.

Female patients and patients with high HADS depression score reported significantly more GI symptoms, whereas patients with low HADS scores did not differ from control subjects. Unexplained GI-symptoms in bipolar patients should be seriously considered to suffer from depression and receive adequate treatment.

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COMMENTS

Background

Symptoms of anxiety and depression as well as increased stress-proneness are frequently occurring in patients with unexplained gastrointestinal (GI) symptoms, however the cause and effect relationship has not been clearly established

Research frontiers

There are many studies performed on patients with functional GI symptoms that investigate the prevalence and characteristics of symptoms of anxiety and depression. These patients often have longstanding and disabling gut symptoms with negative consequences on quality of life which in the long run may have an impact on mood. A different approach for studying how affective syndromes influence the bowel and brain-gut interactions is to set the starting point at the psychiatric patients. In the literature there are relatively few studies using this approach.

Innovations and breakthroughs

The present study supports a relationship between affectivity and gut symptoms. The finding that patients with bipolar disorder with low scores on affectivity do not have more GI symptoms than control subjects whereas patients with bipolar disorder with high scores on affectivity do have GI symptoms support the thesis that mood has an impact on gut function.

Applications

Unexplained GI-symptoms in bipolar patients should be seriously considered to suffer from depression and receive adequate treatment.

Peer-review

The idea of search is significantly studied throughout function bowel disorders, with all types and disorders. Results confirm what we expected, no changes from previous studies.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Interendoscopist variability in proximal colon polyp detection is twice higher for serrated polyps than adenomas

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Abstract

AIM

To assess the interendoscopist variability in the detection of colorectal polyps according to their location and histological type.

METHODS

This study was a retrospective analysis of prospectively collected data from a regional colorectal cancer (CRC) screening program; 2979 complete colonoscopies from 18 endoscopists were included. Variability in performance between endoscopists for detection of at least one adenoma (A), one proximal adenoma (PA), one distal adenoma (DA), and one proximal serrated polyp (PSP) was assessed by using multilevel logistic regression models.

RESULTS

The observed detection rates among the 18 endoscopists ranged from 24.6% to 47.6% (mean = 35.7%) for A, from 19.1% to 39.0% (mean = 29.4%) for DA, from 6.0% to 22.9% (mean = 12.4%) for PA, and from 1.3% to 19.3% (mean = 6.9%) for PSP.

After adjusting for patient-level variables (sex, age), the interendoscopist detection rates variability achieved a significant level for A, PA, and PSP but not for DA (P = 0.03, P = 0.02, P = 0.02 and P = 0.08, respectively). This heterogeneity, as measured by the variance partition coefficient, was approximately threefold higher for PA (6.6%) compared with A (2.1%), and twofold higher for PSP (12.3%) compared with PA.

CONCLUSION

These results demonstrate significant interendoscopist variability for proximal polyp particularly for serrated polyps, but not for distal adenoma detection. These findings contribute to explain the decreased effectiveness of complete colonoscopies at preventing proximal CRCs and the need to carefully assess the proximal colon during scope procedure.

Key words: Colonoscopy; Colorectal cancer; Adenoma; Serrated polyp; Proximal polyp; Detection rate; Quality performance

Core tip: The present study demonstrates high interendoscopist variability in adenoma, proximal adenoma, and proximal serrated polyp detection rates but not in distal adenoma detection rates. The magnitude of interendoscopist variation was wider for proximal serrated polyps as compared to proximal adenoma detection. Altogether, these findings might explain why complete colonoscopies are less effective at preventing proximal than distal colorectal cancers.

Bretagne JF, Hamonic S, Piette C, Viel JF, Bouguen G. Interendoscopist variability in proximal colon polyp detection is twice higher for serrated polyps than adenomas. *World J Gastroenterol* 2016; 22(38): 8549-8557 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i38/8549.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i38.8549

INTRODUCTION

Adenoma detection and removal is the basis for the reduction in colorectal cancer (CRC) incidence and mortality achieved by colonoscopy[1-3]. However, recent studies raised concerns that screening colonoscopies may not decrease CRC incidence and mortality in the proximal colon to the same extent as in the distal colon[4-8]. Although there are multiple plausible explanations for the decreased effectiveness in the proximal colon, the quality of the colonoscopy is a critical issue. Recent studies demonstrated that surrogate indicators for colonoscopy quality performance, such as adenoma detection rates and cecal intubation rates, were predictors of interval CRCs that occur after screening colonoscopies[9-11]. A higher miss rate of proximal adenomas compared to distal adenomas could explain the decreased protective effect of colonoscopy for proximal colon cancer. However, no data are available on the interendoscopist variability of adenoma detection according to the polyp location in the colon, particularly in population-based studies.

Serrated polyps might be another significant contributor to the decreased protective effect of colonoscopies for proximal colon cancer. Serrated lesions can be challenging to visualize because of their morphologic characteristics and could be more likely overlooked as compared to conventional adenomas. Cohort studies demonstrated a wide variation rate among endoscopists of the proximal serrated polyps detection rates[12-14], but no study aimed to compare adenomas detection and serrated polyps detection variability amongst endoscopists, especially for proximal colon location.

This population-based study aimed to test the hypothesis that the variations in adenoma detection rates between colonoscopists are wider for the proximal colon compared with the distal colon, and to compare interendoscopist variability in polyp detection rates in the proximal colon between serrated polyps and adenomas.

MATERIALS AND METHODS

Study population

The study was conducted in "Ille et Vilaine", which has a population of approximately one million and was one of the first French districts to implement a national screening program at the end of 2002. The mass screening is based on biennial guaiac fecal occult blood tests. The target population for the screening includes asymptomatic men and women between 50 and 74 years of age with no other CRC risk factors. Individuals with a family history of CRC or a personal history of CRC or adenomas, those with inflammatory bowel disease, and those who had undergone a total colonoscopy in the previous five years were excluded from the screening program.

There were 96054 (51.8%) and 89309 (46.7%) participants in the first and second rounds, respectively. The proportion of positive tests amongst the participants was 2.58% and 2.26%, respectively. Positive testing was followed by a colonoscopy in 92.6% and 91.4% of the subjects, respectively. Finally, 2295 and 1848 colonoscopies were performed from 2003 to 2007 in the first and second rounds, respectively.

The 18 endoscopists who had performed at least 30 colonoscopies following a positive test in each of the first two rounds of the screening program were included. Fourteen of the 18 endoscopists were in private practice, and 4 worked in public hospitals. Overall, the 18 endoscopists performed 3487 (84.2%) of the 4143 white-light colonoscopies of the 2

screening rounds. Although high cecal intubation rates were recorded for rounds 1 and 2 (96.3% and 95.9%, respectively), we included only complete examinations of the colon in this study. The data from both rounds were pooled because no difference in the colonoscopy findings was noted between the two rounds. We previously reported that individual endoscopists who had participated in the CRC screening program as a factor was not a significant predictor of CRC detection but was a significant predictor of adenoma detection[15]. In the present study, a secondary analysis of the colonoscopy data was done to explore variations in the detection rate of at least one adenoma according to its location in the colon and to compare interendoscopist variability in polyp detection rates in the proximal colon according to histological subtype (serrated polyps and adenomas). The CRC screening program was declared and approved by the CNIL "Commission Nationale de l'Informatique et des Libertés" on August 30th 2002 (n° 812571). Research was approved by the CCTIRS "Comité Consultatif pour le Traitement de l'Information en matière de Recherche dans le domaine de la santé".

Study design and outcomes

This was a cross-sectional study that used data retrieved from a prospectively collected database. Three adenoma detection rates, which were expressed as the proportion of complete colonoscopies with at least one adenoma, were calculated for each endoscopist as follows: The distal adenoma detection rate (DA.DR) for at least one adenoma detected in the distal colon (*i.e.,* below the splenic flexure including the flexure), the proximal adenoma detection rate (PA.DR) for at least one adenoma in the proximal colon (*i.e.,* proximal to the splenic flexure) and the A.DR for at least one colorectal adenoma regardless of its location in the colon. Colonoscopies with CRC, including those with malignant polyps harboring intramucosal carcinoma, were not included in the analysis because additional polyps in these patients were not recorded in the database. The individual detection rates for serrated polyps in the proximal colon (PSP.DR) were also calculated for each endoscopist. Serrated polyps were defined as an entire group of polyps that included traditional hyperplastic polyps, sessile serrated adenomas/polyps and traditional serrated adenomas.

The observed and adjusted (*i.e.,* according to patient age and gender) adenoma detection rates were calculated for each endoscopist and each site. Similarly, the observed and adjusted (*i.e.,* according to patient age and gender) proximal serrated polyp detection rates were calculated for each endoscopist. The variability between endoscopists in the probability to detect one adenoma/one adenoma in the distal colon/one adenoma in the proximal colon/one serrated polyp in the proximal colon was assessed by multilevel logistic regression models.

Additional analyses were performed after defining a proximal polyp as proximal to the hepatic flexure instead of proximal to the splenic flexure. Furthermore, we assessed the interendoscopist variability for polyps of size $\geqslant$ 10 mm.

Statistical analysis

Continuous variables were expressed as the mean, standard error, median and interquartile range, and extremes values; categorical variables were expressed as numbers and percentages. The observed detection rates were compared between males and females and then between age classes using the Wilcoxon test; the use of the Cochran-Mantel Haenszel test permitted endoscopists to be adjustment variables. The patient age- and gender-adjusted adenoma detection rates for each endoscopist (and the corresponding 95%CI) were defined as the observed proportion of colonoscopies with at least one adenoma detected amongst all subjects multiplied by the ratio of the observed to the predicted number of detected adenomas for one endoscopist. The predicted number of detected adenomas for each endoscopist was assessed using logistic regression. Multilevel logistic regression models were used given the hierarchical structure of the sample (*i.e.,* patients are aggregated at the endoscopist level) and binary outcomes (*i.e.,* at least one adenoma/at least one distal adenoma/at least one proximal adenoma/at least one proximal serrated polyp). Each model was a two-level model in which age and gender were included as fixed effects (first or patient level) and in which the endoscopist was introduced as a random effect (second or endoscopist level). The fixed effect results are presented as odds ratios with 95%CI. To determine the proportion of total variance of the outcome that is explained at the endoscopist level, the variance partition coefficient was calculated using the Snijders and Bosker approximation[16,17].

$VPC = \sigma^2_{u0}/(\sigma^2_{u0} + \pi^2/3)$

where σ^2_{u0} is the variance of the endoscopist-level random effect representing the between-endoscopist variability in terms of the outcome. The correlations between adjusted values of polyp detection rates were tested using the Spearman rank test. For all tests, the significance threshold was α = 5%. The analyses were performed using SAS version 9.2 (SAS Institute Inc., Cary, NC, United States).

RESULTS

Distribution of the adenomas and serrated polyps within the population

After excluding incomplete examinations (n = 210) and colonoscopies harboring CRC (n = 298), 2979 colonoscopies were included for the analysis. Of these, 1531 (51.4%) were performed in males and

Table 1 Observed polyp detection rates amongst the 18 colonoscopists

Endoscopist (No.)	Colonoscopies (*n*)	A.DR	DA.DR	PA.DR	PSP.DR
A	273	33.33%	29.67%	7.33%	4.40%
B	272	24.63%	19.49%	6.25%	5.51%
C	213	46.48%	38.97%	17.84%	19.25%
D	210	47.62%	38.10%	18.57%	6.19%
E	207	28.99%	25.12%	7.25%	5.31%
F	185	30.81%	27.57%	5.95%	2.70%
G	172	32.56%	29.07%	9.88%	7.56%
H	164	31.71%	28.05%	8.54%	8.54%
I	160	35.63%	30.00%	11.25%	3.13%
J	157	47.13%	35.67%	22.93%	15.92%
K	148	33.11%	29.05%	11.49%	2.03%
L	148	36.49%	31.76%	11.49%	10.14%
M	135	37.04%	25.19%	17.78%	4.44%
N	135	40.74%	31.11%	14.07%	5.93%
O	132	34.85%	33.33%	6.82%	2.27%
P	105	30.48%	19.05%	21.90%	15.24%
Q	85	38.82%	31.76%	12.94%	4.71%
R	78	32.05%	25.64%	11.54%	1.28%
N	18	18	18	18	18
mean	165.50	35.69%	29.37%	12.43%	6.92%
SD	54.65	6.41%	5.41%	5.36%	5.14%
Median	158.50	34.09%	29.37%	11.49%	5.41%
Q1-Q3	135-207	31.71%-38.82%	25.64%-31.76%	7.33%-17.78%	3.13%-8.54%
Min-Max	78-273	24.63%-47.62%	19.05%-38.97%	5.95%-22.93%	1.28%-19.25%

A.DR: Adenoma detection rate; DA.DR: Distal adenoma detection rate; PA.DR: Proximal adenoma detection rate; PSP.DR: Proximal serrated polyp detection rate.

1448 (48.6%) in females. The overall mean age of the patients was 61.7 ± 7.2 years. The number of colonoscopies performed by each of the 18 endoscopists ranged from 78 to 273 (mean = 165.5 ± 54.7). At least one adenoma was detected in 1057 subjects as follows: 707 (66.9%) in men and 350 (33.1%) in women. Amongst the patients with at least one adenoma, 703 (66.5%) had only distal adenomas, 180 (17.0%) had only proximal adenomas, and 174 (16.5%) had at least one adenoma in both regions. At least one proximal serrated polyp was detected in 210 subjects as follows: 130 (61.9%) in men and 80 (38.1%) in women. The number of colonoscopies harboring both types of proximal polyps, *i.e.,* at least one adenoma and at least one serrated polyp, was 59 (2.0%).

Individual endoscopists' neoplasia detection rates

Table 1 shows the observed detection rates per endoscopist for each of the following four indicators: Adenoma detection rate (A.DR), DA.DR, PA.DR, and proximal serrated polyp detection rate (PSP.DR). The mean detection rates were 35.7%, 29.4%, 12.4% and 6.9%, respectively.

The mean and median values of these four indicators according to gender and age are provided in Tables 2 and 3. For each of the measures related to adenoma detection, the median values were significantly higher in males compared with females, and the values increased with increasing age. The median PSP.DR values did not differ significantly according to age group, but there was a trend for a higher detection rate in males compared with females (7.0% *vs* 4.3%, respectively, $P = 0.06$).

Factors associated to the adenoma and serrated polyp detection rates from multilevel logistic regression

The results of the multilevel logistic regressions are presented in Table 4. Age and gender were significant factors for polyp detection regardless of the indicator used. After adjusting for patient-level variables, the interendoscopist variability achieved a significant level for A.DR, PA.DR, and PSP.DR but not for DA.DR ($P = 0.03$, $P = 0.02$, $P = 0.02$ and $P = 0.08$, respectively). The corresponding variance partition coefficients were as follows: 2.1%, 6.6%, 12.3%, and 1.3%. The heterogeneity between endoscopists was approximately threefold higher for PA.DR compared with A.DR, and twofold higher for PSP.DR compared with PA.DR.

Complementary analyses

The abovementioned results were not affected when the proximal colon was defined as proximal to the hepatic flexure (data not presented).

Amongst the 18 colonoscopists, the median gender- and age-adjusted values for the detection of polyps ⩾ 10 mm were 17.5%, 16.2%, 2.6% and 0.6% for A.DR, DA.DR, PA.DR and PSP.DR, respectively, without significant statistical interendoscopist variability.

The interendoscopist variability amongst the 18 colonoscopists remains significant for the detection rate of proximal polyps of any histological subtype (*i.e.*, proximal serrated polyp and/or proximal

Table 2 Comparison of polyp detection rates (%) between males and females amongst the 18 colonoscopists using the Wilcoxon test

		Males	Females	Total	*P* value
A.DR	*n*	18	18	18	
	mean	46.3	24.5	35.7	
	Median	44.9	23.7	34.1	< 0.0001
	Q1-Q3	40.7-50.0	20.0-27.7	31.7-38.8	
	Min-Max	35.8-67.3	12.5-39.3	24.6-47.6	
DA.DR	*n*	18	18	18	
	mean	38.2	20	29.4	
	Median	37.7	20.6	29.4	< 0.0001
	Q1-Q3	32.4-40.0	17.9-22.7	25.6-31.8	
	Min-Max	27.9-54.8	11.0-29.2	19.0-39.0	
PA.DR	*n*	18	18	18	
	mean	17.8	7.1	12.4	
	Median	15.3	6.6	11.5	< 0.0001
	Q1-Q3	11.0-22.4	3.6-10.8	7.3-17.8	
	Min-Max	8.6-40.0	1.3-18.0	5.9-22.9	
PSP.DR	*n*	18	18	18	
	mean	8.3	5.3	6.9	
	Median	7.0	4.3	5.4	0.06
	Q1-Q3	4.5-10.8	1.5-6.6	3.1-8.5	
	Min-Max	2.2-25.6	0.0-16.9	1.3-19.2	

A.DR: Adenoma detection rate; DA.DR: Distal adenoma detection rate; PA.DR: Proximal adenoma detection rate; PSP.DR: Proximal serrated polyp detection rate.

Table 3 Comparison of polyp detection rates (%) according to age groups amongst the 18 colonoscopists using the Wilcoxon test

		< 55	55-59	60-64	65-69	⩾ 70	Total	*P* value
A.DR	*n*	18	18	18	18	18	18	
	mean	29.5	28.6	36.7	42.3	42.2	35.7	
	Median	28.5	29.6	38.8	43.5	43.0	34.1	< 0.0001
	Q1-Q3	24.4-34.8	21.4-33.3	29.4-41.4	28.6-50.0	37.5-45.5	31.7-38.8	
	Min-Max	16.7-42.9	12.5-42.6	18.4-52.9	21.7-73.0	29.0-53.8	24.6-47.6	
DA.DR	*n*	18	18	18	18	18	18	
	mean	23.9	23.6	31.9	35.3	33.4	29.4	
	Median	22.7	23.8	33.0	39.0	33.3	29.4	0.0003
	Q1-Q3	17.6-31.8	18.5-27.8	29.4-37.5	22.7-44.4	26.5-37.5	25.6-31.8	
	Min-Max	11.1-35.3	12.5-36.8	14.3-42.9	13.6-54.1	23.1-51.6	19.0-39.0	
PA.DR	*n*	18	18	18	18	18	18	
	mean	9.1	8.8	11.7	15.9	17.4	12.4	
	Median	8.3	9.5	9.1	17.5	15.9	11.5	0.0006
	Q1-Q3	5.9-9.3	4.3-11.8	5.0-18.2	11.9-20.0	12.5-20.6	7.3-17.8	
	Min-Max	0-25.0	0-17.9	0-31.0	2.5-32.4	0-46.2	5.9-22.9	
PSP.DR	*n*	18	18	18	18	18	18	
	mean	6.1	6.5	6.2	8.1	8.6	6.9	
	Median	5.0	4.7	1.8	5.8	8.0	5.4	0.37
	Q1-Q3	0.0-7.4	3.2-10.0	0.0-10.0	2.5-10.2	3.2-12.9	3.1-8.5	
	Min-Max	0.0-20.9	0.0-21.4	0.0-23.1	0.0-26.5	0.0-20.6	1.3-19.2	

A.DR: Adenoma detection rate; DA.DR: Distal adenoma detection rate; PA.DR: Proximal adenoma detection rate; PSP.DR: Proximal serrated polyp detection rate.

adenoma) (data not presented). The corresponding variance partition coefficient was 9.6%, which was an intermediary value between the PSP.DR and PA.DR values.

Correlation studies between adjusted values of polyp detection rates

PSP.DR values were not correlated with A.DR values ($\rho = 0.19$, $P = 0.45$), but were significantly correlated with PA.DR values ($\rho = 0.55$, $P < 0.002$). PA.DR values were highly significantly correlated with A.DR values ($\rho = 0.83$, $P < 0.0001$).

DISCUSSION

Colonoscopies are known to display great variability in A.DRs between endoscopists in various settings, including in academic[18], mixed community-academic[19], community practices[20,21] and population-based studies[15,22]. However, no study focused on the variability in A.DRs according to the proximal or distal location of the adenomas in the colon. Although

Table 4 Results of multilevel logistic regression analysis for assessing the interendoscopist variability

			Coefficient (standard error)	*P* value	OR	95%CI
Adenoma detection rate	Patient level	Constant	-2.37 (0.356)	< 0.0001		
		Age	0.036 (0.006)	< 0.0001	1.037	1.025-1.048
		Gender (ref = male)	-1.019 (0.082)	< 0.0001	0.361	0.308-0.424
	Endoscopist level	σ^2u0^1	0.070 (0.032)	0.03		
Distal adenoma detection rate	Patient level	Constant	-2.498 (0.369)	< 0.0001		
		Age	0.033 (0.006)	< 0.0001	1.033	1.022-1.045
		Gender (ref = male)	-0.932 (0.085)	< 0.0001	0.394	0.333-0.466
	Endoscopist level	σ^2u0^1	0.044 (0.025)	0.08		
Proximal adenoma detection rate	Patient level	Constant	-4.169 (0.538)	< 0.0001		
		Age	0.041 (0.008)	< 0.0001	1.042	1.025-1.059
		Gender (ref = male)	-1.065 (0.127)	< 0.0001	0.345	0.269-0.442
	Endoscopist level	σ^2u0^1	0.234 (0.101)	0.02		
Proximal serrated polyp detection rate	Patient level	Constant	-4.049 (0.677)			
		Age	0.025 (0.010)	0.02	1.025	1.004-1.046
		Gender (ref = male)	-0.498 (0.150)	< 0.001	0.608	0.453-0.816
	Endoscopist level	σ^2u0^1	0.460 (0.199)	0.02		

[1]Variance of the endoscopist-level random effect representing the heterogeneity between endoscopists in terms of the outcome.

the present study confirms significant variability for adenoma detection amongst colonoscopists, these data indicate that interendoscopist variability achieves a significant level for proximal adenomas but not distal adenomas detection. Serrated polyps were included to demonstrate that interendoscopist variability was also significant for proximal serrated polyp detection, even higher. These findings which resulted from in-depth statistical analyses using multilevel logistic regressions, demonstrate a higher heterogeneity for proximal serrated polyp than proximal adenoma detection amongst endoscopists. Detection rates for distal serrated polyps were not assessed because we hypothesised that variability between colonoscopists could be related to other factors than the quality of performance by colonoscopists. Some endoscopists might intentionally avoid performing a biopsy or discard small rectal polyps that have the appearance of hyperplastic polyps in the rectosigmoid.

All of these findings contribute to underline that the proximal colon is a difficult issue for colonoscopists. Otherwise, the performances of colonoscopists are more variable for proximal adenomas compared with distal adenomas and within the proximal colon for serrated polyps compared with adenomas. A correlation between adenomas and serrated polyps detection rates is debatable in the literature. No significant correlation between both rates was found similarly to some studies[23,24]. On the opposite, other studies reported a significant correlation between adenoma detection rate and detection rate of polyps with other histological type (sessile serrated polyps, serrated polyps and proximal serrated polyps)[12,14,25-29], but all underlined the poor correlation between A.DR and PSP.DR. Moreover, the significant correlation we found between both proximal polyps detection rates is in accordance with results from one large cohort study[23].

The mean detection rate for proximal adenomas (12.4%) was significantly lower than the 38% rate reported by Kahi *et al*[30] in a recent series of 6681 screening colonoscopies. The anatomical distribution of adenomas in the large bowel is debatable. A right-sided dominance of adenomas has been reported in some studies for both sexes[12] , or for women only[31]. Of note, we did not observe such distribution for adenomas. But, our findings are in line with data of colonoscopies following a positive FOBT in France[32]. Interestingly, data of 2821392 nationwide screening colonoscopies in Germany indicated that only 28.7% of adenomas detected were proximal to the sigmoid colon[33].

The ranges of proximal serrated polyp detection rates amongst endoscopists were 1.3%-19.3% in the present study. Two other studies by Kahi *et al*[13] (1%-18%) and Ijspeert *et al*[29] (2.9%-18.6%) reported similar rates but one study of 7215 screening colonoscopies including 32 endoscopy centers observed lower detection rate of proximal serrated polyps (mean 2.8%, range 0-9.8%)[14]. This discrepancy may be secondary to the selected population, bowel preparation quality, endoscopists' technique or skill. While the rate of clinically relevant serrated polyps was recently reported to be similar in FOBT-based screening cohorts and in primary colonoscopy screening cohorts[14], the strengths of the current cohort remains its homogeneity due to the population selection by a single indication. With regard to the population, our study underlines the fact that the prevalence of proximal serrated polyps does not differ according to age and gender[13,14,24]. The trend for a higher detection rate in males compared with females that we found in the present study is in accordance with recent findings from post-FOBT colonoscopies[34].

These results point out the substantial numbers of undetected lesions in the proximal colon in clinical practice. The wider variability observed for serrated polyps compared to adenomas amongst endosco-

pists support a more subtle appearance of serrated polyps[35,36], particularly of small and diminutive polyps because we did not find any significant variation in the detection rates for proximal polyps of a size greater than 10 mm for either adenomas or serrated polyps. We speculate that education and training are important not solely for adequate mucosal exposure, but also for identification of subtle lesions such as serrated polyps.

Individual endoscopists' adenoma detection rates have been demonstrated to be associated with interval cancer risk[9,11]. The hypothesis that the proximal serrated polyp detection rate or a composite measure for proximal polyp (*i.e.,* adenoma and/or serrated polyp) detection could predict interval CRCs even more accurately than the adenoma detection rate remains to be established.

The present study has several limitations. The preparation quality was not considered in our study. In a recent prospective study, sessile serrated polyps were detected in a significantly smaller proportion of patients with intermediate-quality preparation than high-quality preparation for the whole colon and the right colon[37]. However, it seems unlikely that it could explain the wider magnitude of variation for proximal serrated polyp detection compared with proximal adenoma detection. The withdrawal time was not considered in our study. It has been recently reported that it could affect serrated polyp detection[23,24,38]. Thus, we cannot exclude that a given withdrawal time could affect differently serrated polyp and adenoma detection. Furthermore, patient-related factors known to modify adenoma prevalence, such as smoking, obesity, or aspirin use, were not considered. By contrast, one strength of our study was the exclusion of subjects with a family history of CRC because of their particular distribution of adenomas in the colon[39]. Overall, it seems unlikely that patient-related factors could have explained the magnitude of the variability observed in our study. Nevertheless, as suggested by the variance partition coefficient values found in the current study, other factors than endoscopists could explain interendoscopist variability. Another limitation might be related to the absence of distinction of subtypes of serrated polyps. However, pooling the different histopathological types of serrated polyps is justified when considering the considerable interobserver variation in the differentiation of serrated polyps[12,40,41] and the significant correlation between both detection rates of proximal serrated polyps and clinically relevant serrated polyps[29]. Lastly, we have no information regarding the endoscopes used by the endoscopists during the study period. We hypothesize that all colonoscopies were performed with standard definition endoscopes. Thus, it remains to demonstrate that high definition endoscopes could reduce the variability for serrated polyp detection amongst endoscopists and the gap between proximal serrated polyp and proximal adenoma detection amongst endoscopists.

In conclusion, our findings demonstrate significant variability in the detection of proximal polyps, which included both adenomas and serrated polyps, amongst endoscopists. The heterogeneity was approximately twofold higher for proximal serrated polyps than for proximal adenomas detection. These findings might explain why complete colonoscopies are less effective at preventing proximal CRCs than distal ones. Furthermore, our results question the potential of using a proximal serrated polyp detection rate as a surrogate indicator for interval CRC risk.

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COMMENTS

Background

The variability of detection rate for proximal serrated polyps amongst endoscopists has been reported in the literature. However, no study aimed to compare adenomas and serrated polyps detection variability amongst endoscopists, especially for proximal colon location.

Research frontiers

The authors indicate that interendoscopist variability achieves a significant level for proximal adenomas but not for distal adenomas detection. Moreover, the heterogeneity was approximately twofold higher for proximal serrated polyps than for proximal adenomas detection.

Innovations and breakthroughs

The interendoscopist variability in detection of proximal polyps (serrated polyps and adenomas) had never been compared in previous studies.

Applications

The authors findings might explain why complete colonoscopies are less effective at preventing proximal colorectal cancer (CRCs) than distal ones. Furthermore, our results question the potential of using a proximal serrated polyp detection rate as a surrogate indicator for interval CRC risk.

Terminology

Proximal polyps are defined as polyps located above the splenic flexure. Serrated polyps were defined as an entire group of polyps that included traditional hyperplastic polyps, sessile serrated adenomas/polyps and traditional serrated adenomas.

Peer-review

The study was appropriately designed and analysed. Its result has thoroughly strong impact on routine practice, especially on screening colonoscopy program. The manuscript is clearly constructed and written in appropriate English. There is no major issues to be revised in their paper.

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ORIGINAL ARTICLE

Retrospective Study

Retrospective analysis of hepatitis C infected patients treated through an integrated care model

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Author contributions: Levin JM and Huckins E contributed to the study idea, study design, manuscript writing, and final revision of the article; Dabirshahsahebi S and Bauer M contributed to the literature search, manuscript writing, and final revision of the article.

Institutional review board statement: This study was submitted and reviewed by Dr. Harold F. Bennett, MD, PHD from the DEAN IRB and determined to be exempt from further IRB review since the research involves the study of existing data and identifiers to subjects will be coded.

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

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Abstract

AIM

To determine if our health system's integrated model reflects sustained virologic response (SVR) outcomes similar to those in clinical trial data, maximizes adherence, and averts drug interactions.

METHODS

Subjects with chronic hepatitis C had their medical records reviewed from November 1st, 2014 through March 1st, 2016. Patients eligible for treatment were entered into an integrated care model therapy algorithm. The primary outcome was SVR12 based on intention to treat (ITT) analysis. Inclusion criteria consisted of both treatment naïve and experienced patients over the age of 18 who were at least twelve weeks post-therapy completion with any genotype (GT) or METAVIR score. Secondary outcomes included adherence, adverse events, and number of drug interaction interventions.

RESULTS

At the time of analysis, 133 patients had reached twelve weeks post therapy with ITT. In the ITT analysis 70 patients were GT 1a, 26 GT 1b, 23 could not be differentiated between GT 1a or 1b, 8 GT 2, 4 GT 3, and 2 patients with multiple genotypes. The ITT treatment regimens consisted of 97 sofosbuvir (SOF)/ledipasvir (LDV), 8 SOF/LDV and ribavirin (RBV), 7 SOF

and Simeprevir (SMV), 6 3D and RBV, 1 3D, 11 SOF and RBV, and 1 SOF, peg interferon alpha, and RBV. The overall SVR12 rate was 93% in the ITT analysis with a total of 6 patients relapsing. In patients with cirrhosis, 89% obtained SVR12. All 33 patients who were previous treatment failures achieved SVR12. Drug-drug interactions were identified in 56.4% of our patient population, 69 of which required interventions made by the pharmacist. The most common side effects were fatigue (41.4%), headache (28.6%), nausea (18.1%), and diarrhea (8.3%). No serious adverse effects were reported.

CONCLUSION

Dean Health System's integrated care model successfully managed patients being treated for hepatitis C virus (HCV). The integrated care model demonstrates high SVR rates amongst patients with different levels of fibrosis, genotypes, and HCV treatment history.

Key words: Hepatitis C; Medication adherance; Direct acting antiviral; Sustained viral response; Integrated care model

Core tip: There are new effective options for treating hepatitis C virus. To maximize their effectiveness our health system developed an innovative integrated care model to manage these patients. Through our original therapy algorithm we were able to closely monitor patients from time of insurance approval to the time of obtaining a sustained virologic response (SVR). This real world retrospective study analyses our patient's SVR rate, adherence, and interventions made by the patient care team. Additionally it will provide a model for other systems to improve their care coordination and response with direct acting antiviral treatment.

Levin JM, Dabirshahsahebi S, Bauer M, Huckins E. Retrospective analysis of hepatitis C infected patients treated through an integrated care model. *World J Gastroenterol* 2016; 22(38): 8558-8567 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i38/8558.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i38.8558

INTRODUCTION

Globally, an estimated 185 million people are chronically infected with hepatitis C virus (HCV) infection with about 3.5 million individuals living with chronic HCV in the United States period HCV infection is associated with sizable morbidity and mortality with over 350000 deaths annually[1-3]. Long term effects of chronic HCV can lead to complications such as liver cirrhosis, hepatocellular carcinoma (HCC), and end-stage liver disease requiring liver transplantation[4]. Management of chronic HCV with antiviral therapy is aimed at halting disease progression, preventing cirrhosis decompensation, reducing the risk of HCC, and limiting extrahepatic complications of the infection. The goal of antiviral therapy is to eradicate HCV RNA. Historically, clinical trials of HCV treatment regimens have used a sustained virologic response (SVR) as the primary efficacy endpoint[5]. SVR is defined as undetectable HCV RNA levels 12 wk post-treatment (SVR 12)[6].

Treatment of HCV is evolving and treatment success is often based on the severity of liver fibrosis, presence of cirrhosis, previous treatment failure, and genotype (GT). For years, peg interferon alpha (PEG-IFN)/ribavirin (RBV) combination was the only treatment option. An improved understanding of the HCV genome has led to the development of multiple direct-acting antivirals (DAAs) targeted at specific proteins of the virus, resulting in the disruption of viral replication[7]. New DAAs for HCV are categorized into classes shown in Table 1, defined by their mechanism of action.

Since DAAs target critical steps of HCV replication, selection of resistant mutants is inevitable with monotherapy[9,10]. Combining HCV medications without overlapping resistance patterns, effectively shuts down viral replication which for many patients results in clearance of the virus from the liver. Available guidelines for the therapeutic management of HCV infection include the American Association for the Study of Liver Diseases in conjunction with the Infectious Diseases Society of America AASLD/IDSA, the European Association for the Study of the Liver, the United Kingdom consensus guidelines and the World Health Organization[3,11-13].

Usage of DAAs has been complicated by the high cost of therapy. However, there is also high utilization and costs for the health care system associated with treating HCV-related complications. Achievement of SVR has implications beyond those of viral eradication including improved long-term clinical outcomes, economic benefits and improved health-related quality of life[14]. Achievement of an SVR with DAAs can reduce the risk of advanced liver disease, liver transplant, and liver-related death. Research has shown that the cost associated with liver-related tests, outpatient drugs, and hospitalizations can be significantly lower for patients who achieved SVR than for those without SVR[15].

DAAs have shown remarkable cure rates with SVR12 of 90%-100% in clinical trials[16,17]. A number of phase 3 trials of patients with chronic HCV-GT1 have achieved very high SVR12 rates with different DAA drug combinations. In the ION-2 clinical trial, Ledipasvir (LDV)/Sofosbuvir (SOF) ± RBV was used for 12 or 24 wk in treatment-experienced HCV-GT1 patients with or without cirrhosis[18]. In the OPTIMIST-1 trial, Simeprevir (SMV)/SOF combination was used for 8 and 12 wk in HCV-GT1 treatment-naïve and -experienced patients without cirrhosis[19]. In TURQUOISE-II, the combination

Table 1 Direct-acting antivirals classifications[7,8]

Class (targeting non-structural proteins)	Examples
NS3/4A protease inhibitors	
First generation	telaprevir, boceprevir
Second generation	grazoprevir[1], paritaprevir[2], simeprevir
NS5A inhibitors	ledipasvir[1], ombitasvir[2], daclatasvir, elbasvir[1]
NS5B RNA-dependent RNA polymerase inhibitors	
NS5B nucleoside polymerase inhibitors	sofosbuvir
NS5B non-nucleoside polymerase inhibitors	dasabuvir[1]

[1]Only available as fixed dose combinations; [2]Available in 2 fixed dose combinations.

of ombitasvir/paritaprevir/ritonavir and dasabuvir (3D) + RBV was used for 12 or 24 wk in HCV-GT1 treatment naïve and experienced patients and compensated cirrhosis[20]. The most common adverse events in all four trials were fatigue, headache, and nausea.

In addition to high SVR12 rates with DAAs, durability of SVR and the long term virologic and clinical outcomes with DAA-only regimens have been demonstrated. Data from one of two 3-year registries showed 99.7% (5414/5433) of patients maintaining SVR with 0.3% (19/5433) having emergent virus in the SVR registry[21]. Viral emergence occurred by week 96 in all patients.

In addition to antiviral therapies, general measures in the management of patients with chronic HCV are as follows: psychological counseling, symptom management by dose adjustment of medications, and emphasizing the importance of adherence[5]. The efficacy of DAA therapy is directly proportional to the adherence of these agents. HCV cure rates in real practice are often less than what is seen in highly monitored and controlled clinical trials. Often, there is a decrease in efficacy in intention-to-treat (ITT) real world data due to higher loss to follow up, non-adherence, and insurance barriers. Traditional disconnected models between the physician and pharmacy have demonstrated diminished adherence, ineffective drug interaction management, and lower SVR outcomes compared to those seen in the clinical trials. In order to maximize the benefits of these high cost medications, our health system created an integrated care model between the clinic and pharmacy to maximize the benefits of DAA, minimize potential for drug-drug interactions, provide side effect management, and increase adherence. The purpose of this study is to determine if our health system's integrated model reflects SVR outcomes similar to those seen in clinical trial data, maximize adherence, and avert drug interactions that can impact efficacy. Our hypothesis is that patients treated through our integrated care model will demonstrate SVR rates similar to those seen in the studies based on their associated treatment status and stage of fibrosis. Additionally we anticipate the results of the study to demonstrate an increased number of drug interaction interventions and decreased number of required office visits.

MATERIALS AND METHODS

This retrospective review was conducted at Dean Clinic based in Wisconsin, United States. Patient electronic medical records were reviewed from November 1st, 2014 through March 1st, 2016. Treatment was determined by the ordering physician with recommendations made by the pharmacist based on AASLD/IDSA Guidelines. All therapies were given at FDA approved doses. HCV treatment was managed by a multidisciplinary care team comprised of an infectious disease physician, HCV nurse, and a specialty pharmacist. Patients were referred to pharmacy as treatment candidates by an infectious disease physician. Once referred to pharmacy, patients underwent insurance benefits verification and treatment authorization was submitted to the patients' insurances. Patients that were approved through their insurance followed the integrated therapy algorithm (Figure 1). The initial screening step in the therapy algorithm was for patients' medication lists, laboratory values, and fibrosis measures to be reviewed by the specialty pharmacist. All drug-drug interactions were addressed by the pharmacist and recommendations were relayed to the infectious disease physician and the physician who prescribed the interacting non-HCV medication. The patients were subsequently set up for an antiviral treatment education session where they spoke with a pharmacist and an HCV nurse educator. At the education session the patient was given a therapy calendar with dates for scheduled laboratory tests and appointments with the infectious disease physician. Proper laboratory measurements were performed at baseline and during the course of treatment based on treatment regimen. Throughout the course of treatment there were regular follow-ups scheduled by the pharmacist in addition to an office visit with the infectious disease physician at week 4 of treatment. During the pharmacist follow ups, patients were assessed for adherence, changes to their medications, and side effect management. At the end of therapy the patients were contacted by the nurse and established with post-treatment follow ups. A per protocol (PP) analysis looked at patients that started treatment with a Dean Clinic physician, completed the entire course of therapy, and were able to have an HCV viral load drawn at 12 wk post therapy. The primary outcome was SVR12 based on ITT analysis. Secondary outcomes included adherence, adverse events, and number of drug interaction interventions. Inclusion criteria consisted of both treatment naïve and

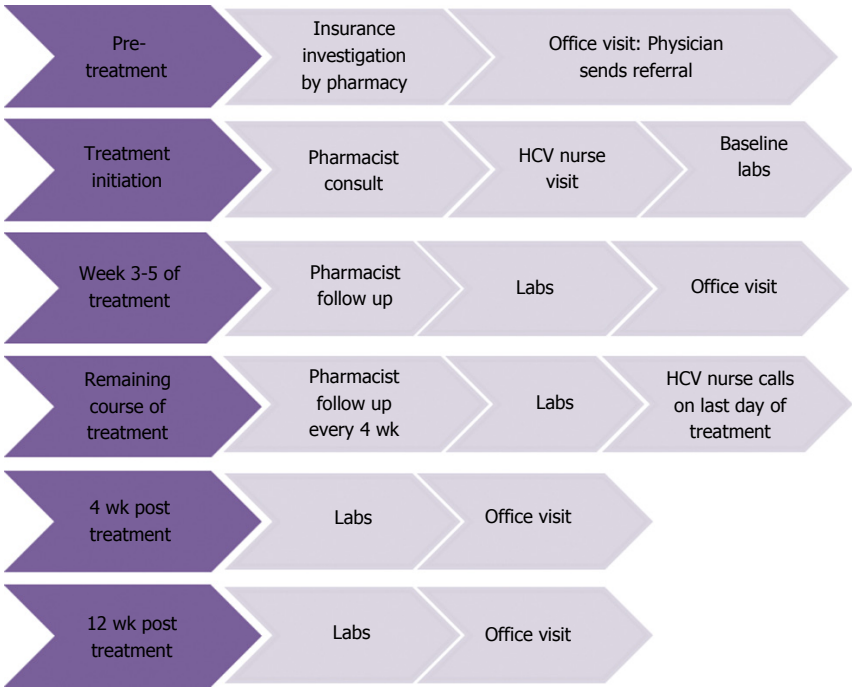


Figure 1 Therapy algorithm.

Table 2 Baseline demographics *n* (%)

Total patients	133 (100)
Median Age	58
Male	89 (66.9)
Cirrhosis	47 (35.3)
Treatment Experienced	33 (24.8)

experienced patients over the age of 18 who were at least twelve weeks post-therapy completion with any GT or METAVIR score. Adverse events were patient-reported during the course of treatment. Adherence rates were monitored using a patient-reported tablet count that was recorded during patient follow ups with the pharmacist as well as the scheduled last day of treatment.

RESULTS

At the time of analysis, 133 patients had reached twelve weeks post hyphen therapy with ITT and 130 patients with PP analyses. Baseline demographics are reported in Table 2. In the ITT analysis, 70 patients were GT 1a, 26 GT 1b, 23 could not be differentiated between GT 1a or 1b, 8 GT 2, and 4 GT 3. Two patients in the undifferentiated GT 1 group had an infection with a second GT. One patient was GT 1 and 2 and the other patient was GT 1 and 4. Two patients in the ITT analysis were lost to follow up after treatment completion. Another patient passed away from unrelated causes after achieving SVR4. A total of 33 (24.8%) patients had undergone previous treatments for hepatitis C. The ITT treatment regimens consisted of 97 SOF/LDV, 8 SOF/LDV and RBV, 7 SOF and SMV, 6 3D and RBV, 1 3D, 11 SOF and RBV, and 1 SOF, PEG-IFN, and RBV.

The overall SVR12 rate was 93% and 95% in patients who had completed the ITT and PP analysis, respectively (Figure 2). SVR12 rates were 89% and 95% for cirrhotic and non-cirrhotic patients, respectively. All treatment-experienced patients (100%) achieved SVR12 in both ITT and PP analyses. Treatment naïve patients with or without cirrhosis achieved an SVR12 rate of 94% in the PP analysis. Efficacy varied based on specific treatment regimens and genotypes (Figure 3). Further analysis was done on the patients who relapsed (n = 6). One of the patients that relapsed had GT 1b with underlying cirrhosis. The patient was treated with LDV/SOF for twelve weeks, and had break in therapy of 5 d due to insurance coverage termination. A second patient with GT 1a HCV who relapsed was treated with LDV/SOF for twelve weeks and had advanced cirrhosis and HCC. A third relapse was seen in a GT 1a cirrhotic African American patient co-infected with HIV, on efavirenz/tenofivir/emtricitabine and was being treated for HCV with LDV/SOF for twelve weeks concomitantly. A fourth GT 1a relapsed patient with cirrhosis was treated with LDV/SOF for twelve weeks and reported reusing diabetic supplies to test blood glucose during the course of treatment. A fifth patient who relapsed had GT 2 without cirrhosis, was treatment-naïve, and was treated with SOF and RBV for twelve weeks with no additional reported variables. The sixth patient relapse case was GT 1a with cirrhosis with no additional reported variables.

Overall SVR12

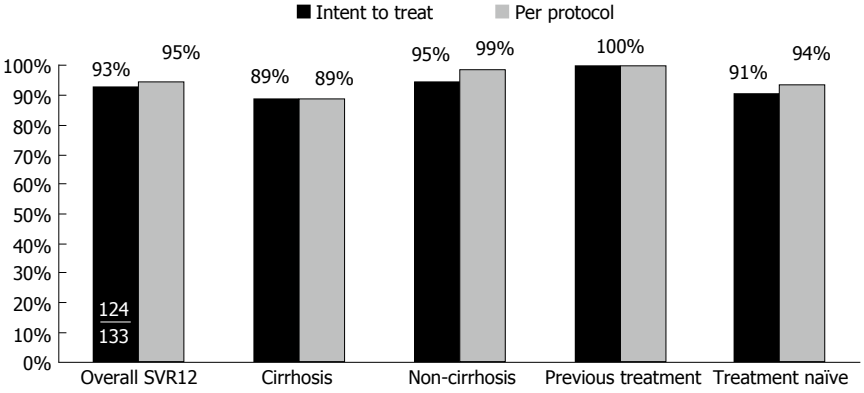
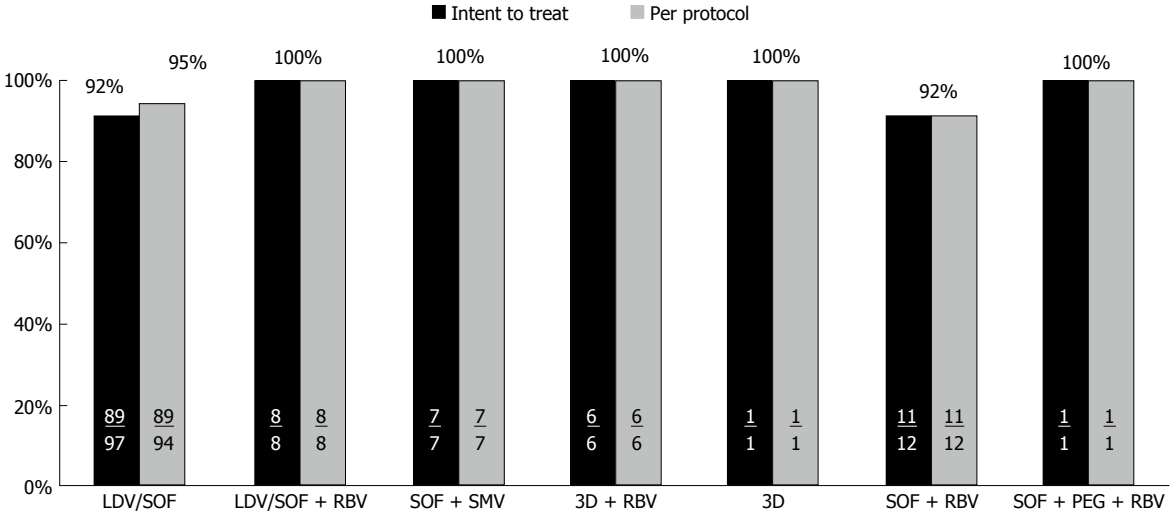


Figure 2 Overall SVR12 rate was 93% and 95% in patients who had completed the intention-to-treat and per protocol analysis respectively. SVR: Sustained virologic response.

SVR12 by treatment regimen



SVR12 by genotype

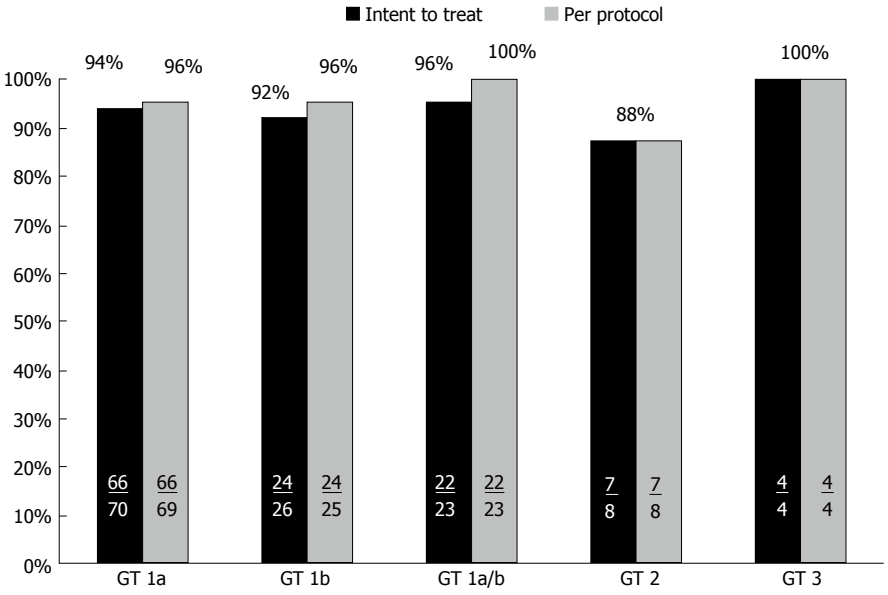


Figure 3 Efficacy varied based on specific treatment regimens and genotypes. A: SVR12 by Treatment regimen; B: SVR12 by genotype. SVR: Sustained virologic respons.

Table 3 Adverse events

Adverse events > 5%	*n* (%)
Fatigue	58 (41.4)
Headache	38 (28.6)
Nausea	24 (18.1)
Diarrhea	11 (8.3)
Dyspepsia	7 (5.3)
Anemia	7 (5.3)

The majority of patients demonstrated adverse effects; however, no patients discontinued DAA therapy prematurely due to adverse effects. The majority of the side effects reported were fatigue (41%) or headache (28.6%), most of which were mild to moderate in severity. A full list of adverse effects with a prevalence greater than 5% is reported in Table 3.

Drug-drug Interactions were identified in 56.4% of our patient population, 69 of which required interventions made by the pharmacist. The most prevalent drug-drug interaction intervention was dosing of proton pump inhibitors (PPIs) with LDV regimens (28.6%). The recommendation was made to discontinue or decrease the dose of the PPI to 20 mg omeprazole equivalent to be taken at the exact same time as the LDV/SOF. If this was not achievable, the patient was not a candidate for this therapy. Additional stomach pH related drug-drug interaction interventions included histamine 2 receptor antagonists (5.3%) or short acting antacids (9.8%). Other medication interventions (< 5%) included drug-drug interactions with phenobarbital, phenytoin, carbamazepine, milk thistle, St. John's wort, fluoxetine, clonazepam, amlodipine, and inhaled corticosteroids.

A total of 79.1% of patients had adherence rates that were 100% on the treatment algorithm. There were 17.1% of patients that missed three or less doses. One patient (0.8%) had more than 3 doses missed. Additionally, 2.4% of patients were lost to follow up after treatment without a documented adherence rate.

DISCUSSION

HCV treatment guidelines emphasize the importance of addressing adherence, adverse effects, and drug interactions with HCV regimens as clinically indicated. However, no specific recommendations are made regarding follow-up methods. Thus, effective real-world care models need to be identified for the newer DAA therapies to ensure the best HCV treatment outcomes are achieved in real-world practice settings. Our study describes an integrated multidisciplinary care team model with SVR12 rates comparable to those seen in controlled clinical trial settings. Overall SVR12 among patients in the current study was 93% in the ITT cohort and 95% in the PP cohort. Among patients with cirrhosis our SVR12 rates remained high at 89% for both PP and ITT cohorts, despite this patient population generally being more difficult to treat. Another patient population that achieved notably high SVR12 rates in our study was the treatment-experienced cohort with a 100% SVR12 rate for PP and ITT analyses. This cohort achieved a higher SVR12 rate compared to our treatment-naïve patients of which 91% in the ITT cohort and 94% in the PP cohort achieved SVR12. This was an unexpected finding we cannot explain. This was additionally unexpected because more patients in the treatment-experienced cohort were cirrhotic compared to the treatment-naïve cohort (57.6% and 28.9% cirrhotic, respectively).

SVR12 achievement rates were similar to clinical trial results based on the specific treatment regimen as well. Patients who completed LDV/SOF regimens achieved 95% SVR12 PP (92% ITT) in our study. The ION-1 study included GT 1 treatment-naïve patients with or without cirrhosis treated with a fixed-dose combination of LDV/SOF with or without RBV[16]. SVR12 rates were 99% with LDV/SOF. ION-2 included GT 1 treatment-experienced patients with or without cirrhosis treated with a fixed-dose combination of LDV/SOF with or without RBV[18]. SVR12 rates were 96% with LDV/SOF. The addition of RBV did not significantly impact SVR12 rates in our study or in ION-1 or -2; SVR12 remained high.

The SMV/SOF regimen resulted in 100% SVR12 PP (100% ITT) in our study patients. OPTIMIST-1 and OPTIMIST-2 investigated SMV/SOF among GT 1 treatment-naïve and treatment-experienced patients[19,22]. Patients without cirrhosis were included in OPTIMIST-1 and the resulting SVR12 was 97%. OPTIMIST-2 included patients with cirrhosis and the SVR12 was 84%. Although only seven patients total received the SMV/SOF regimen among our study patients, five out of the seven were cirrhotic. Our SVR12 rates of 100% were unexpectedly higher than those seen in the OPTIMIST trials.

Patients who completed the 3D plus RBV regimen achieved 100% SVR12 PP (100% ITT) in our study. Two of the six patients were cirrhotic. The SAPPHIRE Ⅰ and SAPPHIRE Ⅱ clinical trials included patients that were treatment-naïve and treatment-experienced, respectively, without cirrhosis treated with 3D plus RBV for 12 wk. SVR12 was 96% for both studies[17,23]. In the TURQUOISE Ⅱ trial, 92% of treatment-naïve or experienced patients with cirrhosis who received 3D plus RBV for 12 wk achieved SVR12[20].

Compared to other real-world analyses of newer DAA treatments, our response rates are either higher than or similar to other studies, demonstrating the effectiveness of our model. A real-world analysis of treatment-naïve or experienced patients with HCV GT 1 with or without cirrhosis was conducted on patients in the HCV-TARGET cohort treated with SMV/SOF with or without RBV[24]. The overall SVR12 rate for SMV/SOF without RBV was 85%, which was lower than the SVR12 of 100% (PP and ITT) seen in our study for

patients who were treated with the SMV/SOF regimen.

A real-world study from Israel included treatment-naïve or experienced HCV GT 1 patients with stage 3 or 4 fibrosis treated with 3D with or without RBV. Amongst the patients who completed therapy and retested 12 wk after completion, SVR12 rates were 97.8%[25]. Seven patients in our study received treatment with 3D plus RBV and only two were cirrhotic. Our SVR12 rates with this regimen were 100% for both PP and ITT analyses.

Another real-world effectiveness study from a large integrated health care system in the United States enrolled patients with GT 1 infection and receiving LDV/SOF with or without RBV. Patients were treatment-naïve or experienced and both cirrhotic and noncirrhotic. SVR12 for LDV/SOF was 93% in the ITT analysis[26]. The overall SVR12 in our study for patients treated with LDV/SOF was similar at 92% in the ITT analysis. The addition of RBV did not significantly impact SVR12 rates in either study.

Six patients in our study relapsed. One patient with GT 1b and underlying cirrhosis may have relapsed due to a 5-d break in therapy, another with GT 1a and cirrhosis was due to reinfection from reusing diabetes supplies, and one patient with GT 1a and cirrhosis relapsed for unknown reasons. The other three cases warrant further discussion. The GT 1a infected patient with advanced cirrhosis and HCC treated with LDV/SOF for 12 wk in the current study would also have been treated with RBV if evidence from the SOLAR-1 and SOLAR-2 Phase 2 trials were available at the time of treatment course selection, which may have prevented the relapse. SOLAR-1 and SOLAR-2 enrolled patients with HCV GT 1 or 4 with cirrhosis and moderate to severe hepatic impairment (Child-Pugh class B and C) with and without a history of previous liver transplant[27,28]. Patients were treated with 12 or 24 wk of a fixed-dose combination of LDV/SOF once daily plus RBV. SVR12 was 87% in non-transplant patients treated for 12 wk in SOLAR-1. In SOLAR-2, SVR12 was approximately 86% after 12 wk of treatment in non-transplant patients with GT 1.

The African American patient with GT 1a, cirrhosis, and HIV coinfection relapsed after 12 wk of treatment with LDV/SOF for an undermined reason. A recent study, ION-4, enrolled patients with HCV GT 1 or 4 coinfected with HIV-1. All patients received a 12-wk, fixed-dose combination of LDV/SOF for their HCV treatment regimen[29]. Thirty-four percent of patients in this study were black. Black patients had a lower SVR12 rate than other races (90% *vs* 99%, $P < 0.001$). Of note, 10 of the 335 patients in ION-4 relapsed and all were black. Seven of the relapsed patients had the TT allele in the gene encoding IL28B and 8 were receiving efavirenz as part of their HIV treatment regimen. Black race and presence of the TT allele were both significantly associated with relapse in ION-4. Among black patients in ION-4, 13% relapsed if they were also taking efavirenz and only 4% relapsed if they were taking other antiretroviral regimens. However, the difference was not found to be significant. It is possible that the patient in our case possesses the TT allele; however, we did not test patients in our study for the presence of this allele. Concomitantly taking efavirenz could have provoked the relapse in our patient, even though the role efavirenz plays in reduced effectiveness of HCV treatment remains unclear.

The non-cirrhotic, treatment-naive patient with GT2 who relapsed after being treated with 12 wk of SOF and RBV was somewhat surprising to us. The VALENCE trial confirmed that this same regimen is 96.7% effective in naïve, non-cirrhotic patients with GT2[30]. We cannot provide an explanation for why this particular patient relapsed.

In our study, 130 patients completed the analysis PP and 133 were in the ITT analysis. The high percentage of PP patients represents a high engagement between patient and clinical staff monitoring in our model. Furthermore, in our model, a high percentage (79.1%) of patients were 100% adherent on their treatment regimen and only one patient missed more than three doses. Other real-world studies looking at adherence demonstrated about 14% of patients were non-adherent to their treatment regimen and 18% had gaps in therapy of greater than 14 d[31]. A second study reported that 89.3% of patients completed treatment and 9% were non-adherent to therapy in a real-world setting[25].

The specialty pharmacist in our model identified drug interactions in 56.4% of patients. Sixty-nine drug interaction interventions were made with the most prevalent intervention being PPI dosing changes. Overall, drugs to lower gastric pH accounted for about 44% of all drug interaction interventions made. A study from Europe of drug-drug interactions identified that between 12%-19% of patients being treated for HCV were taking a drug that was contraindicated with one or more drugs in their HCV treatment regimen[32]. This same study showed that 29%-39% of patients were on two or more drugs that were either contraindicated or required additional monitoring or dose reduction with their HCV regimen. Similarly to our study, a high percentage (27%-38%) of interacting drugs in the Marra *et al*[32] study were drugs that target the gastrointestinal tract. The frequency and severity of drug-drug interactions with HCV therapies supports the workflow in our model where a specialty pharmacist consistently screened all patients for drug interactions.

Adverse reactions reported by our patients were consistent with those reported in DAA clinical trials and real-world experience with fatigue, headache, and nausea being the most common[24]. No serious adverse events were recorded. Drop-out rates due to adverse effects tend to be low with the newer generation DAAs, but no patients discontinued treatment for this reason in our study. One possible reason for this may be due

to close follow-up by the pharmacist on adverse effects and management strategies.

Our study had some notable limitations. A major limitation is the lack of a control group to allow a statistical comparison of the effectiveness of our integrated model compared to a non-integrated model. Only qualitative comparisons to clinical trial data and other real-world data could be made. A second limitation is that this is a single-center study and results may not be generalizable to patient populations with different demographics. The population at our site is primarily Caucasian and insured. A third limitation is that the methodology of fibrosis determination was not standardized in our protocol. A fourth limitation was that adherence was self-reported by patients *via* tablet counts. There are inherent limitations with using patient-reported information in a study. The high adherence rates reported in our study likely reflected reality as shown by the high rates of SVR12 in our patients.

The results of this study have demonstrated the need to continue to manage patients using the integrated care model in our current practice. However, the limitations of this study have showed that future research is needed to find causation for patients that relapsed on DAAs. In the scope of our practice, follow up studies will be pursued to assess the impact of adherence and how new technology may assist in increasing adherence to therapy. Additionally, future studies at our practice will analyze if there is correlation of NS5A resistance associated variants and treatment efficacy in our patient population. Furthermore, additional focus will be put on the financial savings that the integrated care model has on the system and the patient.

In conclusion, there is a scarcity of published trials that describe real-world integrated care models for successful treatment of patients with the newer DAA HCV therapies. Dean Health System's integrated care model helped successfully manage the patients being treated for HCV. The results of our study demonstrated favorable outcomes despite not being able to statistically compare across other studies. The integrated care model demonstrates high SVR rates amongst patients with different levels of fibrosis, genotypes, and HCV treatment history. The integrated care model assisted in catching and evading potential drug interactions that may have impacted treatment efficacy and tolerability. Overall, the evidence from this retrospective analysis demonstrates the benefits and value of treating HCV patients in an integrated care delivery model.

COMMENTS

Background

New direct-acting antivirals (DAAs) for hepatitis C virus (HCV) infected patients have produced sustained sustained virologic response (SVR) rates of 90%-100% in clinical trials. The efficacy of DAA therapy is directly proportional to the adherence to these agents. Traditional disconnected models between the provider and pharmacy have demonstrated diminished adherence, ineffective drug interaction management, and lower SVR outcomes compared to those seen in the clinical trials. In order to maximize the benefits of these high cost medications, our health system created an integrated care model between the clinic and pharmacy to maximize benefits of DAA therapy.

Research frontiers

The adherence to DAA therapy and their efficacy are typically lower in less monitored environments models as shown in a few prior real world reports compared to controlled clinical trials. The results of the present study suggest our health system's integrated model reflects SVR outcomes similar to those in clinical trial data.

Innovations and breakthroughs

In this study, the model was a useful tool for improving adherence rates and achieving 100% SVR12 for cirrhotic and non-cirrhotic patients on 3D plus RBV regimen. In previous 3D plus RBV clinical trials cirrhotic and non-cirrhotic patients treated showed lower SVR12 rates of up to 96%. Compared to other real-world analyses of newer DAA treatments, the response rates were either higher than or similar to other studies, demonstrating the effectiveness of this model.

Applications

The authors integrated care model between clinic staff and pharmacy helped better manage the HCV patients. This model demonstrated high cure rates, maximized adherence, and aversion of a high volume of potential drug interactions that may have impacted treatment efficacy and tolerability. Future research is needed to find causation for patients that relapsed on DAAs and follow-up studies will be pursued to assess the impact of adherence and how new technology may assist in increasing adherence to therapy.

Terminology

Integrated care model: Worldwide trend in health care reforms and new organizational arrangements focusing on more systematic coordination and integrated forms of care provision. Integrated care may be seen as a response to the fragmented delivery of health services. Medication adherence: the extent to which patients take medications as prescribed by their health care providers.

Peer-review

This manuscript was very interesting.

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ORIGINAL ARTICLE

Retrospective Study

Uncovering the uncertainty: Risk factors and clinical relevance of P1 lesions on small bowel capsule endoscopy of anemic patients

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Abstract

AIM

To identify risk factors for P1 lesions on small bowel capsule endoscopy (SBCE) and to describe the natural history of anemic patients with such type of lesions.

METHODS

One hundred patients were consecutively selected for a case-control analysis performed between 37 cases with P1 lesions and 63 controls with negative SBCE. Age, gender, comorbidities and regular medication were collected. Rebleeding, further investigational studies and death were also analyzed during the follow-up.

RESULTS

No significant differences on gender, median age or Charlson index were found between groups. Although no differences were found on the use of proton pump inhibitors, acetylsalicylic acid, anticoagulants or antiplatelet agents, the use of non-steroidal anti-inflammatory drugs (NSAID) was associated with a higher risk of P1 lesions (OR = 12.00, 95%CI: 1.38-104.1). From the 87 patients followed at our center, 39 were submitted to additional studies for investigation of iron-deficiency anemia (IDA), and this was significantly more common in those patients with no findings on SBCE (53.7% *vs* 30.3%, P = 0.033). A total of 29 patients had at least one rebleeding or IDA recurrence episode and 9 patients died of non-anemia related causes but no differences were found between cases and controls.

CONCLUSION
P1 lesions are commonly found in patients with IDA submitted to SBCE. The use of NSAID seems to be a risk factor for P1 lesions. The outcomes of patients with P1 lesions do not differ significantly from those with P0 lesions or normal SBCE.

Key words: P1 lesions; Iron-deficiency anemia; Small bowel capsule endoscopy; Risk factors; Rebleeding

Core tip: Despite the high diagnostic yield of small bowel capsule endoscopy (SBCE) in the study of iron-deficiency anemia (IDA), the clinical relevance and bleeding potential of findings such as red spots or mucosal erosions (P1 lesions) remain uncertain. We found that P1 lesions were commonly found in the SBCE of patients with IDA and their presence was associated with non-steroidal anti-inflammatory drugs use. The outcomes of patients with P1 lesions do not differ significantly from those with P0 lesions or normal SBCE. An algorithm with a stepwise approach to the patients with IDA who are submitted to SBCE is proposed.

Cúrdia Gonçalves T, Barbosa M, Rosa B, Moreira MJ, Cotter J. Uncovering the uncertainty: Risk factors and clinical relevance of P1 lesions on small bowel capsule endoscopy of anemic patients. *World J Gastroenterol* 2016; 22(38): 8568-8575 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i38/8568.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i38.8568

INTRODUCTION

Iron-deficiency anemia (IDA) is a highly common medical condition occurring in 2%-5% of adult men and postmenopausal women in developed countries[1]. These patients are often referred to gastroenterologists, accounting for up to 13% of all referrals[1]. Except for premenopausal women in whom the menstrual blood loss is the commonest cause of IDA, blood loss from the gastrointestinal (GI) tract is the most frequent reason for developing IDA. Consequently, the study of the GI tract is the cornerstone in the initial approach of these patients. While upper endoscopy and colonoscopy may reveal the causative lesion in 70%-80% of patients, there is still a reasonable proportion of patients in which these procedures will be unrevealing[2].

Recent guidelines on IDA recommend an empirical trial of iron supplementation before the study of small bowel[1]. Nonetheless, this strategy may delay a definitive diagnosis, which may be unacceptable in some subgroups of patients, particularly those with other associated GI symptoms. With the advent of small bowel capsule endoscopy (SBCE) and device-assisted enteroscopy, in the setting of normal upper and lower GI tract, the study of small bowel should be strongly considered. In fact, in these patients, about 75% of them will have a potentially bleeding lesion in the small bowel which can explain IDA[3]. Due to its wide availability, patient acceptance and safety, SBCE is recommended by current guidelines as a first-line examination, before consideration of other diagnostic modalities[2].

The diagnostic yield of SBCE in patients with IDA is variable among studies, but has been reported to be as high as 66%[4]. Although SBCE may identify different lesions, IDA cannot be equally attributable to all of them. There are certainly some lesions such as angioectasias or tumours, whose bleeding potential is greater than that of scarce red spots, submucosal veins or small bowel diverticula. Saurin *et al*[5] proposed a classification of lesions in 3 groups according to their bleeding potential: P0 lesions included visible submucosal veins, diverticula without the presence of blood, or nodules without mucosal breaks, which are believed to have no bleeding potential; P1 lesions, such as red spots on the intestinal mucosa or small or isolated erosions, were regarded as having uncertain hemorrhagic potential; and P2 lesions are those considered to have a high potential for bleeding, and include angioectasia, ulcers, tumours or varices. While several studies have focused on the clinical significance of P2 lesions and their risk factors[6,7], little is known about P1 lesions. What are the characteristics of patients with P1 lesions? Are there any risk factors for these lesions? What is the IDA recurrence rate of patients with P1 lesions? What should be the best approach in patients with IDA and P1 lesions found on SBCE? These were some unanswered questions that warranted further investigation.

Therefore, the present study was designed to better characterize patients with P1 lesions, namely their natural history, to identify risk factors for their presence in the small bowel of patients examined by SBCE, and to propose a stepwise approach to these patients.

MATERIALS AND METHODS

Study design

This was a single-center, retrospective case-control study. All the 222 patients referred to our center for the investigation of IDA between September 2008 and August 2013 were reviewed. Anemia was defined according to the World Health Organization criteria, *i.e.* hemoglobin level of < 12 g/dL in nonpregnant women and < 13 g/dL in men[1], while IDA was defined when the previous hemoglobin values were associated with a ferritin level of < 15 μg/L[8]. Premenopausal women were cautiously observed by a gynecologist and gynecologic causes for IDA were ruled out previous to SBCE referral. Patients with IDA and P1 lesions on

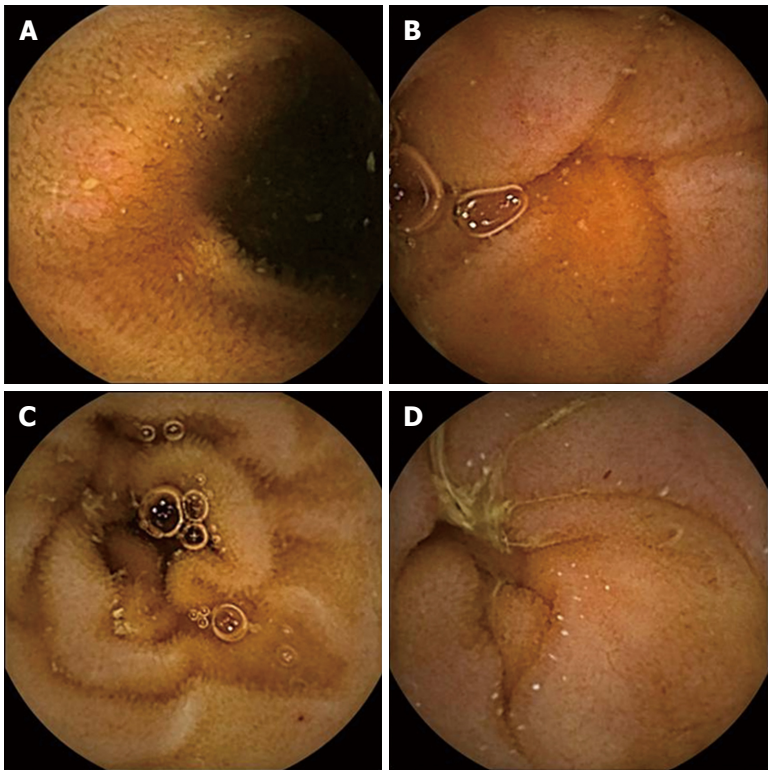

Figure 1 P1 lesions found on small bowel capsule endoscopy findings. A: isolated erosion; B: Isolated erosion; C: Red spot; D: Red spot.

SBCE were considered cases, while those patients with P0 lesions or negative examinations were included in the control group. Patients with P2 lesions were excluded of study analysis. Data from each patient was collected by reviewing medical records and included demographic data such as age and gender, as well as clinical data namely comorbidities and regular medications. The burden of comorbidities was assessed using the Charlson Comorbidity Index[9]. This index estimates the risk of death due to comorbid disease, and includes a total of 22 variables, scored 1, 2, 3 or 6, depending on the risk of dying associated with each one[9]. Relevant medication for analysis included proton pump inhibitors (PPIs), acetylsalicylic acid and other antiplatelet agents, anticoagulants, and non-steroidal anti-inflammatory drugs (NSAIDs). During the follow-up period, the performance of additional diagnostic exams for the study of IDA, IDA recurrence and death were assessed. IDA recurrence was consistently defined as hospitalization due to symptomatic anemia, decrease of > 2 g/dL in the hemoglobin value, transfusion of red blood cells, or melena or hematochezia with non-diagnostic upper endoscopy and colonoscopy, occurring > 30 d after the initial episode. This study was approved by the local Ethical Committee.

SBCE procedure

After a clear liquid diet in the previous day and a 12 h fasting period, patients were instructed to swallow the capsule (PillCam SB2 or SB3, Given Imaging, Yoqneam, Israel) with a simethicone solution to reduce bubble formation as previously reported[10]. No other specific small bowel preparation was used. To minimize the possibility of incomplete examination, capsule location was assessed using the Real Time Viewer System one hour after the beginning of the exam, and if it was still in the gastric cavity, 10 mg of domperidone were given to the patient[11]. Patients were allowed to drink clear fluids 2 h after the passage of the capsule to the stomach and to have a light snack 4 h from the beginning of the examination. Each SBCE video was analyzed by two SBCE experts (with experience of reporting more than 500 SBCE) at a speed of 12 frames per second. When no consensus was reached regarding the SBCE findings, the video was simultaneously reviewed by both experts so no discrepancy remained. No cases of capsule retention or aspiration were observed.

SBCE findings

The SBCE findings were classified according to the system reported by Saurin *et al*[5], which divides the small bowel lesions in three distinct groups: angioectasias, varices, ulcerations and tumours represent P2 lesions; red spots and small or isolated erosions are considered P1 lesions; submucosal veins, diverticula and nodules are included in the P0 lesions group. When none of these findings were found, the SBCE examination was considered negative. Examples of some P1 lesions found on SBCE are shown in Figure 1.

Table 1 Demographic and clinical characteristics of patients

Characteristic, *n* (%)	Cases (*n* = 37)	Controls (*n* = 63)	*P* value
Age, mean ± SD	57.2 ± 15.6	55.4 ± 19.5	0.609
Female gender	29 (78.4)	46 (73.0)	0.55
Mean Comorbidity Charlson Index, mean ± SD	4.1 ± 2.8	3.9 ± 3.2	0.612
PPI	8 (21.6)	17 (27.0)	1.000
Acetylsalicylic acid	15 (40.5)	14 (22.2)	0.051
Other antiplatelet agents	5 (13.5)	12 (19.0)	0.477
Anticoagulants	9 (24.3)	8 (12.7)	0.135
NSAID	6 (16.2)	1 (1.6)	**0.01**

PPI: Proton pump inhibitors; NSAID: Non-steroidal anti-inflammatory drugs.

Table 2 Follow-up characterization

Characteristic, *n* (%)	Cases (*n* = 33)	Controls (*n* = 54)	*P* value
Follow-up duration, mean ± SD	31.7 ± 17.2	38.2 ± 15.9	0.075
Further diagnostic examinations	10 (30.3)	29 (53.7)	**0.033**
IDA recurrence	9 (21.6)	20 (37.0)	0.349
Death	4 (12.1)	5 (9.3)	0.725

IDA: Iron-deficiency anemia.

Statistical analysis

Quantitative data was expressed as mean ± standard deviation. Univariate analysis was performed using the Student's *t* test for continuous variables and the χ^2 or Fisher's exact test for categorical variables. A *P* value of < 0.05 was considered to denote statistical significance. Statistical analysis was performed using the IBM SPSS Statistics for Windows version 20.0 (Armonk, New York, Unites States).

RESULTS

Patients' characteristics

Out of the 222 patients referred to our center for SBCE for the study of IDA from September 2008 until August 2013, 122 had P2 lesions on examination and were excluded from the final analysis. From the remaining 100 patients, 37 were found to have P1 lesions on small bowel (29 had small or isolated erosions, and 8 had red spots) and were included in the case group, while 63 had P0 lesions or negative examinations and were regarded as controls. The baseline characteristics of the analyzed patients are summarized in Table 1. Concerning demographic characteristics, namely mean age and gender, no significant differences were found between cases and controls. P1 lesions were not associated with a heavier burden of comorbidities as shown by the absence of significant differences in the mean Comorbidity Charlson Index between cases and controls. Regarding the regular medication, no differences were found between groups in the consumption of PPI, acetylsalicylic acid, other anti-platelet agents or anticoagulants. Contrarily, the use of NSAID was significantly higher in patients with IDA and P1 lesions (*P* = 0.01, OR = 12.0, 95%CI: 1.38-104.1).

Patients' follow-up

While 13 patients had follow-up intervals shorter than 12 mo and were excluded from this subanalysis, the remaining 87 patients had longer follow-up periods. Globally, the mean follow-up interval was 34.0 ± 16.6 mo (range from 12 to 72 mo). Data related to follow-up of cases and controls, namely its duration, submission to further diagnostic modalities, IDA recurrence and death are presented in Table 2. Thirty-three cases (37.9%) and 54 controls (62.1%) had a follow-up longer than 12 mo. No significant differences were found in the duration of follow-up between groups. The strategy of requiring further diagnostic modalities was significantly more common in the control group (*P* = 0.033). In general, in the sum of 39 cases and controls submitted to further examinations, a total of 33 upper endoscopies, 37 colonoscopies, 2 SBCE, and 3 99-mTc labeled red-blood cell scintigraphies were performed. Despite a final diagnosis could not be established in 31 (75.8%) patients, a definitive diagnosis was reached in the remaining: 4 patients had colonic angioectasia, 1 patient had Cameron's lesions, 1 patient had gastric antral vascular ectasia, 1 patient had a duodenal angioectasia, and 1 patient had a benign gastric ulcer. During the follow-up, a total of 29 patients (9 cases and 20 controls) had rebleeding, but no significant differences were found in the rebleeding rate between groups. The mean interval time between SBCE and the rebleeding episode was 17.8 mo. A total of 9 patients (4 cases and 5 controls) died during the follow-up. In all of them the cause of death was not directly attributed to IDA: 3 patients died of sepsis, 2 of terminal cirrhosis, 1 of terminal chronic kidney disease, 1 of terminal heart failure, 1 had hemorrhagic stroke, and 1 had malignant mesothelioma.

DISCUSSION

The role of SBCE in the study of IDA is currently unquestionable, as shown in different international guidelines[2,12]. Despite lacking the potential for therapeutic intervention, due to its safety, acceptance, availability, and diagnostic yield, SBCE is nowadays a first-line procedure for the study of small bowel causes for IDA. The type of lesions that can be found in patients with IDA submitted to SBCE is highly variable and include angioectasia, small bowel tumours, villous atrophy, ulcers, erosions, strictures, varices[13-15]. As the bleeding potential is not the same for all types of lesions, there was a need to classify them according their hemorrhagic potential. The most widely accepted

classification system is the one proposed by Saurin *et al*[5].

P2 lesions are known to have a high bleeding potential, with some studies reporting a rebleeding rate of up to 36.8%[16]. Different studies have also reported several factors that are associated with P2 lesions on SBCE of patients with IDA, namely NSAID and antiplatelet use, higher transfusion requirements, moderate to severe chronic kidney disease, older age, hypertension, hypercholesterolemia, and anticoagulants[6,7,17,18].

While the natural history and risk factors for P2 lesions are relatively well established, the presence of P1 lesions on the SBCE of patients with IDA and its associated uncertainty may pose serious concerns regarding the best management in this clinical scenario.

Regarding the features of patients presenting with P1 lesions, we found that although women were more commonly submitted to SBCE for the study of IDA, P1 lesions had no gender predominance. The mean age of patients in both cases and controls was in the sixth decade of life, reflecting the increasing prevalence of IDA in people older than 50 years old[19]. However, unlike P2 lesions which seem to be more common in older patients, the presence of P1 lesions does not seem to be influenced by age. It is well known that several conditions such as ischemia, collagen vascular diseases, metabolic disorders or systemic inflammatory diseases may be associated with small bowel mucosal damage. In this study we aimed to investigate if any particular condition or a higher comorbidity burden as a whole were responsible for P1 lesions found on SBCE. We found that neither any of the 22 conditions contemplated in the Charlson Comorbidity Index nor the mean Charlson Comorbidity Index value were significantly more common in the case group. Many drugs like NSAID, corticosteroids, digoxin, ferrous salts, immunosuppressors, or enteric-coated potassium have been also linked to small bowel lesions. From the analyzed medication, we found that only NSAID chronic use was significantly more common in patients with P1 lesions and IDA, being associated with a 12 fold increased risk. In fact, it has been reported that up to 75% of NSAID users will have some degree of small bowel mucosal damage, which can lead to anemia or protein loss[20,21]. The proposed mechanism is an increased mucosal permeability induced by NSAID which may lead to inflammation that manifests subsequently as reddened, edematous folds, and areas of denuded villi, petechiae, erosions and ulcers[22]. We had also particular interest in assess the potential association between the use of PPI and P1 lesions. While the gastroprotective effect of PPI is well established, being mediated not only by their anti-secretory properties, but also by inhibition of neutrophil functions and antioxidant actions[23], the effect of PPI in small bowel mucosa is still poorly understood. Some authors advocate that PPI may not be able to repair NSAID-induced small bowel mucosal damage in part because of significant lesion in mechanical barrier function and reduction in epidermal growth factor[24]. Others argue that PPI therapy may even worsen the NSAID-induced injury due to their ability to affect enteric microbial populations[25]. We found no relation between the use of PPI and P1 lesions.

We aimed to characterize the natural history of patients with IDA and P1 lesions on SBCE. According to our results, the mean follow-up time did not differ significantly between cases and controls, suggesting that the presence of P1 lesions on SBCE do not influence the decision to keep the patients under regular surveillance. Regarding IDA recurrence, we found that although no significant differences were found between groups it occurred in about a fifth of patients with P1 lesions and in about a third of patients with P0 lesions or negative SBCE, which is conformable with our previously published results[26]. In fact, it is now known that patients with a negative SBCE (that includes in most studies patients with P1 and P0 lesions) may still be at risk of rebleeding, which can be as high as 50%[27]. Until definitive guidelines about this topic are published, the approach to patients without P2 lesions on SBCE is left to case-by-case decision, but generally relies on one of two strategies: submitting patients to more examinations either to further explore potentially bleeding causes in the GI tract or alternative causes for IDA, or instead assume a "wait and see" policy. In our series, despite a final diagnosis was reached only in a minority of patients who were further studied, the causative lesion for IDA was within reach of conventional upper and lower endoscopic examinations in all of them, a possibility that have been well established in other studies[28,29]. We verified that the number of patients submitted to further diagnostic testing during the follow-up was significantly higher when P0 lesions or no lesions were found on small bowel CE, which means that P1 lesions were often interpreted as lesions that could justify patient's anemia, not requiring further investigation.

As it was our intention, and according to the presented results, we propose in Figure 2 an algorithm with a stepwise approach to the patients with IDA who are submitted to SBCE. Upon the performance of SBCE for the study of IDA, patients should be classified in one of the 3 groups according to the SBCE findings. On one hand, patients with P2 lesions should receive medical, endoscopic or surgical therapy directed to the specific finding. Additionally, these patients should discontinue NSAID and have their indications for antiplatelets and anticoagulants reassessed as they may be associated with continuous mucosal damage and rebleeding. On the other hand, patients with P1 lesions should be encouraged to stop NSAID use as they are associated with a 12 fold increased risk of small bowel mucosal damage, potentially leading to IDA and protein loss. Independently of the SBCE findings, and as the risk of IDA recurrence is not negligible even in patients with P0 lesions or a negative

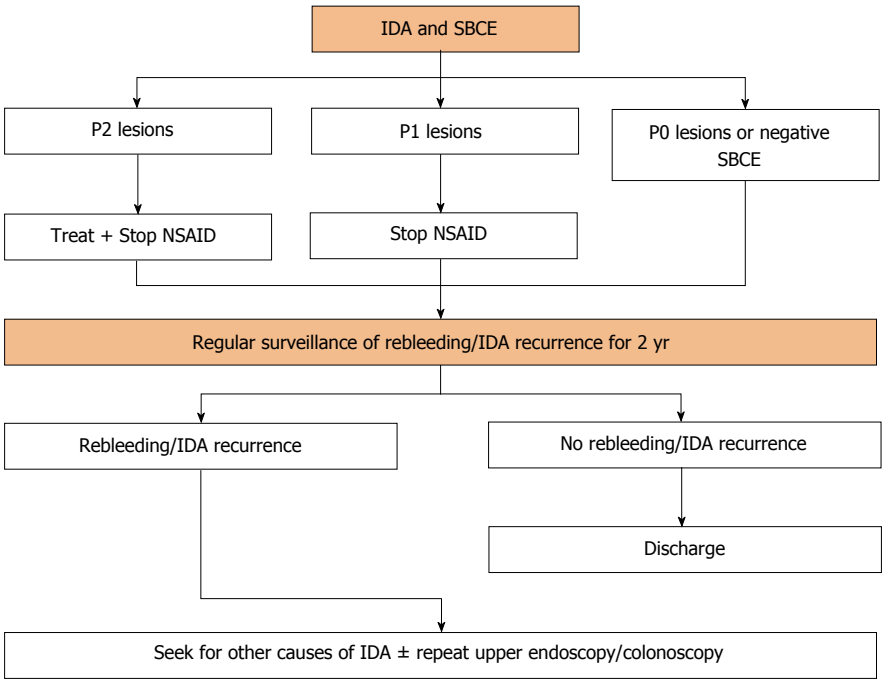

Figure 2 Approach to patients with iron-deficiency anemia submitted to small bowel capsule endoscopy. IDA: Iron-deficiency anemia; SBCE: Small bowel capsule endoscopy; NSAID: Non-steroidal anti-inflammatory drug.

small bowel CE, patients should receive regular surveillance for rebleeding or IDA recurrence during 2 years without advancing immediately to further investigations. The definition of a 2-year surveillance period is somehow arbitrary, but is in accordance with the current evidence which suggests that most patients will rebleed within the first 2 years after the index event[26]. If rebleeding or IDA recurrence do not occur during the proposed 2-year period, patients may be discharged from the Gastroenterology Clinic. However, if the patient rebleeds or presents recurrent IDA anytime during the follow-up interval, further investigation is suggested. Investigation of alternative causes of IDA such as malnutrition, haematological diseases, chronic liver or renal disease should be thoroughly seek. At this point, if GI bleeding is still strongly suspected, repeat conventional upper endoscopy and colonoscopy may prove valuable as they are cheaper, safer, readily available, and can identify a definitive diagnosis, if not in all, in the great majority of patients. Only when upper endoscopy and colonoscopy are negative, should patients be submitted to other diagnostic modalities such as device-assisted enteroscopy, computed tomography enterography or magnetic resonance enterography.

In conclusion, P1 lesions are commonly found in the SBCE of patients with IDA, particularly after the fifth decade of life. Their presence is associated with NSAID use and may in some cases represent a subtle form of NSAID enteropathy. The outcomes of patients with P1 lesions do not differ significantly from those with P0 lesions or normal SBCE, but keeping these patients under regular surveillance for at least 2 years seems to be a prudent approach. Although immediate further investigation after SBCE may not be warranted for all patients, at least those with recurrent IDA should be investigated for alternative non-gastrointestinal causes of IDA, as P1 lesions in the small bowel seem to have little or no clinical relevance.

COMMENTS

Background

Small bowel capsule endoscopy (SBCE) remains a crucial diagnostic instrument for the study of iron-deficiency anemia (IDA). Despite its high diagnostic yield, the clinical relevance and bleeding potential of findings such as red spots or isolated mucosal erosions (P1 lesions) remain uncertain.

Research frontiers

The present study was designed to better characterize patients with P1 lesions, namely their natural history, to identify risk factors for their presence in the small bowel of patients examined by SBCE, and to propose a stepwise approach to these patients.

Innovations and breakthroughs

P1 lesions are commonly found in the SBCE of patients with IDA, particularly after the fifth decade of life. Their presence is associated with non-steroidal anti-inflammatory drugs (NSAIDs) use and may in some cases represent a subtle form of NSAID enteropathy. The outcomes of patients with P1 lesions do not differ significantly from those with P0 lesions or normal SBCE. Although immediate further investigation after SBCE may not be warranted for all patients, at least those with recurrent IDA should be investigated for alternative non-gastrointestinal causes of IDA, as P1 lesions in the small bowel seem to have little or no clinical relevance.

Applications

An algorithm with a stepwise approach to the patients with IDA who are submitted to SBCE is proposed.

Terminology

SBCE findings can be classified in 3 groups of lesions according to their bleeding potential: P0 lesions included visible submucosal veins, diverticula without the presence of blood, or nodules without mucosal breaks, which are believed to have no bleeding potential; P1 lesions, such as red spots on the intestinal mucosa or small or isolated erosions, were regarded as having uncertain hemorrhagic potential; and P2 lesions are those considered to have a high potential for bleeding, and include angioectasia, ulcers, tumours or varices.

Peer-review

All to many times researchers are faced with the challenge of how to interpret the significance of a red spot seen on wireless capsule endoscopy but there is a dearth of literature on advising the risk of a rebleeding or how to properly manage the patient. When controlling for the Charleston index, the use of NSIADs appeared to be associated with a higher risk of P1 lesions translating to a 12 fold increased risk. Interestingly, P1 lesions did not have a higher risk of rebleeding whereas P2 lesions have a 36.8% rate. Moreover, from a management perspective, this study enlightens our awareness regarding NSAID use and its relationship to possible rebleeding risk. The algorithm was by the authors also presented very interesting.

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ORIGINAL ARTICLE

Retrospective Study

Combination of three-gene immunohistochemical panel and magnetic resonance imaging-detected extramural vascular invasion to assess prognosis in non-advanced rectal cancer patients

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Author contributions: Li XF wrote the paper; Li XF and Jiang Z contributed equally to this work; Li XF and Jiang Z performed the research; Li XF and Shen BZ designed the research; Jiang Z and Gao Y analyzed the data; Li CX contributed new reagents or analytic tools.

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Abstract

AIM

To identify a small, clinically applicable immunohistochemistry (IHC) panel that could be combined with magnetic resonance imaging (MRI)-detected extramural vascular invasion (EMVI) for assessment of prognosis concerning the non-advanced rectal cancer patients prior to operation.

METHODS

About 329 patients with pathologically confirmed rectal carcinoma (RC) were screened in this research, all

of whom had been examined *via* an MRI and were treatment-naïve from July 2011 to July 2014. The candidate proteins that were reported to be altered by RC were examined in tissues by IHC. All chosen samples were adopted from the fundamental cores of histopathologically confirmed carcinomas during the initial surgeries.

RESULTS

Of the three proteins that were tested, c-MYC, PCNA and TIMP1 were detected with relatively significant expression in tumors, 35.9%, 23.7% and 58.7% respectively. The expression of the three proteins were closely connected with prognosis (P = 0.032, 0.003, 0.021). The patients could be classified into different outcome groups according to an IHC panel (P < 0.01) *via* these three proteins. Taking into consideration known survival covariates, especially EMVI, the IHC panel served as an independent prognostic factor. The EMVI combined with the IHC panel could categorize patients into different prognostic groups with distinction (P < 0.01).

CONCLUSION

These studies argue that this three-protein panel of c-MYC, PCNA, coupled with TIMP1 combined with MRI-detected EMVI could offer extra prognostic details for preoperative treatment of RC.

Key words: Rectal cancer; Magnetic resonance imaging; Prognosis; Immunohistochemistry; Extramural vascular invasion

Core tip: Magnetic resonance imaging (MRI) to determine the stage of rectal cancer has been shown to be accurate and reproducible. The patients who showed MRI-detected extramural vascular invasion (EMVI) positivity had worse survival outcomes than those with EMVI negativity. However, the survival rates of patients with an EMVI-positive histology varies dramatically, which suggests that this factor does not relate to each individual tumor in terms of the biology or molecular features. The ideal staging system evolves from the consideration of them and the correlation of the prognosis with patient-specific tumor biomarkers. In this study, we hypothesized that the combination of imaging evidence of EMVI and biomolecular factors could provide additional predictive value in clinical practice, especially for non-advanced cancer patients.

Li XF, Jiang Z, Gao Y, Li CX, Shen BZ. Combination of three-gene immunohistochemical panel and magnetic resonance imaging-detected extramural vascular invasion to assess prognosis in non-advanced rectal cancer patients. *World J Gastroenterol* 2016; 22(38): 8576-8583 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i38/8576.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i38.8576

INTRODUCTION

The transanal approach may be used to effectively excise rectal carcinoma (RC), which is usually found in the lower part of the rectum. Transanal excision of RCs, however, may be associated with poor locoregional control[1,2] .Therefore, accuracy of preoperative staging is vital in modern rectal cancer management. Magnetic resonance imaging (MRI) to determine the stage of rectal cancer has been shown to be accurate and reproducible[3,4]. MRI enables planning for primary tumor resection.

Recent development in MRI provides the distinguishment of extramural vascular invasion (EMVI) prior to surgery being performed[5]. EMVI is described as blood vessels invaded by malignant cells surpassing the muscularis propria of rectal cancer[6]. Chand *et al*[7] showed that EMVI leads to worse survival outcomes[6]. However, the survival rates of patients with an EMVI-positive histology varies dramatically, which suggests that this factor does not relate to each individual tumor in terms of the biology or molecular features.

The ideal staging system will evolve from the consideration of them and the correlation of the prognosis with patient-specific tumor biomarkers[8,9]. Based on previous studies, we hypothesized that the combination of imaging evidence of EMVI and biomolecular factors could provide additional predictive value in clinical practice, especially for non-advanced cancer patients.

In this study, three candidate proteins were screened in paraffin-embedded tissue samples *via* immunohistochemistry (IHC). When combined with MRI-detected EMVI, this panel could help clinicians determine the prognosis for patients with RC without regional lymph node involvement and distant metastasis.

MATERIALS AND METHODS

Patients and tissue specimens

This study was approved by the institutional review board of Harbin Medical University, and consent was obtained from all of the patients in written form. Patients with pathologically confirmed rectal adenocarcinoma who were treatment-naïve were screened in this research, and were examined through preoperative rectal MRI at the Second Afflict Hospital of Harbin Medical University from July 2011 to July 2014. Patients who were found to have a synchronous regional lymph node and/or distant metastases at the initial staging were excluded from the study. All chosen samples were adopted from the crucial cores of histopathologically confirmed carcinomas during the initial surgeries. Two pathologists performed diagnosis on all of the lesions and the findings were reviewed by an expert colorectal cancer pathologist independently; additionally, the stage of tumor was determined according to the system of the International Union

Against Cancer.

MRI protocol

The MRI examination of patients with RC was carried out using a 3.0 T Philips Achieva TX (Philips Medical Systems, Holland) system with an 8-channel body phased array coil. Each rectal magnetic resonance examination was performed as follows: To decrease the colonic motility, an intramuscular injection of 20 mg of scopolamine butylbromide (Buscopan; Boehringer Ingelheim, Germany) was administered prior to the MRI. Approximately 100-120 mL of saline was rectally administered using an enema syringe. High-resolution magnetic resonance images were obtained, including high-resolution oblique axial T2-weighted turbo spin-echo, oblique coronal T2-weighted turbo spin-echo and sagittal T2-weighted turbo spin-echo coupled with the following parameters: TE (echo time) 81 ms-185 ms, TR (repetition time) 3900-5600 ms, thickness of 3 mm, spacing of 3 mm, matrix of 256 × 256 to 320 × 320, field of view of 250 × 250 to 199 × 199, and echo train length of 17-35.

Image analysis

All magnetic resonance images required at least three abdominal radiologists to analyze the images separately with no clinical information about the patients. The MRI analysis involved tumor morphology, the presence of EMVI, circumferential resection margin, lymph node involvement and tumor stage. The EMVI grading score was adopted from the system proposed by Smith *et al*[10]. According to this scoring system, scores of 0 to 2 were defined as EMVI-negative disease, and scores of 3 and 4 corresponded to EMVI-positive disease. A score of 3 EMVI included a tumor with a vein that did not change its contour and may have only slightly expanded the vessel. A score of 4 EMVI described an irregularly expanded vein, which indicated the vessel wall was invaded. To achieve a consensus agreement, EMVI negative cases were re-evaluated together by three abdominal radiologists.

IHC

To select the study samples, we employed a usual histological categorization according to the WHO classification of tumors. A 3-tiered histological grading system played an important role in this research. The tumor-node-metastasis stage was evaluated, based on the guideline of the 2002 International Union Against Cancer classification. Monoclonal mouse anti-human c-MYC, PCNA and TIMP-1 antibodies were introduced from Santa Cruz Biotechnology (United States). The slides were deparaffinized, rehydrated, immersed in 3% hydrogen peroxide solution for 10 min, heated in citrate buffer (pH 6.0) for 25 min at 95 ℃, and cooled for 60 min at room temperature. The slides were washed with PBS (pH 7.4) between each incubation step. Then, the slides were incubated separately with the primary antibodies. Immunoperoxidase staining was performed using the 2-step Envision Method (DAKO, Denmark) abiding by the manufacturer's instructions, and visualized with 3,3 9-diaminobenzidine tetrachloride (Sigma, United States). Negative controls were slides with no primary antibodies.

Cytoplasm and membrane staining were mensurated for the TIMP1 antibody, and c-MYC and PCNA staining showed nuclear localization. Two pathologists counted the positive cells. The scores of the results according to the immunohistochemical staining were obtained with the examiners being completely ignorant of any information related to the patient's clinical data. When it comes to clinicopathological association, the researchers applied a 4-tiered scoring system (negative to 3+), taking into consideration the proportion of positive cells and staining intensity as represented before[11].

Statistical analysis

The statistical analyses were carried out *via* the SPSS software program (standard version 19.0; SPSS, United States). The distinctions between groups were determined according to the Mann-Whitney *U* test or the Kruskal-Wallis *H* test. The correlation among categorical data underwent statistical analysis using the χ^2 test. Kaplan-Meier curves resulted from the log-rank test for survival analyses. The clinical end point in the research, the period from cancer confirmed to death starting at RC or last contact, was overall survival. Multivariate Cox proportional hazards regression analysis served in the detection of independent prognosticators, which had a conspicuous influence on patient survival. A difference was regarded to be significant under the condition that the *P*-value was less than 0.05.

RESULTS

Characteristics of the study population

A total of 329 RC specimens were collected from patients who underwent an operation. The median patient age was 62 years (range, 21-82 years), and the subjects included 122 (37.1%) females and 207 (62.9%) males. All patients underwent curative resection with tumor-free margins. Among all patients, 99 underwent adjuvant chemotherapy. All tumors were histologically diagnosed, graded, and grouped as lowly and moderately differentiated (n = 319) or highly differentiated (n = 10). The follow-up interval ranged from 1 to 58 mo from the date of diagnosis.

MRI findings

There were 53 cases (16.1%) of EMVI-positive RCs on the initial preoperative MRI scans. Of them, 30 patients (56.6%) had an EMVI score of 3, and 23 patients (43.4%) had an EMVI score of 4. Score 3 MRI-detected EMVI only occurred in small vessels. Score 4 was only observed in medium and large vessels, which was consistent with previous findings[12].

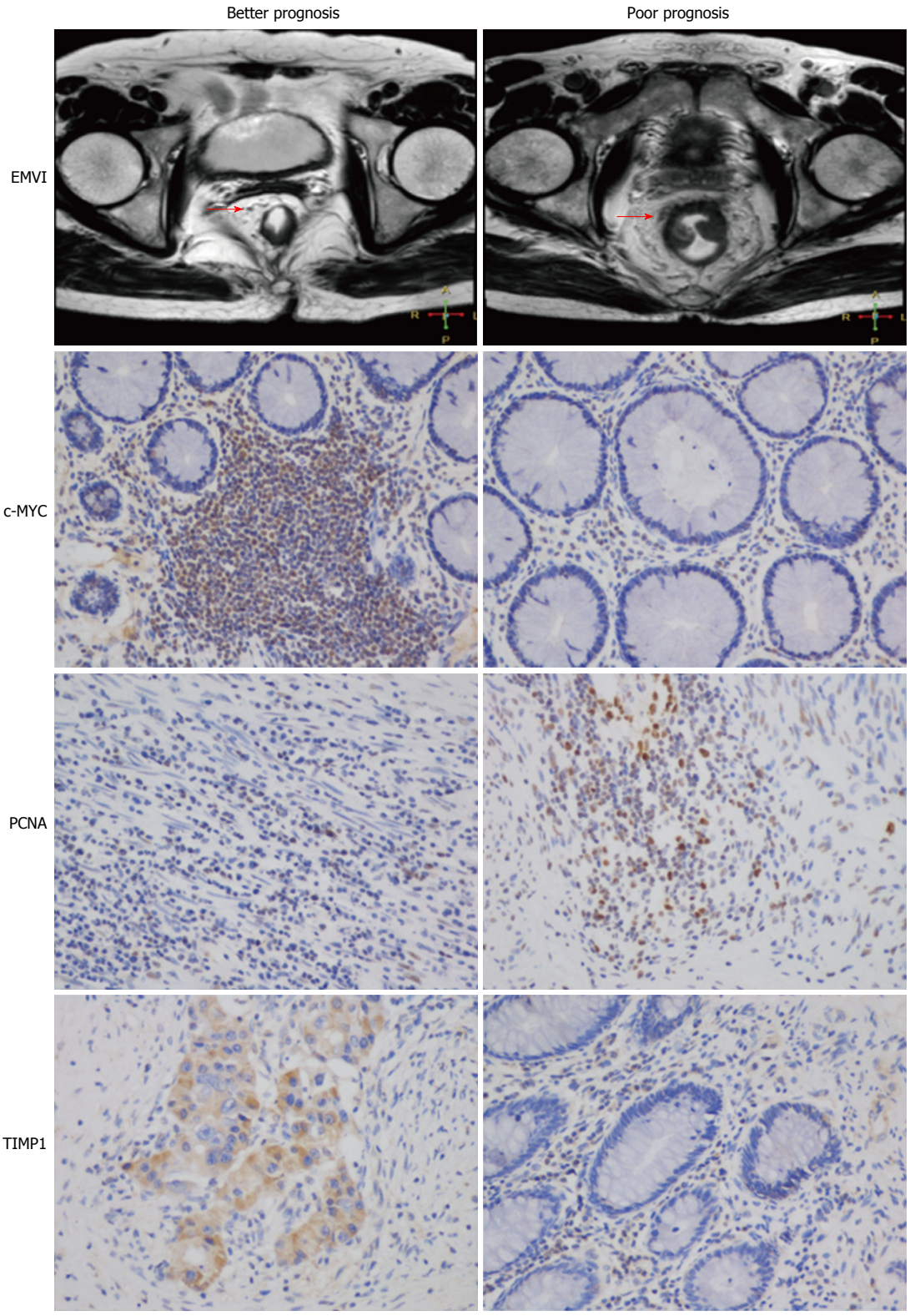

Figure 1 Representative examples of extramural vascular invasion and immunohistochemistry images in distinct prognostic groups. Overexpression of c-MYC and TIMP1 is a marker of better prognosis, whereas PCNA is a marker of poor prognosis, which was consistent with known biological roles.

Relationships between protein expression and clinicopathologic characteristics

For TIMP1, positive staining was found mainly in the cytoplasm and membrane of the neoplasm cells, and nuclear localization of c-MYC and PCNA was generally observed. Immunostaining of the three proteins was low in some samples but strong in others. Representative examples of IHC expression of TIMP1, c-MYC, and PCNA performed in the study cohorts are shown in Figure 1.

Table 1 Relationship between expression of proteins and clinicopathologic parameters

Variable	*n*	c-MYC expression		*P* value[1]	PCNA expression		*P* value[1]	TIMP1 expression		*P* value[1]
		Strong (*n* = 118)	Low (*n* = 211)		Strong (*n* = 78)	Low (*n* = 241)		Strong (*n* = 193)	Low (*n* = 136)	
Sex										
Male	207	69	136		47	160		119	88	
Female	122	49	73	0.236	31	81	0.324	74	48	0.573
Age, median (range)	62 (21-82)									
Histology grade, differentiation										
Well	10	4	6		2	8		5	5	
Moderate	143	48	95		36	107		57	86	
Poor	176	66	110	0.738	40	136	0.844	131	45	< 0.01
Pathologic T stage										
T1	24	9	15		5	19		11	13	
T2	78	30	48		7	71		41	37	
T3	74	28	46		15	59		53	21	
T4	153	51	102	0.848	51	102	< 0.01	88	65	0.045
Lymphovascular invasion										
Yes	153	56	97		37	116		90	63	
No	176	62	114	0.796	41	135	0.850	103	73	0.956
Perineural invasion										
Yes	127	45	82		32	95		88	39	
No	202	73	129	0.897	46	156	0.615	105	97	< 0.01
Chemotherapy										
Yes	99	43	56		24	75		58	41	
No	230	75	155	0.060	54	176	0.881	135	95	0.985
EMVI										
Positive	53	18	35		13	40		32	21	
Negative	276	100	176	0.752	65	211	0.878	161	115	0.782
Location										
Below peritoneal reflection	120	41	79		31	89		71	49	
At peritoneal reflection	43	22	21		4	39		25	18	
Above peritoneal reflection	166	55	111	0.080	43	123	0.059	97	69	0.990

[1]*P* value was obtained by χ^2 test. EMVI: Extramural vascular invasion.

There were no significant differences concerning age, sex, chemotherapy and tumor location. However, a significant association was detected between the expression levels of PCNA, TIMP1 and pT ($P < 0.01$; $P = 0.045$). Moreover, the overexpression of TIMP1 was associated with the histological grading and perineural invasion ($P < 0.01$; $P < 0.01$). Table 1 shows the relationships between the clinicopathological features and protein expression levels.

Independent prognostic value of three-protein IHC panel

Due to the fact that it illustrated that the three-protein panel and MRI-detected EMVI was of significant predictive value for survival in the long run, multivariate Cox regression analysis was implemented to decide whether these parameters supplied further information of prognosis independent of known clinicopathologic features which could affect the prognosis. As summarized in Table 2, each parameter had a prognostic significance independently ($P < 0.05$). Additionally, the three-protein panel displayed greater significance as an independent prognostic factor (HR = 2.110, 95%CI: 1.631-2.556, $P < 0.01$). The panel combined with the EMVI status could rank patients in a more accurate way ($P < 0.01$) (Figure 2).

DISCUSSION

Nowadays, local excision has won growing popularity as a strategy for treating RC in that it removes the necessity for colostomy, lowers the threatening perioperative risks for patients, and ensures optimistic functional outcomes[13,14]. This surgical procedure, however, requires a thorough analysis of its oncologic risks since standard resection protocols used to eliminate the rectum with its surrounding lymph nodes have gained favorable oncologic results, and are considered as the gold standard[15]. Hence, proper preoperative staging is necessary. However, staging remains challenging owing to the variable accuracy and limitations of imaging. Patient-specific tumor biomarkers could function as a useful aid to present clinicopathologic parameters for risk stratification. This study identified a three-protein panel for the prognostic assessment of non-advanced RC patients that could supplement current diagnostic systems.

Table 2 Multivariate Cox regression analysis of factors predicting survival time of patients without regional lymph node and distant metastasis

Variable	HR	95%CI	*P* value
Sex (male *vs* female)	1.312	0.784-2.159	0.354
Pathologic T stage (T3-4 *vs* T1-2)	1.226	0.817-2.657	0.012
Lymphovascular invasion (yes *vs* no)	1.413	0.716-1.971	0.241
Perineural invasion (yes *vs* no)	1.539	0.992-2.321	0.114
EMVI (positive *vs* negative)	3.071	2.784-5.754	< 0.01
c-MYC expression (low *vs* strong)	1.138	1.003-2.421	0.032
PCNA expression (strong *vs* low)	2.582	1.748-4.373	0.003
TIMP1 expression (low *vs* strong)	2.643	1.869-5.821	0.021
IHC panel (2-3 markers high *vs* 0-1 marker high)	2.110	1.631-2.556	< 0.01

EMVI: Extramural vascular invasion; IHC: Immunohistochemistry.

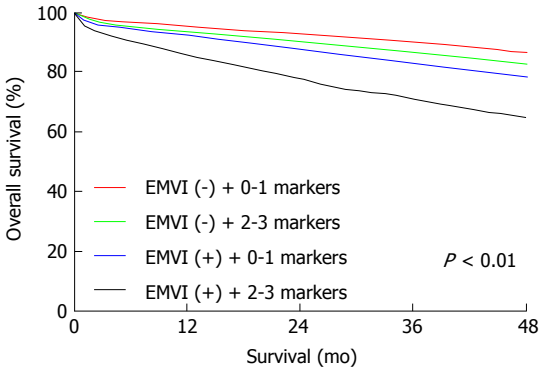

Figure 2 Immunohistochemistry panel combined with magnetic resonance imaging-detected extramural vascular invasion enhanced the accuracy of prognoses for patients with rectal carcinoma. Combination of EMVI and the IHC panel separated patients into distinct prognostic groups ($P < 0.01$). EMVI: Extramural vascular invasion; IHC: Immunohistochemistry.

The c-MYC proto-oncogene encodes a transcription factor that has a vital effect on cell proliferation, differentiation, apoptosis, metabolism, and survival[16,17]. In recent study on adenocarcinoma of the colorectum, patients with a c-MYCGCN gain had low rates of survival compared to those without the gain, which was found to be a poor independent prognostic factor[18]. However, the correlation between expression of c-MYC in colorectal cancer and prognosis was contradicted in previous studies[19-21]. The conflicting results presumably occurred as c-MYC expression is controlled by a complicated regulatory pathway, engaging multi-interactive activities with other molecules[22]. In the present study, we clarified that c-MYC overexpression determined by IHC solely was significantly linked with higher rates of survival in colorectal cancer patients when evaluated by multivariate analysis.

PCNA, which is a non-histamine nuclear protein, has been confirmed to connect with the degree of malignancy, vascular infiltration, distant metastasis and survival[23-25]. TIMPs have been proposed to be important inhibitors of tumor invasion of the extracellular matrix based on *in vitro* and animal studies[26,27]. Pellegrini *et al*[28] put forward that, from the phase of pre-invasive node to the phase of invasion, detections of serum levels of CEA and TIMP1 in colorectal cancer patients simultaneously could serve as markers for disease progression in prognosis and diagnosis.

It has been established that patients may experience a drastically increased risk of distant metastasis, given pathological EMVI at the moment of primary resection[29]. The high-resolution MRI provides the preoperative identification of EMVI[30]; preoperative MRI-detected EMVI status for rectal cancer is a crucial procedure, especially in the screening of patients for neoadjuvant treatment[31]. A microRNA panel has been shown to have considerable clinical value in the early diagnosis of colorectal cancer[32]. Through the current research, we identified a three-protein prognostic panel independent of clinicopathological features. What's more important, the three-protein panel combined with MRI-detected EMVI could rank patients into subgroups with diverse prognoses ($P < 0.01$), which may benefit clinical intervention.

In conclusion, we have identified a three-protein panel in a considerable number of rectal tissue specimens that determines prognosis with accuracy independent of clinical prognostic parameters, and it has magnificent clinical value for surgical decision-making for treating RC.

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COMMENTS

Background

Non-advanced rectal cancer (RC) can be treated using various strategies. Accuracy of preoperative staging is vital for modern management of RC. To accurately classify prognostic risks, this study set out to identify a small, clinically applicable immunohistochemistry (IHC) panel that could be combined with magnetic resonance imaging (MRI)-detected extramural vascular invasion (EMVI) for preoperative prognostic assessment of RC patients.

Research frontiers

MRI is important for the local staging of rectal cancer, the patients who showed MRI-detected EMVI positivity had worse survival outcomes than the EMVI-negative cases. Few prior reports contain analyses of the combination of imaging evidence of EMVI and biomolecular factors in RC. The results of this study suggest that the three-protein panel of c-MYC, PCNA and TIMP1 combined with MRI-detected EMVI could provide additional prognostic information for preoperative treatment of RC.

Innovations and breakthroughs

In this study, c-MYC, PCNA and TIMP1 were detected with high expression in 35.9%, 23.7% and 58.7% of tumors, respectively. Significant associations were found between the expression of these proteins and the prognosis ($P = 0.032$,

0.003, 0.021). Applying these three proteins as an IHC panel could be used to classify patients into different subgroups ($P < 0.01$). Upon adjustment for known survival covariates, including EMVI, the IHC panel remained an independent prognostic factor. The combination of EMVI and the IHC panel could separate patients into distinct prognostic groups ($P < 0.01$).

Applications

These findings suggest that the three-protein panel of c-MYC, PCNA and TIMP1 combined with MRI-detected EMVI could provide additional prognostic information for preoperative treatment of RC.

Terminology

EMVI is defined as the presence of malignant cells within the blood vessels beyond the muscularis propria of rectal cancer.

Peer-review

This is an excellent study about the combination of three-gene immunohistochemical panel and MRI-detected EMVI to assess prognosis in non-advanced rectal cancer patients.

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ORIGINAL ARTICLE

Observational Study

Racial/ethnic disparities in hepatocellular carcinoma treatment and survival in California, 1988-2012

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Abstract

AIM

To describe racial/ethnic differences in treatment and survival among liver cancer patients in a population-based cancer registry.

METHODS

Invasive cases of primary hepatocellular carcinoma, *n* = 33270, diagnosed between January 1, 1988-December 31, 2012 and reported to the California Cancer Registry were analyzed by race/ethnicity, age, gender, geographical region, socio-economic status, time period of diagnosis, stage, surgical treatment, and survival. Patients were classified into 15 racial/ethnic groups: non-Hispanic White (White, *n* = 12710), Hispanic (*n* = 8500), Chinese (*n* = 2723), non-Hispanic Black (Black, *n* = 2609), Vietnamese (*n* = 2063), Filipino (*n* = 1479), Korean (*n* = 1099), Japanese (*n* = 658), American Indian/Alaskan Native (AIAN, *n* = 281), Laotian/Hmong

(*n* = 244), Cambodian (*n* = 233), South Asian (*n* = 190), Hawai`ian/Pacific Islander (*n* = 172), Thai (*n* = 95), and Other Asian (*n* = 214). The main outcome measures were receipt of surgical treatment, and cause-specific and all-cause mortality.

RESULTS

After adjustment for socio-demographic characteristics, time period, and stage of disease, compared to Whites, Laotian/Hmong [odds ratio (OR) = 0.30, 95%CI: 0.17-0.53], Cambodian (OR = 0.65, 95%CI: 0.45-0.96), AIAN (OR = 0.66, 95%CI: 0.46-0.93), Black (OR = 0.76, 95%CI: 0.67-0.86), and Hispanic (OR = 0.78, 95%CI: 0.72-0.84) patients were less likely, whereas Chinese (OR = 1.58, 95%CI: 1.42-1.77), Koreans (OR = 1.45, 95%CI: 1.24-1.70), Japanese (OR = 1.41, 95%CI: 1.15-1.72), and Vietnamese (OR = 1.26, 95%CI: 1.12-1.42) were more likely to receive surgical treatment. After adjustment for the same covariates and treatment, cause-specific mortality was higher for Laotian/Hmong [(hazard ratio (HR) = 1.50, 95%CI: 1.29-1.73)], Cambodians (HR = 1.35, 95%CI: 1.16-1.58), and Blacks (HR = 1.07, 95%CI: 1.01-1.13), and lower for Chinese (HR = 0.82, 95%CI: 0.77-0.86), Filipinos (HR = 0.84, 95%CI: 0.78-0.90), Vietnamese (HR = 0.85, 95%CI: 0.80-0.90), Koreans (HR = 0.90, 95%CI: 0.83-0.97), and Hispanics (HR = 0.91, 95%CI: 0.88-0.94); results were similar for all-cause mortality.

CONCLUSION

Disaggregated data revealed substantial racial/ethnic differences in liver cancer treatment and survival, demonstrating the need for development of targeted interventions to mitigate disparities.

Key words: Disparities; Treatment; Survival; Liver cancer; Hepatocellular carcinoma

Core tip: We found substantial racial/ethnic differences in treatment and survival in our analysis of 33270 cases of hepatocellular carcinoma from the world's largest cancer registry in a single geo-political jurisdiction, diagnosed over a 25-year period and disaggregated into 15 racial/ethnic categories. Such granularity provides more precise identification of populations at risk by race/ethnicity, age, gender, socio-economic status, and stage of disease so that targeted interventions to mitigate disparities can be developed.

Stewart SL, Kwong SL, Bowlus CL, Nguyen TT, Maxwell AE, Bastani R, Chak EW, Chen MS Jr. Racial/ethnic disparities in hepatocellular carcinoma treatment and survival in California, 1988-2012. *World J Gastroenterol* 2016; 22(38): 8584-8595 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i38/8584.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i38.8584

INTRODUCTION

Cancer of the liver and intrahepatic bile duct, of which approximately 80% is hepatocellular carcinoma (HCC)[1], led the 17 most common cancer sites with a 3.1% average annual increase in mortality rates between 2008 and 2012 among both men and women in the United States[2]. In contrast, mortality rates declined an average of 1.8% per year among men and 1.4% among women during the same time period for all cancer sites combined[2]. HCC's prominence is further exemplified by the quadrupling of its incidence from 1.5 to 6.2 per 100000 between 1973 and 2011[3]. Worldwide, liver cancer has become the second leading cause of cancer deaths[4].

The principal risk factors for HCC are chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections[5]. After tobacco use, HBV infection, because of its etiological linkage to liver cancer, is the next most important cause of cancer worldwide[6,7]. HCV-related HCC has become the fastest increasing cause of cancer mortality in the United States[8]. In addition to alcohol consumption[8], other risk factors contributing to the increase in HCC in the United States are metabolic syndrome including diabetes[9,10] and obesity[8], which are risk factors for non-alcoholic steatohepatitis (NASH)[11]. There is evidence that the peak of the HCC epidemic may be near[3,12]. However, because HCC disproportionately affects populations of color[2]-African Americans, American Indians/Alaska Natives, Asian Americans, Hispanics, and Pacific Islanders-and because many of these racial/ethnic populations are increasing at faster rates than the population as a whole, the burden of liver cancer will continue to increase[2,13,14] unless detected earlier and properly treated or prevented[15].

Although the 5-year relative survival rate for liver cancer has risen in recent years, from 3.4% in 1975-77 to 18.1% in 2006-2012[16], it remains lower than that of most other common cancers[16]. Treatments have become more effective[11], and a larger proportion of patients are being diagnosed with early stage disease[17]. However not all HCC patients have benefited from these improvements[18]; consequently, racial/ethnic, socioeconomic, and geographic disparities in mortality persist[12,17].

Previous analyses of HCC survival and treatment characteristics have typically reported on HCC cases aggregated by ethnicity (Hispanic or non-Hispanic) accompanied by broad racial categories, *e.g.*, American Indian/Alaska Native, Asian, Black, Native Hawaiian and Other Pacific Islander[19-21], or focused on Black-White comparisons[22]. Our previous study of more than 6000 HCC cases diagnosed in California in 1988-2007, which reported on specific Asian ethnicities, found substantial inter-ethnic variation in survival but did not include comparison with other racial/ethnic groups[23]. Rarely have HCC survival and

treatment characteristics been characterized for 15 race/ethnic groups in a large geographically contiguous area over a period of 25 years.

The purpose of this study was to identify disparities in treatment and survival by race and ethnicity among more than 33000 California residents diagnosed with HCC from 1988-2012, and determine the extent to which variables such as age, gender, stage at diagnosis, and socioeconomic status explain these disparities.

MATERIALS AND METHODS

The data source for our study is the California Cancer Registry (CCR), the world's largest population-based registry with ethnic-specific data in a single contiguous political jurisdiction. The CCR covers the entire state of California and includes three Surveillance, Epidemiology, and End Results (SEER) regions: the Greater Bay Area, Los Angeles County, and Greater California. The CCR has achieved the highest standards for cancer registry quality established by the North American Association of Central Cancer Registries (NAACCR) and the National Program of Cancer Registries (NPCR) for completeness and quality. Reporting of cancer cases to the CCR has been legislatively mandated in California since 1985. The CCR includes data from all cancer cases (except basal and squamous cell carcinoma of the skin and carcinoma *in situ* of the cervix), and its completeness is estimated to be 95% or greater.

The CCR follows standardized data collection and quality-control procedures in terms of racial/ethnic categorizations and cancer diagnoses[24]. Race/ethnicity information for the HCC cases is primarily based on information contained in the patient's medical record. This information may be based on self-identification by the patient, on the assumptions by an admissions clerk or other medical personnel, or by inference using race/ethnicity of parents, birthplace, maiden name, or last name. To better identify Hispanics and Asian ethnic groups, cases were run through NAACCR Hispanic and Asian Identification Algorithm[25,26]. Cases are classified as non-Hispanic White (White), non-Hispanic Black (Black), Hispanic, American Indian/Alaskan Native, Asian American, and Native Hawaiian/Pacific Islander. Asian race is further divided into twelve groups, the nine largest in California in rank order according to their 2010 U.S. Census populations are as follows: Filipino, Chinese (including Taiwanese), Vietnamese, South Asian (Asian Indian, Pakistani, Bangladeshi, Sri Lankan), Korean, Japanese, Hmong and Laotian, Cambodian, and Thai.

In our study, Laotian and Hmong have been combined into one group because the majority of foreign-born Hmong were born in Laos[27], and older Hmong individuals may classify themselves as Laotian because they were formerly citizens of Laos[28]. South Asians, whose land of origin is the Indian subcontinent[29,30], are comprised of Asian Indian, Pakistani, other South Asian, Bangladeshi, Bhutanese, Nepalese, Sikh, and Sri Lankan. We combined cases from smaller or unknown Asian ethnic groups into an Other Asian category. Excluded from our analyses were 107 HCC cases with unknown race.

The analysis included all invasive hepatocellular carcinoma (HCC) cases diagnosed between January 1, 1988 and December 31, 2012 and reported to the CCR as of December 2015. We used the *International Classification of Diseases for Oncology, Third Edition* site code (C22.0) and histology code (8170) to identify patients with HCC among all patients with primary liver cancer. Eligibility was restricted to HCC as the first primary cancer in order to eliminate survival differences due to the effects of other cancers. Only cases with diagnostic confirmation of HCC were included in our study (92.3%). Diagnostic confirmation of HCC was defined as having positive histology (56.7%), positive radiological test (27.6%), cytology (11.2%), laboratory test/marker study (4.2%), or direct visualization (0.3%). A total of 33270 invasive HCC cases that met the above requirements were analyzed for this study.

Patient vital status was updated using both passive and active follow-up methods. Passive follow-up methods included annual record linkages with the California State death file, National Death Index, Social Security Death Master File, Medicare and Medicaid, California Department of Motor Vehicles, Voter Registration, and National Change of Address. Active follow-up methods required contacting physician's offices, hospitals, patient's relatives, and patients. The follow-up period for this study began at HCC diagnosis and ended at the earlier of the date of death or last follow-up and December 31, 2013 (the end of the latest full year of case follow-up at the time these data were reported).

Statistical analysis

We used χ^2 tests to examine bivariate relationships between race/ethnic groups and the variables displayed in Table 1. These variables included time period of diagnosis divided into five consecutive five-year intervals; age at diagnosis (< 50, 50-59, 60-69, 70-79, and 80 years or older); gender; geographical region (Los Angeles County, Greater San Francisco Bay Area, Central California, Northern California, and San Diego-Imperial-Orange Counties); stage of diagnosis (remote, regional, local, and unstaged); type of surgery (none, local, resection/transplant); and socioeconomic status (SES) on the basis of neighborhood income levels in quintiles. In categorizing type of surgery, resection and transplant were combined because SEER did not begin coding transplantation as a separate category until 1998.

Individual patient-level SES data are not collected by the CCR, and neighborhood SES was calculated using two methods. For cases diagnosed from 1988 through 2005, the index of SES was a composite

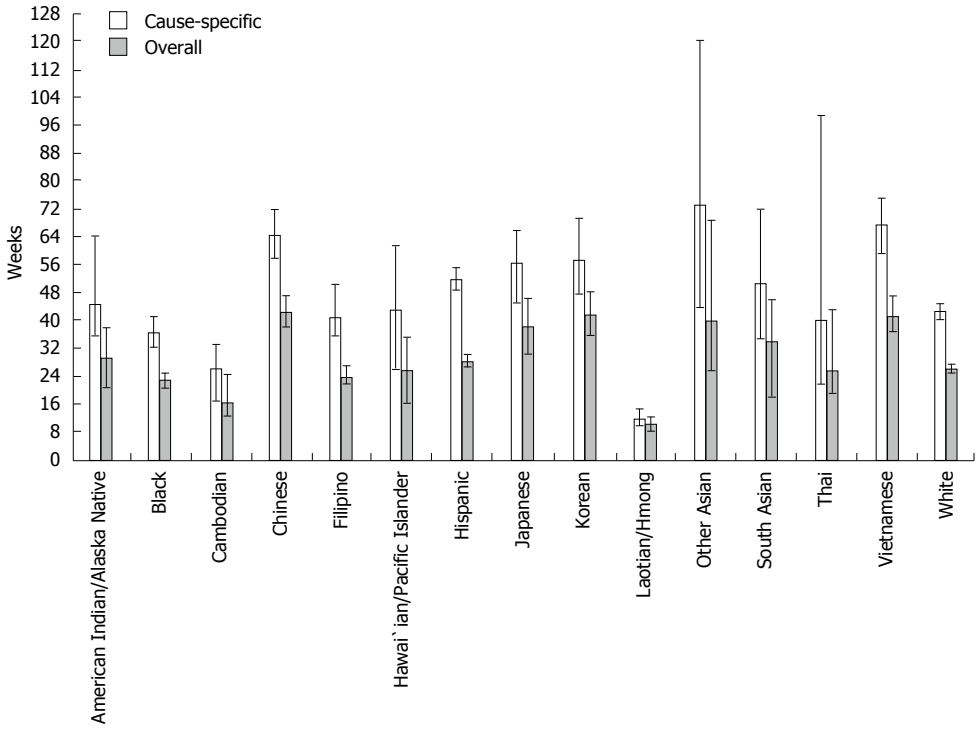

Figure 1 Median survival for patients with hepatocellular carcinoma by race/ethnicity in California, 1988-2012. Note: Error bars are limits of 95%CI.

variable created by principal components analysis using a number of variables from 1990 and 2000 Census data at the block group level. The Census variables used in creating the aggregate SES measure included: education index, median household income, proportion below 200% of the poverty level, median rent, median house value, proportion with a blue collar job, and proportion older than 16 in the workforce without a job. Block group quintiles based on statewide measurement of the SES variable were used in the analysis[31]. For cases diagnosed from 2006 through 2012, a composite variable was also created by principal components analysis using variables from the American Community Survey (ACS) at the block group level. The index used the following variables: education index, percent persons with a ratio of household income to poverty line 2 or higher (percent persons above 200% poverty line), percent persons with a blue collar job, percent persons employed, median rental, median value of owner-occupied housing unit, and median household income. The SES index could not be calculated if any of the seven components were missing. Missing values were imputed using multiple imputation, and the SES index was based on the imputed data[32].

The main difference between the two SES indexes is that the index based on the ACS used the inverse complement of two variables used from the 2000 Census: percent unemployed (2000 Census) and percent less than 200% of poverty line (ACS). Cases missing Census block group due to incomplete address at time of diagnosis (4.9% of patients) were randomly allocated to census block groups within county of residence because excluding these cases has been shown to bias results. Each case was assigned a neighborhood SES quintile, based on the distribution of SES across census block groups in California.

Logistic regression was used to evaluate the association between race/ethnicity and receipt of surgical treatment (any *vs* none) before and after adjustment for time period of diagnosis, age, gender, geographic region, SES quintile, and stage at diagnosis; because prioritization for transplantation for HCC changed in 2002[33], we included an interaction between time period and stage in the multivariable model, allowing estimation of stage effects for each time period and time period effects at the referent level of stage. Odds ratios (OR) and 95%CI are shown in Table 2.

Kaplan-Meier methods were used to estimate cause-specific and overall survival curves for each of the race/ethnic groups, and the log-rank test was used to assess racial/ethnic differences in survival. Median survival times with 95%CI are presented in Figure 1. Cox proportional hazards models were used to evaluate the association between race/ethnicity and survival, before and after adjustment for the effects of time period of diagnosis, age, gender, geographic region, SES quintile, stage at diagnosis, and type of surgery. Both cause-specific and all cause hazard ratios were calculated. Using non-Hispanic White as the referent group, hazard ratios (HR) and 95%CI were calculated for death from HCC. Survival time was measured in weeks from the date of diagnosis to death or censoring. People who were still alive on December 31, 2013 were censored

Table 1 Demographic and tumor characteristics by race/ethnic groups among patients with hepatocellular carcinoma in California, 1988-2012 (*n* = 33270)

	American Indian/ Alaska Native (*n* = 281)		Black (*n* = 2609)		Cambodian (*n* = 233)		Chinese (*n* = 2723)		Filipino (*n* = 1479)		Hawai`ian/ Pacific Islander (*n* = 172)		Hispanic (*n* = 8500)		Japanese (*n* = 658)		Korean (*n* = 1099)		Laotian/ Hmong (*n* = 244)		Other Asian (*n* = 214)		South Asian (*n* = 190)		Thai (*n* = 95)		Vietnamese (*n* = 2063)		White (*n* = 12710)	
	n	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Age at diagnosis																														
< 50	47	16.7	323	12.4	63	27.0	437	16.0	219	14.8	30	17.4	1170	13.8	30	4.6	172	15.7	65	26.6	44	20.6	13	6.8	29	30.5	326	15.8	1181	9.3
50-59	102	36.3	936	35.9	78	33.5	579	21.3	324	21.9	58	33.7	2604	30.6	116	17.6	284	25.8	62	25.4	58	27.1	44	23.2	27	28.4	533	25.8	3705	29.2
60-69	90	32.0	833	31.9	57	24.5	744	27.3	339	22.9	46	26.7	2424	28.5	219	33.3	346	31.5	72	29.5	59	27.6	65	34.2	22	23.2	596	28.9	3640	28.6
70-79	34	12.1	391	15.0	19	8.2	689	25.3	403	27.2	30	17.4	1657	19.5	206	31.3	233	21.2	31	12.7	44	20.6	45	23.7	13	13.7	471	22.8	2831	22.3
⩾ 80	8	2.8	126	4.8	16	6.9	274	10.1	194	13.1	8	4.7	645	7.6	87	13.2	64	5.8	14	5.7	9	4.2	23	12.1	-[1]	-[1]	137	6.6	1353	10.6
Gender																														
Male	214	76.2	1971	75.6	182	78.1	2078	76.3	1111	75.1	138	80.2	6368	74.9	285	43.3	761	69.2	188	77.0	155	72.4	138	72.6	73	76.8	1622	78.6	9762	76.8
Female	67	23.8	638	24.4	51	21.9	645	23.7	368	24.9	34	19.8	2132	25.1	373	56.7	338	30.8	56	23.0	59	27.6	52	27.4	22	23.2	441	21.4	2948	23.2
Socioeconomic status (SES)																														
1 - Low SES	75	26.7	867	33.2	113	48.5	377	13.8	204	13.8	30	17.4	2975	35.0	49	7.4	176	16.0	129	52.9	31	14.5	13	6.8	12	12.6	316	15.3	1599	12.6
2	82	29.2	586	24.8	42	18.0	428	15.7	286	19.3	42	24.4	2248	26.4	118	17.9	184	16.7	68	27.9	38	17.8	32	16.8	31	32.6	541	26.2	2512	19.8
3	72	25.6	483	18.5	33	14.2	488	17.9	367	24.8	40	23.3	1621	19.1	153	23.3	188	17.1	26	10.7	42	19.6	38	20.0	20	21.1	481	23.3	2977	23.4
4	36	12.8	409	15.7	26	11.2	660	24.2	402	27.2	34	19.8	1054	12.4	164	24.9	255	23.2	19	7.8	63	29.4	60	31.6	18	18.9	400	19.4	2942	23.1
5 - High SES	16	5.7	203	7.8	19	8.2	770	28.3	220	14.9	26	15.1	602	7.1	174	26.4	296	26.9	-[1]	-[1]	40	18.7	47	24.7	14	14.7	325	15.8	2680	21.1
Region																														
San Francisco-Oakland	44	15.7	753	28.9	31	13.3	1482	54.4	517	35.0	62	36.0	1329	15.6	171	26.0	178	16.2	28	11.5	79	36.9	57	30.0	14	14.7	701	34.0	2637	20.7
Central California	78	27.8	335	12.8	24	10.3	78	2.9	109	7.4	21	12.2	2129	25.0	65	9.9	67	6.1	75	30.7	39	18.2	36	18.9	11	11.6	104	5.0	2705	21.3
Northern California	100	35.6	323	12.4	38	16.3	152	5.6	126	8.5	22	12.8	635	7.5	92	14.0	34	3.1	85	34.8	13	6.1	40	21.1	7	7.4	116	5.6	2578	20.3
San Diego-Imperial-Orange	33	11.7	206	7.9	22	9.4	173	6.4	263	17.8	28	16.3	1258	14.8	112	17.0	197	17.9	43	17.6	19	8.9	29	15.3	8	8.4	760	36.8	2208	17.4
Los Angeles	26	9.3	992	38.0	118	50.6	838	30.8	464	31.4	39	22.7	3149	37.0	218	33.1	623	56.7	13	5.3	64	29.9	28	14.7	55	57.9	382	18.5	2582	20.3
Stage at diagnosis																														
Local	109	38.8	987	37.8	96	41.2	1179	43.3	584	39.5	73	42.4	3825	45.0	288	43.8	450	40.9	71	29.1	102	47.7	84	44.2	38	40.0	947	45.9	5355	42.1
Regional	85	30.2	668	25.6	61	26.2	606	22.3	384	26.0	43	25.0	2010	23.6	142	21.6	267	24.3	52	21.3	48	22.4	53	27.9	22	23.2	498	24.1	2988	23.5
Remote	62	22.1	682	26.1	55	23.6	645	23.7	362	24.5	44	25.6	1812	21.3	149	22.6	242	22.0	88	36.1	49	22.9	40	21.1	24	25.3	437	21.2	2905	22.9
Unstaged	25	8.9	272	10.4	21	9.0	293	10.8	149	10.1	12	7.0	853	10.0	79	12.0	140	12.7	33	13.5	15	7.0	13	6.8	11	11.6	181	8.8	1462	11.5
Time period of diagnosis																														
1988-1992	9	3.2	217	8.3	22	9.4	334	12.3	161	10.9	14	8.1	557	6.6	84	12.8	114	10.4	29	11.9	9	4.2	12	6.3	7	7.4	128	6.2	1257	9.9
1993-1997	14	5.0	319	12.2	30	12.9	435	16.0	195	13.2	25	14.5	809	9.5	108	16.4	178	16.2	41	16.8	11	5.1	15	7.9	12	12.6	271	13.1	1681	13.2
1998-2002	49	17.4	472	18.1	45	19.3	543	19.9	304	20.6	32	18.6	1461	17.2	139	21.1	267	24.3	56	23.0	51	23.8	29	15.3	21	22.1	429	20.8	2252	17.7
2003-2007	88	31.3	683	26.2	61	26.2	695	25.5	408	27.6	41	23.8	2340	27.5	166	25.2	270	24.6	49	20.1	50	23.4	61	32.1	16	16.8	575	27.9	3208	25.2
2008-2012	121	43.1	918	35.2	75	32.2	716	26.3	411	27.8	60	34.9	3333	39.2	161	24.5	270	24.6	69	28.3	93	43.5	73	38.4	39	41.1	660	32.0	4312	33.9
Type of surgery																														
None	238	84.7	2214	84.9	198	85.0	2007	73.7	1220	82.5	138	80.2	7062	83.1	491	74.6	805	73.2	230	94.3	158	73.8	145	76.3	75	78.9	1536	74.4	10117	79.6
Local	20	7.1	150	5.7	13	5.6	184	6.8	60	4.1	10	5.8	550	6.5	58	8.8	83	7.6	7	2.9	16	7.5	18	9.5	-[1]	-[1]	194	9.4	925	7.3
Resection/transplant	23	8.2	245	9.4	22	9.4	532	19.5	199	13.5	24	14.0	888	10.4	109	16.6	211	19.2	7	2.9	40	18.7	27	14.2	16	16.8	333	16.1	1668	13.1

[1]Less than 5 cases. χ^2, $P < 0.0001$ for racial/ethnic differences in all variables tabulated.

on that date; in cause-specific survival analyses, because the outcome of interest was death due to HCC, people who died of other causes before that date were censored at date of death. All analyses were performed using SAS version 9.3 (SAS Institute Inc., Cary, NC); statistical significance was assessed at the 0.05 level (2-sided).

RESULTS

Patient characteristics

A total of 33270 patients in the designated race/ethnic groups were diagnosed with HCC in California in 1988-2012 and reported to the CCR as of December 31, 2015. The largest number were identified as non-Hispanic White (n = 12710), followed by Hispanic (n = 8500), Chinese (n = 2723), non-Hispanic Black (n = 2609), Vietnamese (n = 2063), Filipino (n = 1479), and Korean (n = 1099). As shown in Table 1, the distributions of all patient characteristics differed significantly by race ethnicity (P < 0.0001). There was a male predominance of cases in all groups (69%-80%), except Japanese (43%). Overall, 12% were under age 50 at diagnosis, with highest proportions under 50 among Thai (31%), Cambodians (27%), and Laotian/Hmong (27%). There were substantial disparities in SES: those most likely to live in lowest quintile neighborhoods were Laotian/Hmong (53%), Cambodians (48%), Hispanics (35%), and Blacks (33%). There were also disparities in stage at diagnosis and receipt of treatment. Those least likely to be diagnosed with local stage tumors (43% overall) were Laotian/Hmong (29%), Blacks (38%), AIANs (39%), and Filipinos (39%). Those least likely to receive a resection or transplant (13% overall) were Laotian/Hmong (3%), AIANs (8%), Blacks (9%), and Cambodians (9%).

Receipt of surgical treatment

Compared to Whites, Laotian/Hmong (OR = 0.24, 95%CI: 0.14-0.41), Cambodians (OR = 0.69, 95%CI: 0.48-0.99), Blacks (OR = 0.70, 95%CI: 0.62-0.78), AIAN (OR = 0.71, 95%CI: 0.51-0.98), Hispanics (OR = 0.79, 95%CI: 0.74-0.85), and Filipinos (OR = 0.83, 95%CI: 0.72-0.95) were less likely, and Koreans (OR = 1.43, 95%CI: 1.24-1.64), Chinese (OR = 1.39, 95%CI: 1.27-1.53), Other Asians (OR = 1.38, 95%CI: 1.02-1.88), Vietnamese (OR = 1.34, 95%CI:1.20-1.49), and Japanese (OR = 1.33, 95%CI: 1.11-1.59) were more likely to receive surgical treatment (Table 2). After adjustment for demographic characteristics, time period, and stage of disease, Laotian/Hmong (OR = 0.30, 95%CI: 0.17-0.53), Cambodian (OR = 0.65, 95%CI: 0.45-0.96), AIAN (OR = 0.66, 95%CI: 0.46-0.93), Black (OR = 0.76, 95%CI: 0.67-0.86), and Hispanic (OR=0.78, 95%CI 0.72-0.84) patients were less likely, whereas Chinese (OR = 1.58, 95%CI:1.42-1.77), Koreans (OR = 1.45, 95%CI: 1.24-1.70), Japanese (OR = 1.41, 95%CI: 1.15-1.72), and Vietnamese (OR = 1.26, 95%CI: 1.12-1.42) were more likely to receive surgical treatment. The odds of treatment decreased with age (over 80 *vs* under 50: OR = 0.20, 95%CI: 0.17-0.24) and increased with SES (highest vs. lowest quintile: OR = 1.84, 95%CI: 1.66-2.05); males were less likely than females to be treated (OR = 0.82, 95%CI: 0.77-0.88). The time X stage interaction was statistically significant (P = 0.0014): patients with local stage disease had a greater advantage over those with remote stage disease in 2003-2012 (ORs = 13.0 and 13.6) than in 1988-2002 (ORs = 8.28, 8.81, and 8.07).

Survival

Kaplan-Meier analysis: Both cause-specific and overall survival differed significantly by race/ethnicity (log-rank P < 0.0001). Cause-specific median survival in weeks (Figure 1) was lowest for Laotian/Hmong (11.6, 95%CI: 9.6-14.4), followed by Cambodians (26.1, 95%CI: 16.7-33.1), Blacks (36.3, 95%CI: 32.1-41.0), Thai (39.9, 95%CI: 21.7-98.7), Filipinos (41.0, 95%CI: 35.4-50.0), Whites (42.4, 95%CI: 40.1-44.6), Hawai`ians/Pacific Islanders (42.7, 95%CI: 25.7-61.4), AIANs [44.6, 95%CI: (35.6-64.1)], South Asians (50.6, 95%CI: 34.4-72.0), Hispanics (51.9, 95%CI: 48.4-55.3), Japanese (56.3, 95%CI: 44.9-65.9), Koreans (57.4, 95%CI: 47.6-69.4), Chinese (64.4, 95%CI: 57.7-71.7), Vietnamese (67.3, 95%CI: 59.1-75.0), and Other Asians (73.3, 95%CI: 43.7-120.3). Results were similar for all cause survival.

Cox proportional hazards models: Compared to Whites, higher cause-specific mortality was experienced by Laotian/Hmong [hazard ratio (HR) = 1.91, 95%CI: 1.65-2.21], Cambodians (HR = 1.38, 95%CI: 1.18-1.60), and Blacks (HR = 1.12, 95%CI: 1.06-1.18), and lower mortality by Other Asians (HR = 0.80, 95%CI 0.67-0.96), Chinese (HR = 0.81, 95%CI: 0.77-0.86), Vietnamese (HR = 0.83, 95%CI: 0.79-0.89), Koreans (HR = 0.87, 95%CI: 0.80-0.93), and Hispanics (HR = 0.91, 95%CI: 0.88-0.95) (Table 3). After adjustment for demographics, time period, stage of disease, and treatment, mortality remained higher for Laotian/Hmong (HR = 1.50, 95%CI: 1.29-1.73), Cambodians (HR = 1.35, 95%CI: 1.16-1.58), and Blacks (HR = 1.07, 95%CI: 1.01-1.13), and was lower for Chinese (HR = 0.82, 95%CI: 0.77-0.86), Filipinos (HR = 0.84, 95%CI: 0.78-0.90), Vietnamese (HR = 0.85, 95%CI: 0.80-0.90), Koreans (HR = 0.90, 95%CI: 0.83-0.97), and Hispanics (HR = 0.91, 9 95%CI: 0.88-0.94). Lower mortality was associated with younger age, female gender, earlier stage disease, receipt of surgical treatment, higher SES, and later time period of diagnosis. Results were similar for all-cause mortality.

Table 2 Factors associated with receipt of surgical treatment for hepatocellular carcinoma in California, 1988-2012 (n = 33270)

	Receipt of surgical treatment			
	Unadjusted OR	95%CI	Adjusted[1] OR	95%CI
American Indian/Alaska Native	0.71[a]	0.51-0.98[a]	0.66[a]	0.46-0.93[a]
Black	0.70[a]	0.62-0.78[a]	0.76[a]	0.67-0.86[a]
Cambodian	0.69[a]	0.48-0.99[a]	0.65[a]	0.45-0.96[a]
Chinese	1.39[a]	1.27-1.53[a]	1.58[a]	1.42-1.77[a]
Filipino	0.83[a]	0.72-0.95[a]	0.92	0.79-1.07
Hawai`ian/Pacific Islander	0.96	0.66-1.40	0.94	0.62-1.40
Hispanic	0.79[a]	0.74-0.85[a]	0.78[a]	0.72-0.84[a]
Japanese	1.33[a]	1.11-1.59[a]	1.41[a]	1.15-1.72[a]
Korean	1.43[a]	1.24-1.64[a]	1.45[a]	1.24-1.70[a]
Laotian/Hmong	0.24[a]	0.14-0.41[a]	0.30[a]	0.17-0.53[a]
Other Asian	1.38[a]	1.02-1.88[a]	1.27	0.91-1.78
South Asian	1.21	0.86-1.70	1.13	0.78-1.63
Thai	1.04	0.63-1.71	1.04	0.61-1.78
Vietnamese	1.34[a]	1.20-1.49[a]	1.26[a]	1.12-1.42[a]
White	1.00		1.00	
Age at diagnosis				
< 50			1.00	
50-59			0.83[a]	0.76-0.92[a]
60-69			0.76[a]	0.69-0.84[a]
70-79			0.47[a]	0.42-0.52[a]
⩾ 80			0.20[a]	0.17-0.24[a]
Gender				
Female			1.00	
Male			0.82[a]	0.77-0.88[a]
Socioeconomic Status				
1 - Low SES			1.00	
2			1.25[a]	1.13-1.37[a]
3			1.29[a]	1.17-1.42[a]
4			1.65[a]	1.49-1.82[a]
5 - High SES			1.84[a]	1.66-2.05[a]
Region				
Los Angeles			1.00	
San Francisco-Oakland			0.81[a]	0.74-0.88[a]
Central California			0.96	0.87-1.05
Northern California			1.09	0.98-1.20
San Diego-Imperial-Orange			1.34[a]	1.22-1.47[a]
Stage at diagnosis: 1988-1992				
Remote			1.00	
Regional			3.30[a]	2.29-4.75[a]
Local			8.28[a]	6.09-11.26[a]
Unstaged			0.27[a]	0.15-0.48[a]
Stage at diagnosis: 1993-1997				
Remote			1.00	
Regional			2.88[a]	2.02-4.11[a]
Local			8.81[a]	6.79-11.43[a]
Unstaged			0.47[a]	0.27-0.79[a]
Stage at diagnosis: 1998-2002				
Remote			1.00	
Regional			2.61[a]	2.01-3.40[a]
Local			8.07[a]	6.49-10.04[a]
Unstaged			0.78	0.52-1.17
Stage at diagnosis: 2003-2007				
Remote			1.00	
Regional			4.44[a]	3.44-5.73[a]
Local			12.97[a]	10.16-16.56[a]
Unstaged			0.93	0.58-1.49
Stage at diagnosis: 2008-2012				
Remote			1.00	
Regional			4.50[a]	3.43-5.92[a]
Local			13.6[a]	10.48-17.66[a]
Unstaged			1.12	0.68-1.87
Time period of diagnosis: remote stage tumors				

1988-1992	1.00	
1993-1997	0.75	0.53-1.06
1998-2002	0.89	0.64-1.23
2003-2007	0.83	0.58-1.17
2008-2012	0.59[a]	0.41-0.85[a]

[1]Adjusted for all other factors presented in the table using multivariable logistic regression. [a]$P < 0.05$. Time period X stage interaction $P = 0.0014$.

Table 3 Factors associated with survival from hepatocellular carcinoma in California, 1988-2012 (n = 33270)

	Cause-specific survival				All cause survival			
	Unadjusted HR	95%CI	Adjusted[1] HR	95%CI	Unadjusted HR	95%CI	Adjusted[1] HR	95%CI
American Indian/ Alaska Native	0.92	0.79-1.07	0.90	0.77-1.04	0.94	0.82-1.07	0.91	0.80-1.04
Black	1.12[a]	1.06-1.18[a]	1.07[a]	1.01-1.13[a]	1.12[a]	1.07-1.17[a]	1.06[a]	1.01-1.11[a]
Cambodian	1.38[a]	1.18-1.60[a]	1.35[a]	1.16-1.58[a]	1.31[a]	1.15-1.51[a]	1.27[a]	1.11-1.46[a]
Chinese	0.81[a]	0.77-0.86[a]	0.82[a]	0.77-0.86[a]	0.75[a]	0.72-0.79[a]	0.76[a]	0.72-0.79[a]
Filipino	0.95	0.88-1.01	0.84[a]	0.78-0.90[a]	0.98	0.92-1.04	0.88[a]	0.83-0.93[a]
Hawai`ian/ Pacific Islander	0.95	0.78-1.15	0.98	0.81-1.19	0.97	0.82-1.14	1.00	0.85-1.18
Hispanic	0.91[a]	0.88-0.95[a]	0.91[a]	0.88-0.94[a]	0.98	0.95-1.01	0.95[a]	0.92-0.99[a]
Japanese	0.95	0.86-1.04	0.93	0.84-1.02	0.88[a]	0.81-0.96[a]	0.86[a]	0.79-0.94[a]
Korean	0.87[a]	0.80-0.93[a]	0.90[a]	0.83-0.97[a]	0.80[a]	0.74-0.85[a]	0.82[a]	0.76-0.88[a]
Laotian/Hmong	1.91[a]	1.65-2.21[a]	1.50[a]	1.29-1.73[a]	1.74[a]	1.52-1.98[a]	1.37[a]	1.20-1.57[a]
Other Asian	0.80[a]	0.67-0.96[a]	0.98	0.82-1.17	0.80[a]	0.68-0.93[a]	0.96	0.82-1.12
South Asian	0.91	0.75-1.09	0.88	0.74-1.06	0.93	0.79-1.09	0.91	0.78-1.06
Thai	1.00	0.77-1.29	1.08	0.84-1.40	0.96	0.77-1.21	1.04	0.82-1.30
Vietnamese	0.83[a]	0.79-0.89[a]	0.85[a]	0.80-0.90[a]	0.79[a]	0.75-0.84[a]	0.80[a]	0.76-0.84[a]
White	1.00		1.00		1.00		1.00	
Age at diagnosis								
< 50			1.00				1.00	
50-59			1.01	0.96-1.06			1.04	1.00-1.09
60-69			1.06[a]	1.01-1.11[a]			1.08[a]	1.04-1.13[a]
70-79			1.29[a]	1.23-1.35[a]			1.29[a]	1.24-1.35[a]
≥ 80			1.54[a]	1.45-1.64[a]			1.57[a]	1.49-1.65[a]
Gender								
Female			1.00				1.00	
Male			1.10[a]	1.07-1.14[a]			1.09[a]	1.06-1.12[a]
Socioeconomic status								
1 - Low SES			1.00				1.00	
2			0.95[a]	0.92-0.99[a]			0.94[a]	0.91-0.98[a]
3			0.91[a]	0.88-0.95[a]			0.90[a]	0.87-0.94[a]
4			0.88[a]	0.84-0.92[a]			0.86[a]	0.83-0.89[a]
5 - High SES			0.81[a]	0.77-0.85[a]			0.79[a]	0.75-0.82[a]
Region								
Los Angeles			1.00				1.00	
San Francisco-Oakland			1.03	0.99-1.07			1.00	0.97-1.03
Central California			1.06[a]	1.02-1.11[a]			1.07[a]	1.03-1.11[a]
Northern California			1.14[a]	1.08-1.19[a]			1.08[a]	1.04-1.12[a]
San Diego-Imperial-Orange			1.09[a]	1.05-1.14[a]			1.08[a]	1.04-1.13[a]
Stage at diagnosis								
Remote			1.00				1.00	
Regional			0.70[a]	0.67-0.73[a]			0.71[a]	0.69-0.74[a]
Local			0.39[a]	0.38-0.41[a]			0.44[a]	0.43-0.46[a]
Unstaged			0.76[a]	0.72-0.79[a]			0.77[a]	0.74-0.80[a]
Time period of diagnosis								
1988-1992			1.00				1.00	
1993-1997			0.86[a]	0.82-0.91[a]			0.90[a]	0.86-0.95[a]

1998-2002	0.75[a]	0.71-0.79[a]	0.77[a]	0.74-0.81[a]
2003-2007	0.69[a]	0.66-0.73[a]	0.72[a]	0.68-0.75[a]
2008-2012	0.54[a]	0.51-0.57[a]	0.57[a]	0.54-0.60[a]
Type of surgery				
None	1.00		1.00	
Local	0.40[a]	0.38-0.43[a]	0.43[a]	0.41-0.46[a]
Resection or transplant	0.26[a]	0.25-0.28[a]	0.29[a]	0.28-0.31[a]

[1]Adjusted for all other factors presented in the table using Cox proportional hazards regression models. [a]$P < 0.05$.

DISCUSSION

We found substantial racial/ethnic variation in receipt of curative treatment, even after accounting for stage of disease and SES, which also varied considerably. These results are consistent with those of others[34], who also found that Blacks and Hispanics were less likely and Asians as a whole were more likely to receive treatment (although less likely to receive a transplant) than Whites. Patients with local stage disease were more likely to receive curative treatment than those with distant stage disease, and this advantage increased after changes to transplant guidelines in 2002 allowing for and increasing prioritization of transplantation in cases of local stage disease. Others have found that the change in guidelines did not lead to decreasing disparities in treatment[35].

We found that even after accounting for treatment differences, disparities in survival remained, with Blacks, Laotian/Hmong, and Cambodians experiencing significantly higher mortality than Whites, Hispanics, and most other Asian ethnicities. Those other Asian ethnic groups, such as Chinese and Vietnamese, have been the focus of longer histories of HBV screening than Blacks, Laotian/Hmong, and Cambodians and perhaps are the beneficiaries of earlier detection. Other studies have found Black-White differences to persist after adjustment for receipt of surgical treatment[19,22,36]. One study noted that even among transplant patients, Blacks had shorter and Asian/Pacific Islander patients had longer survival compared to Whites, with causes of death that suggested variation in the amount of immunosuppression accounted for differences in survival[19]. Consistent with others[37], we found that females had a survival advantage.

Differences in survival may be in part due to differences in comorbidities, stage of underlying liver disease, and etiology of HCC. A study using National Health and Nutrition Examination (NHANES) data found that risk factors for liver disease varied by race/ethnicity and gender, with Mexican Americans more likely than Blacks and Whites to have elevated aminotransferase activity, and Blacks more likely than Mexican Americans and Whites to have hepatitis B or C infection. Among men, Mexican Americans were more likely than Whites to be heavy/binge drinkers; among women, Mexican Americans and Blacks were more likely to be obese or diabetic but less likely to be heavy/binge drinkers than Whites[38]. Among East and Southeast Asians, HBV is the most common cause of HCC[1,20], except for Japanese, among whom HCV is more common[1]. HBV-associated HCC can occur without cirrhosis, which may confer a survival advantage as the typical complications of portal hypertension are not present[3].

HCC in the setting of a non-cirrhotic liver (NCL) is rare and has different etiologic, genetic, and pathologic characteristics from cirrhotic HCC, including a lower prevalence of HBV, HCV, and alcohol abuse, a lower rate of p53 mutation, and more advanced tumor stage at diagnosis[39]. Risk factors for the development of HCC in NCL include metabolic syndrome and non-alcoholic fatty liver disease, which may co-exist with viral hepatitis or alcohol abuse[40]. Hepatic resection is generally the best treatment choice for HCC patients with NCL, leading to better overall and disease-free survival than those of cirrhotic patients; in fact, survival after resection among NCL patients with non-advanced tumors is comparable to that of cirrhotic patients with early tumors who receive liver transplantation[39].

Compared to other etiologies, HCV-related HCC has been associated with poorer overall and recurrence-free survival after surgery[41]. Among patients with cirrhosis, those with chronic HCV experienced lower survival at 1, 3, and 5 years after liver transplantation compared to those without HCV. An accelerated progression to cirrhosis in HCV patients post-transplant may be responsible for this phenomenon seen in an era when treatment with interferon-based therapies was minimally effective in this population[42]. These outcomes will need to be revisited in the era of highly effective direct acting antiviral medications[41]. There is also evidence of racial differences in protein expression in HCV-associated HCC, indicating a possible biological mechanism for some disparities[43].

Other disparities in survival are likely due to differences in access to care and quality of treatment[18,36], as well as knowledge and attitudes regarding liver disease[44-46]. It is clear that gaps in both patient and provider knowledge lead to decreased screening and vaccination rates among those at risk for chronic hepatitis B[47-51]. Trends in earlier stage at diagnosis and leveling off of liver cancer incidence rates among Asians as a whole have been attributed to HBV testing and surveillance of those chronically infected[3,21]. Now that

all-oral, curative treatment for HCV is available, HCV testing of people born in 1945-1965 is recommended[52]. Nevertheless, barriers to HBV and HCV testing and treatment remain[21,53-55]. However, attempts to intentionally link HBV screening results with linkage to care, while not optimized yet, are promising[56].

In summary, this paper reports on and analyzes 33270 HCC cases among Californians who were diagnosed over a 25-year period from 1988 to 2012. To our knowledge, these data represent the largest and most racially/ethnic diverse study of HCC cases collected through a registry with a Gold Certification (highest award) from the North American Association of Central Cancer Registries. Previously published reports of HCC cases utilizing the California Cancer Registry were focused on specific population groups, *e.g.,* Asian Americans[23] or combined analyses of two population groups, *e.g.,* Asian Americans and Hispanics[57] or were otherwise limited in numbers of cases, geographic scope, time period. Papers utilizing the SEER cancer registries, including those in California, were limited to a focus on a single racial category, *e.g.,* American Indians and Alaska Natives[58] or did not analyze as many disaggregated ethnic groups. Our findings underscore the need for disaggregation-those least likely to be treated and those with the highest mortality were Asian, as were those most likely to be treated and those with the lowest mortality. Our analyses provided greater granularity by including as separate categories: Cambodian, Chinese, Filipino, Hawai`ian/Pacific Islander, Japanese, Korean, Laotian/Hmong, Other Asian, South Asian, Thai, and Vietnamese, almost all of whom are at higher risk for HCC compared to the population-at-large. The greater granularity also enabled us to specifically identify populations-at-risk who share common socio-ethnic characteristics. Thus, the findings from this paper offer the potential for more precise targeting of interventions by ethnic group, and hence language preference, and geographical area.

Despite the advantages of being able to access and analyze the largest cancer registry in a geographically contiguous political jurisdiction, we recognize several limitations. We were not able to assess racial/ethnic differences in receipt of transplant over this time period because the CCR did not distinguish between transplant and resection until 1998. The CCR does not include data on risk factors, such as exposure to viral infections, cirrhosis, alcohol consumption, or documentation of an individual's metabolic syndrome/diabetes or body mass index as a measure of obesity. These latter risk factors are increasingly influential in HCC etiology[11]. No data are captured regarding the patients' English fluency or other potential measures of acculturation and access to care. The aggregate socioeconomic status variables are not measures of the individual patients but rather that of their Census block group[31,32]. Although the CCR employs extensive follow-up procedures it is possible that some patients returned to their home countries to die[59]. Finally, there was limited power to detect treatment and survival differences for racial/ethnic groups with small numbers of cases.

In conclusion, nonetheless these findings demonstrate substantial racial/ethnic disparities in HCC treatment and survival that were not explained by disease stage, time period of diagnosis, or socio-demographic factors. Continued effort is required to improve access and attitudes towards HBV and HCV testing and follow-up, address other etiological risk factors such as alcoholism and obesity, develop targeted therapies, and provide high quality treatment to all patients.

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COMMENTS

Background

Cancer of the liver and intrahepatic bile duct, of which approximately 80% is hepatocellular carcinoma (HCC), led the 17 most common cancer sites with a 3.1% average annual increase in mortality rates between 2008 and 2012 among both men and women in the United States.

Research frontiers

The authors' previous study of more than 6000 HCC cases diagnosed in California in 1988-2007, which reported on specific Asian ethnicities, found substantial inter-ethnic variation in survival but did not include comparison with other racial/ethnic groups. Rarely have HCC survival and treatment characteristics been characterized for 15 race/ethnic groups in a large geographically contiguous area over a period of 25 years.

Innovations and breakthroughs

This paper reports on and analyzes 33270 HCC cases among Californians who were diagnosed over a 25-year period from 1988 to 2012. To the best knowledge of the authors, these data represent the largest and most racially/ethnic diverse study of HCC cases collected through a Gold Certification (highest award) North American Association of Central Cancer Registries.

Applications

Nonetheless these findings demonstrate substantial racial/ethnic disparities in HCC treatment and survival that were not explained by disease stage, time period of diagnosis, or socio-demographic factors.

Peer-review

Non-cirrhotic liver with HCC has a different prognosis from a liver cirrhosis and HCV-related cirrhosis has a different survival rates in comparison to other etiologies.

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ORIGINAL ARTICLE

Observational Study

Pancreatic neuroendocrine tumor and solid-pseudopapillary neoplasm: Key immunohistochemical profiles for differential diagnosis

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Abstract

AIM

To reveal better diagnostic markers for differentiating neuroendocrine tumor (NET) from solid-pseudopapillary neoplasm (SPN), focusing primarily on immunohistochemical analysis.

METHODS

We reviewed 30 pancreatic surgical specimens of NET (24 cases) and SPN (6 cases). We carried out comprehensive immunohistochemical profiling using 9 markers: Synaptophysin, chromogranin A, pan-cytokeratin, E-cadherin, progesterone receptor,

vimentin, α-1-antitrypsin, CD10, and β-catenin.

RESULTS

E-cadherin staining in NETs, and nuclear labeling of β-catenin in SPNs were the most sensitive and specific markers. Dot-like staining of chromogranin A might indicate the possibility of SPNs rather than NETs. The other six markers were not useful because their expression overlapped widely between NETs and SPNs. Moreover, two cases that had been initially diagnosed as NETs on the basis of their morphological features, demonstrated SPN-like immunohistochemical profiles. Careful diagnosis is crucial as we actually found two confusing cases showing disagreement between the tumor morphology and immunohistochemical profiles.

CONCLUSION

E-cadherin, chromogranin A, and β-catenin were the most useful markers which should be employed for differentiating between NET and SPN.

Key words: Neuroendocrine tumor; Pancreas; Solid-pseudopapillary neoplasm; Immunohistochemistry; Diagnosis

Core tip: Neuroendocrine tumor (NET) and solid-pseudopapillary neoplasm (SPN) are two types of pancreatic tumor that were sometimes confused in differential diagnosis. We reviewed 30 pancreatic surgical specimens of NET (24 cases) and SPN (6 cases). We carried out comprehensive immunohistochemical profiling using 9 markers. E-cadherin staining in NETs, and nuclear labeling of β-catenin in SPNs were the most sensitive and specific markers. Dot-like staining of chromogranin A might indicate the possibility of SPNs rather than NETs. Moreover, two cases that had been initially diagnosed as NETs on the basis of their morphological features, demonstrated SPN-like immunohistochemical profiles.

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INTRODUCTION

Neuroendocrine tumor (NET) and solid-pseudopapillary neoplasm (SPN) of the pancreas differ from each other significantly in terms of tumor aggressiveness, treatment, and prognosis. NETs are considered to have malignant potential, as 40%-90% of them (excluding insulinoma) show gross invasive growth or metastasis[1,2], whereas SPNs have low grade malignancy, exhibiting local invasion or metastasis in only 20% of cases[3]. Consequently, the five-year survival rate for patients with NETs is worse than that for patients with SPNs (65% of NETs *vs* 95% of SPNs)[3,4]. Surgical resection is the mainstay treatment for both types of tumor, irrespective of whether they are localized or metastatic. For patients with unresectable NET, chemotherapy using agents such as somatostatin analogues, α-interferon, sunitinib, or mTOR inhibitor can be applied[5,6]. On the other hand, clinical trials have yet to yield an effective form of chemotherapy for unresectable SPNs[7]. Due to these differences in clinical features and strategies, careful differentiation between NETs and SPNs is needed, as this can have a crucial bearing on outcome.

Morphological structures have often been the key guide for differentiating SPNs from NETs. SPNs have a distinctive appearance, exhibiting solid and pseudopapillary growth patterns. Grossly, they appear as a large encapsulated mass, containing yellowish solid areas, with cystic zones that are frequently necrotic or hemorrhagic[8]. Microscopically, this heterogeneous growth pattern demonstrates solid areas with alternating pseudopapillary structures, together with cystic spaces made up of poorly cohesive monomorphic cells with abundant degenerative changes[9,10]. However, SPNs can show considerable morphological overlap with NETs. That is, a proportion of NET cases may show cystic and necrotic areas composed of discohesive cells, and a proportion of SPN cases may show a predominantly solid growth pattern without pseudopapillary structures[9,11]. In a review of a case series, Liu *et al*[12] reported that three of 14 NETs had cystic components, whereas one of 10 SPNs did not exhibit cystic areas. Accordingly, it was not possible to distinguish these two types of tumor simply on the basis of their gross or microscopic features.

In addition to morphological evaluation, immunohistochemical analysis plays a crucial role in differentiating these two tumor types. Klimstra *et al*[13,14] have reported a simple algorithm for diagnostic evaluation of pancreatic neoplasms, and demonstrated that immunohistochemstry based on the direction of tumor differentiation can be useful for establishment of tumor entities. Basturk *et al*[15] reviewed earlier reports indicating that synaptophysin, chromogranin A, pan-cytokeratin, and E-cadherin were markers for NETs, whereas progesterone receptor, vimentin, α-1-antitrypsin, CD10, and nuclear labeling of β-catenin were markers for SPNs. However, the expressions of these markers show overlap between NETs and SPNs[12,16,17]. For example, synaptophysin, a neuroendocrine marker expressed in most NETs, is also expressed in a number of SPNs[18,19]. As the sensitivity and specificity of the markers used in earlier studies have varied, more practical procedures for

Table 1 Immunohistochemical procedures

Antigen (Clone)	Manufacturer	Antigen retrieval solution/temperature/time	Dilution	Incubation temperature/time
Synaptophysin (27G12)	Nichirei Biosciences, Tokyo, Japan	TB/105 ℃/10 min	Prediluted	RT/30 min
Chromogranin A (polyclonal)	Dako Japan, Tokyo, Japan	CB/115 ℃/10 min	1:500	RT/30 min
Pan-cytokeratin (AE1/AE3)	Dako Japan, Tokyo, Japan	TB/105 ℃/10 min	1:100	RT/30 min
E-cadherin (36B5)	Thermo Scientific, Yokohama, Japan	TB/121 ℃/10 min	1:20	RT/60 min
Progesterone receptor (IE2)	Roche Diagnostics, Tokyo, Japan	CB/115 ℃/10 min	Prediluted	RT/30 min
Vimentin (V9)	Dako Japan, Tokyo, Japan	CB/100 ℃/15 min	1:100	RT/30 min
α-1-antitrypsin (polyclonal)	Dako Japan, Tokyo, Japan	No retrieval	1:100	RT/30 min
CD10 (56C6)	Leica Microsystems, Tokyo, Japan	TB/105 ℃/10 min	1:40	RT/30 min
β-catenin (β-Catenin-1)	Dako Japan, Tokyo, Japan	TB/105 ℃/10 min	1:200	RT/30 min
Ki-67 (MIB-1)	Dako Japan, Tokyo, Japan	CB/100 ℃/30 min	1:25	4 ℃/overnight

TB: Tris-HCl buffer; CB: Citrate buffer; RT: Room temperature.

correct diagnosis are anticipated.

In the present study, we reviewed the morphological and immunohistochemical profiles of 30 pancreatic tumors including 24 cases of NET and 6 cases of SPN. We comprehensively surveyed the usefulness of 9 markers in order to derive better diagnostic procedures for differentiating between the two tumor types. As a result, we discovered two confusing cases which showed both NET-like morphology and SPN-like immunohistochemical profiles, indicating the difficulties in differential diagnosis.

MATERIALS AND METHODS

Patients and tissue samples

Surgical specimens of primary NETs (24 cases) and SPNs (6 cases) of the pancreas were obtained at the Department of Surgery, University of Tsukuba Hospital, Tsukuba, Japan, between 2002 and 2011. All of the patients provided informed consent for analysis of their tissue samples in accordance with the ethics committee of University of Tsukuba Hospital. The specimens had been originally diagnosed by at least two pathologists in accordance with the World Health Organization (WHO) classification[10,20]. The grading of the NETs was re-evaluated on the basis of the WHO classification 2010, using the mitotic count and Ki-67 labeling index. Cases of Grade 1 and Grade 2 NET were included in the present study, but Grade 3 cases (neuroendocrine carcinoma) were excluded.

Immunohistochemical procedures

We performed immunohistochemical staining for synaptophysin, chromogranin A, pan-cytokeratin, E-cadherin, progesterone receptor, vimentin, α-1-antitrypsin, CD10, β-catenin, and Ki-67. Briefly, the resected tissues were fixed in 10% formalin and embedded in paraffin blocks, and the most representative block was chosen for each case. Each block was cut into serial sections 2 μm thick, and then deparaffinized with xylene and rehydrated with ethanol. After antigen retrieval and blocking of endogenous peroxidase activity with hydrogen peroxide, the sections were incubated with the primary antibodies (listed in Table 1, along with the antigen retrieval methods, dilutions, and incubation methods). All antigens except for progesterone receptor were detected using the EnVision+ System-HRP (Dako Japan, Tokyo, Japan); progesterone receptor was detected using an ultraView DAB universal kit (Roche Diagnostics, Tokyo, Japan). After visualization with diaminobenzidine chromogen, the sections were counterstained with hematoxylin, dehydrated with ethanol, made transparent with xylene, and mounted. All specimens were also stained with hematoxylin and eosin (H&E).

Interpretation of immunohistochemical staining and morphological features

Synaptophysin, chromogranin A, pan-cytokeratin, E-cadherin, vimentin, and α-1-antitrypsin were assessed for membranous and/or cytoplasmic staining. For progesterone receptor and β-catenin staining, nuclear labeling was evaluated as positive expression. All immunohistochemical markers except for Ki-67 were classified as strongly positive (++), weakly positive (+), or negative (-). Briefly, the distribution (no stain: 0-1%, focal: 2%-50%, or diffuse: 51%-100%) and the intensity (weak or strong) of cells in the stained sections were determined separately. Diffuse distribution with strong intensity was evaluated as strongly positive. Diffuse-weak, focal-strong or focal-weak combinations were all evaluated as weakly positive. No stain was evaluated as negative. For Ki-67 staining, nuclear labeling index of Ki-67 per 500-2000 cells was counted. Mitotic count was evaluated in at least 50 high-power fields using HE staining. Gross morphologic features were evaluated by analyzing pictures of the resected tumors and H&E-stained sections.

RESULTS

Clinicopathological and morphological characteristics

The clinicopathological data for the examined cases are listed in Table 2. Postoperatively, 24 cases had

Table 2 Clinicopathological and morphological characteristics of neuroendocrine tumor (24 cases) and solid-pseudopapillary neoplasm (6 cases)

Case	Age (yr)	Sex	Clinical findings	Tumor location	Operation	Diameter (cm)	Mitotic count	Ki-67 (%)	Grade for NETs	Solid or Cystic	PP pattern
NET-1	31	F	MEN1	H	PD	4.5	0	0	1	Solid and Cystic	Absent
NET-2	57	F	MEN1	H, T	EC, DP	5.3	0	1	1	Solid	Absent
NET-3	51	F	MEN1	H, B, T, L	EC, DP, LR	6.0	0	0	1	Solid	Absent
NET-4	45	F	MEN1	B, T	DP	1.4	0	0	1	Solid and Cystic	Absent
NET-5	42	F	insulinoma	B	EC	1.0	0	0	1	Solid	Absent
NET-6	62	F	insulinoma	H	EC	1.2	0	0	1	Solid	Absent
NET-7	73	M	insulinoma	H	PD	1.5	0	0	1	Solid	Absent
NET-8	74	F	insulinoma	T	DP	1.0	0	0	1	Solid	Absent
NET-9	67	M	insulinoma	B	EC	0.9	1	0	1	Solid	Absent
NET-10	51	F	insulinoma	B	MP	1.1	7	2	2	Solid	Absent
NET-11	45	M		H	EC	2.3	0	0	1	Solid	Absent
NET-12	68	F		H	EC	0.5	0	0	1	Solid	Absent
NET-13	77	M		T	DP	0.9	1	0	1	Solid	Absent
NET-14	57	M		H	PD	1.5	1	0	1	Solid	Absent
NET-15	45	M		H	EC	0.9	2	0	1	Solid	Absent
NET-16	51	F		H	PD	2.5	0	1	1	Solid	Absent
NET-17	59	M		H	MP	1.8	1	1	1	Solid	Absent
NET-18	59	F		T	EC	2.3	1	1	1	Solid and Cystic	Absent
NET-19	40	F		T	DP	3.3	0	4	2	Solid and Cystic	Absent
NET-20	53	M		T	DP	5.5	2	3	2	Solid	Absent
NET-21	39	M		H, B, T	TP	16	3	0	2	Solid	Absent
NET-22	51	M		H	PD	7.0	7	4	2	Solid	Absent
NET-23	58	F		B	EC	1.8	1	0	1	Solid	Absent
NET-24	32	M		H	PD	2.3	0	0	1	Solid	Absent
SPN-1	26	F		H	EC	8.5	0	0	-	Solid and Cystic	Present
SPN-2	34	F		T	DP	7.0	1	1	-	Solid and Cystic	Present
SPN-3	20	F		T	DP	7.5	0	0	-	Solid and Cystic	Present
SPN-4	23	F		T	DP	13	0	0	-	Solid and Cystic	Present
SPN-5	43	F		B	MP	3.8	0	0	-	Solid and Cystic	Present
SPN-6	27	M		T	DP	4.3	0	0	-	Solid and Cystic	Present

NET: Neuroendocrine tumor; SPN: Solid-pseudopapillary neoplasm; MEN: Multiple endocrine neoplasia; H: Pancreatic head; B: Pancreatic body; T: Pancreatic tail; L: Liver; PD: Pancreatoduodenectomy; MP: Middle pancreatectomy; DP: Distal pancreatectomy: TP: Total pancreatectomy; EC: Enucleation; LR: Liver resection; PP: Pattern pseudopapillary pattern; -: Not assessed.

been originally diagnosed as NET (NET-1 to NET-24), and 6 as SPN (SPN-1 to SPN-6). The mean age of the patients was 54 (range 31-77) years in the NET group, and 28.8 (range 20-43) years in the SPN group. In the NET group, 11 patients were male and 13 were female, whereas in the SPN group one was male and 5 were female. In the NET group, 4 cases (NET-1 to NET-4) were clinically diagnosed as multiple endocrine neoplasia type 1 (MEN1), 6 (NET-5 to NET-10) were diagnosed as insulinoma with clinical symptoms (*e.g.,* hypoglycemia), and the other 14 (NET-11 to NET-24) did not have any genetic background, symptoms or blood examination data attributable to hormone hypersecretion. Preoperative enhanced computed tomography were performed in all cases, most of NET cases showed the enhancement of tumor in early phase, but SPN cases did not. Preoperative biopsy using ultrasonography was not performed in all cases. The mean tumor diameter of the surgical specimens was 3.0 (range 0.5-16) cm in the NET group, and 7.4 (range 3.8-13) cm in the SPN group. Assessment of tumor grading of NETs according to the mitotic count and Ki-67 labeling index showed that 19 cases were Grade 1, and 5 were Grade 2. In the SPN group, mitosis or Ki-67 labeling of cells was scarcely evident in the sections. At the end of 2012, metastasis and/or recurrence were found in 6 cases of NETs (NET-2, NET-3, NET-15, NET-20, NET-21, and NET-22), whereas they did not occur in SPN cases. Metastatic or recurrent NETs were treated by reoperation, chemotherapy and/or radiotherapy after initial operation.

In the NET group, 20 cases showed a predominantly solid growth pattern, and 4 cases had cystic areas. In the SPN group, all 6 cases showed a solid and cystic growth pattern. The cystic lesions of SPNs were typically larger than those of NETs. NETs were composed of relatively uniform cells forming various organoid histological patterns, characterized by nesting, trabecular, glandular, gyriform, tubuloacinar, or pseudorosette arrangements. All of the cases in the SPN group showed pseudopapillary structures with poorly cohesive cells, whereas no such features were evident in the NET group.

Immunohistochemical findings

The immunohistochemical profiles are summarized in Table 3. Synaptophysin was positive in all NETs (100%), whereas in 4 SPNs (67%). Chromogranin A

Table 3 Immunohistochemical profiles of neuroendocrine tumor and solid-pseudopapillary neoplasm

Case	Syn	CgA	CK	E-cad	PgR	Vim	αATP	CD10	βcat (N/C)
NET-1	++	++	++	++	-	-	+	++	-
NET-2	++	++	++	++	++	-	+	++	-
NET-3	++	++	++	++	++	-	++	++	-
NET-4	++	++	++	++	++	++	-	-	-
NET-5	++	++	+	++	++	-	-	-	-
NET-6	++	++	+	++	++	-	-	-	-
NET-7	++	++	++	++	++	-	++	-	-
NET-8	++	++	+	++	++	-	++	-	-
NET-9	++	++	-	++	-	-	-	-	-
NET-10	++	++	+	++	++	++	++	-	-
NET-11	++	++	-	++	++	-	+	-	-
NET-12	++	++	+	+	++	++	-	-	-
NET-13	++	++	++	++	++	++	-	-	-
NET-14	++	++	++	++	-	++	++	-	-
NET-15	++	++	++	++	++	-	-	-	-
NET-16	++	++	+	+	++	-	++	++	-
NET-17	++	++	++	++	++	-	-	++	-
NET-18	++	++	++	++	++	++	-	++	-
NET-19	++	++	++	++	++	++	-	-	-
NET-20	++	++	++	++	++	++	++	-	-
NET-21	++	++	++	++	++	-	++	-	-
NET-22	++	++	++	++	-	-	++	-	-
NET-23	++	+ (dot)	-	-	++	++	++	++	++
NET-24	++	+ (dot)	-	-	++	++	++	++	++
SPN-1	-	+ (dot)	-	-	++	++	++	++	++
SPN-2	-	+ (dot)	-	-	++	++	++	++	++
SPN-3	++	+ (dot)	+	-	++	++	++	++	++
SPN-4	++	+ (dot)	-	-	++	++	++	++	++
SPN-5	++	+ (dot)	-	-	++	++	++	++	++
SPN-6	++	+ (dot)	-	-	++	++	++	++	++

NET: Neuroendocrine tumor; SPN: Solid-pseudopapillary neoplasm; Syn: Synaptophysin; CgA: Chromogranin A; CK: Pan-cytokeratin; E-cad: E-cadherin; PgR: Progesterone receptor; Vim: Vimentin; αATP: α-1-antitrypsin; βcat (N/C): β-catenin (nuclear/cytoplasmic staining); ++: Strongly positive; +: Weakly positive; -: Negative; dot: Dot-like pattern.

was positive in all NETs (100%); however, two cases (NET-23 and NET-24) showed a dot-like pattern, whereas 22 cases showed diffuse staining. On the other hand, all SPNs (100%) showed a dot-like pattern of chromogranin A immunostaining. Pan-cytokeratin was positive in 20 NETs (83%), but in only one (17%) of the SPNs. E-cadherin was positive in 22 NETs (92%), with the exception of NET-23 and NET-24, whereas all the SPNs were negative for E-cadherin. Progesterone receptor, vimentin, α-1-antitrypsin, CD10, and β-catenin (nuclear/cytoplasmic expression) were positive in all SPNs (100%), whereas among the NETs, progesterone receptor was positive in 20 (83%), vimentin was positive in 10 (42%), α-1-antitrypsin was positive in 14 (58%), CD10 was positive in 8 (33%), and β-catenin was positive in 2 (8%).

The majority of NETs were positive for synaptophysin, chromogranin A, pan-cytokeratin, E-cadherin, and progesterone receptor. Some cases of NET were positive for vimentin, α-1-antitrypsin, CD10, and β-catenin. On the other hand, all SPNs showed the same immunohistochemical profiles, being positive for progesterone receptor, vimentin, α-1-antitrypsin, CD10, and β-catenin. Moreover, they were also negative for E-cadherin, and showed a dot-like pattern of chromogranin A immunostaining. In the NET group we examined two cases (NET-23 and NET-24) that showed distinctive expression in comparison with the others; although these cases had been originally diagnosed as NETs, they showed the same profiles as SPNs (positive for progesterone receptor, vimentin, α-1-antitrypsin, CD10 and β-catenin, a dot-like chromogranin A pattern, and negative for E-cadherin). Microscopically, these cases did not show a pseudopapillary pattern. Representative staining patterns in a typical case of NET (NET-21), confusing case (NET-24), and a typical case of SPN (SPN-5) are shown in Figure 1.

DISCUSSION

Differentiating between pancreatic NETs and SPNs is crucial but still challenging even with precise morphological and histological analysis. Several immunohistochemical markers are reported to be useful for the differential diagnosis; however, not all of these markers are conclusive. Our immunohistochemical review of surgical specimens of NETs and SPNs demonstrated that E-cadherin, and nuclear labeling of β-catenin were the most sensitive and specific among the examined markers for differentiating the two type of tumors.

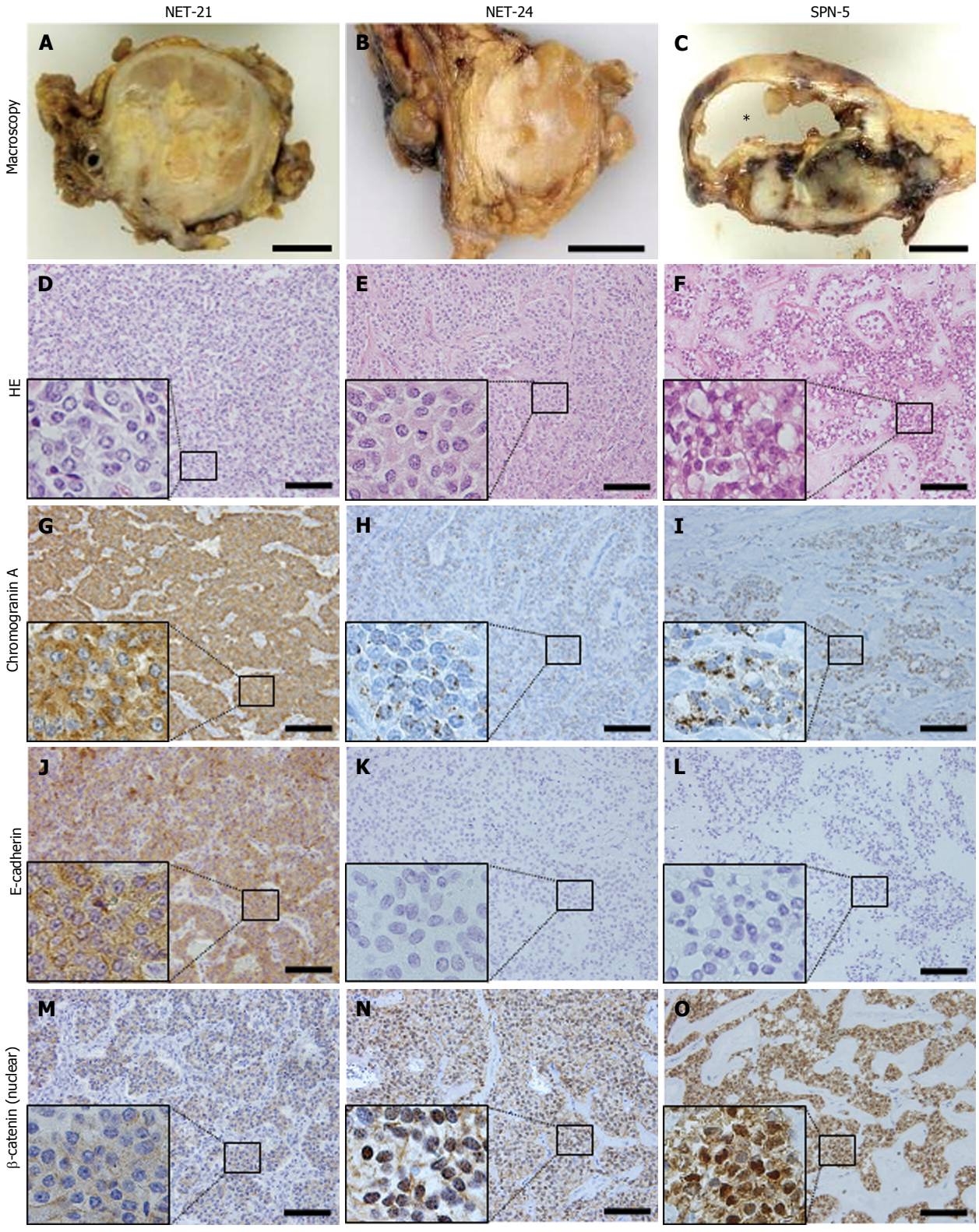

Figure 1 Macroscopic features and representative staining of a typical case of neuroendocrine tumor (case NET-21: A, D, G, J, M), a confusing case (case NET-24: B, E, H, K, N), and a typical case of solid-pseudopapillary neoplasm (case SPN-5: C, F, I, L, O). The typical NET case showed a solid growth pattern (A) with homogeneous cells, being strongly positive for chromogranin A and E-cadherin, and did not show nuclear labeling of β-catenin. In contrast, the typical SPN case showed a solid and cystic growth pattern (C) containing pseudopapillary structures formed by poorly cohesive cells, with a dot-like pattern of chromogranin A, negativity for E-cadherin, and nuclear labeling for β-catenin. NET-24, originally diagnosed as NET, mimicked the macroscopic features of NET, but exhibited the same immunohistochemical profile as SPN. HE, hematoxylin and eosin stain. Asterisk, cystic lesion of SPN case. Scale bars, 1 cm (macroscopic image) and 100 μm (microscopic image). Small boxes indicate representative magnified fields.

As earlier studies suggested, NETs in our case series also showed more malignant potential and needed various postoperative treatments compared with SPNs; therefore, differential diagnosis of the two tumors should be performed carefully. The key gross and microscopic features for differential diagnosis between NETs and SPNs are that SPNs typically show a mixture of solid and cystic growth areas composed of pseudopapillary structures, as was the case in all the SPNs we examined, whereas NETs generally show a solid growth pattern without pseudopapillae (Table 2). However, it should be noted that NETs sometimes show areas of cystic degeneration (NET-1, NET-4, NET-18 and NET-19). Clinical characteristics other than histopathology may sometimes be helpful for differential diagnosis between NETs and SPNs (Table 2). Clinical symptoms may be key feature of NETs, as some are associated with hypersecretion of hormones including insulin, glucagon, somatostatin, or gastrin[21]. Genetic background, such as MEN1, or von Hippel-Lindau disease, may also suggest a high likelihood of NET[22]. Although these features play a substantial role in differential diagnosis, their usefulness may sometimes be limited, and in fact in the present series of 24 NET cases diagnosis on this basis was made in only 10, including 4 cases of MEN1 (NET-1 to NET-4) and 6 cases of functional insulinoma (NET-5 to NET-10). The other 14 NET cases (NET-11 to NET-24) required further detailed differential diagnosis on the basis of pathology. Although SPNs occur predominantly in young female patients, this is not always pathognomonic, since males can also be sometimes affected[23], as was the case for SPN-6. Thus, more objective diagnostic modalities need to be developed.

Here we addressed the usefulness of immunohistochemical expression of various markers, either alone or in combination, for differential diagnosis between NETs and SPNs. Synaptophysin and chromogranin A, which are representative well-known neuroendocrine markers, were usually strongly positive in the majority of NETs. However, these are not specific markers for NETs, as 4 of the 6 SPNs we examined were also positive for synaptophysin, as reported earlier[18]. In contrast, chromogranin A has been regarded as typically negative in SPNs[16,18]. Here we found that all SPNs were weakly positive for chromogranin A, and that its distribution was quite unique, exhibiting a dot-like pattern formed by a relatively small number of chromogranin granules. Jirásek *et al*[24] have suggested that this expression of chromogranin A might reflect weak differentiation from a neuroendocrine lineage. In fact several studies have reported positivity for chromogranin A in SPNs[12,17,25]; these cases might be resulted in the criteria that dot-like pattern was evaluated as positive expression of chromogranin A. E-cadherin, a major epithelial adhesion molecule, was generally expressed in the majority of NETs, whereas it was not expressed in SPNs[26]. Loss of E-cadherin may explain the histological characteristics of SPNs, which show a pseudopapillary pattern perhaps resulting from loss of cell cohesiveness. Abnormal accumulation of β-catenin in the nucleus, caused by prolonged degradation of mutated β-catenin protein correlated with loss of E-cadherin, was observed in 95% of SPNs[27], whereas NET was generally negative (except for cases NET-23 and NET-24, as discussed in the next paragraph). Progesterone receptor, vimentin, α-1-antitrypsin, and CD10, also known to be markers for SPNs, were expressed in all of the SPNs we examined, but were less specific. Therefore, the main message of our present study is that membranous/cytoplasmic expression of E-cadherin in NETs, and nuclear staining for β-catenin in SPNs were useful immunohistochemical markers, which should be routinely applied to the differential diagnosis between NETs and SPNs. In addition, chromogranin A immunostaining should be interpreted carefully since a dot like pattern of chromogranin A might indicate the possibility of SPNs rather than NETs.

The two confusing cases in this series (NET-23 and NET-24) had been initially diagnosed as NETs because they did not show the typical morphological structure of SPN, *i.e.,* solid and cystic growth and a pseudopapillary pattern. However, they expressed SPN-like immunohistochemical profiles. The issue here is whether these two cases were truly NETs or truly SPNs. The WHO classification already states that a few SPNs display a solid growth pattern and lack pseudopapillary structures[9,10], and recommends that immunohistochemistry including nuclear β-catenin staining is potentially helpful for diagnosis of these SPNs. Moreover, hyaline globules, which are an architectural feature known to occur predominantly in SPNs rather than in NETs[28], were actually observed in all 6 of the present cases of SPN. Cases NET-23 and NET-24 also exhibited hyaline globules (data not shown), in addition to nuclear β-catenin staining. Therefore, we consider that these two cases might be SPNs lacking pseudopapillary structures which had been initially diagnosed as NETs.

The limitation of our study is that the number of cases we evaluated was small because SPN is rare pancreatic tumor. However, our present data should be useful for improving our routine diagnostic approach for NETs. In our institution, NETs have been initially diagnosed on the basis of gross and microscopic features, followed by supplementary immunohistochemistry for neuroendocrine markers including synaptophysin and chromogranin A. However, application of only neuroendocrine markers to "morphologically" NET-like cases is insufficient, since a substantial proportion of SPN cases mimicking the morphology of NETs will be present among these NET-like cases. Therefore, we propose that the immunohistochemical analysis should be extended to SPN-specific markers such as β-catenin, even if the tumors appear to have a NET-like morphology.

In conclusion, we have carried out comprehensive immunohistochemical profiling of 24 cases of NET and 6 cases of SPN of the pancreas. E-cadherin and β-catenin are the most useful immunostaining markers for differentiating between NETs and SPNs. On the other hand, we also found two cases which showed disagreement between the tumor morphology and immunohistochemical profiles. These cases strongly indicate the careful and precise assessments are crucial for differential diagnosis between NETs and SPNs.

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COMMENTS

Background

Neuroendocrine tumor (NET) and solid-pseudopapillary neoplasm (SPN) are two types of pancreatic tumor that were sometimes confused in differential diagnosis. Morphological structures and immunohistochemical profiles have often been the key guide for differentiating SPNs from NETs. However, morphological or immunohistochemical features show overlap between NETs and SPNs.

Research frontiers

NET and SPN sometimes show malignant character such as liver metastasis; however additional therapy after resection is different between the two tumors.

Innovations and breakthroughs

The immunohistochemical review of surgical specimens of NETs and SPNs demonstrated that E-cadherin, and nuclear labeling of β-catenin were the most sensitive and specific among the examined markers for differentiating the two type of tumors.

Applications

The authors propose that the immunohistochemical analysis should be extended to SPN-specific markers such as β-catenin, even if the tumors appear to have a NET-like morphology.

Terminology

SPN of the pancreas is an uncommon low grade malignant neoplasm. Exact histogenesis of SPN remains uncertain. It is well known for its predilection in young women.

Peer-review

In the present study, the authors reviewed the morphological and immunohistochemical profiles of 30 pancreatic tumors including 24 cases of NET and 6 cases of SPN. The authors comprehensively surveyed the usefulness of 9 markers in order to derive better diagnostic procedures for differentiating between the two tumor types.

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ORIGINAL ARTICLE

Prospective Study

Contrast-enhanced ultrasonography in the evaluation of incidental focal liver lesions: A cost-effectiveness analysis

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Abstract

AIM

To determine whether contrast-enhanced ultrasonography (CEUS) as the first-line method is more cost-effective in evaluating incidentally discovered focal liver lesions (FLLs) than is computed tomography (CT) and magnetic resonance imaging (MRI).

METHODS

Between 2010 and 2015, our prospective study enrolled 459 patients with incidentally found FLLs. The biological nature of FLLs was assessed by CEUS in all patients. CT or MRI examinations were added in unclear cases. The sensitivity and specificity of CEUS were calculated. The total costs of CEUS examinations and of the added examinations performed in inconclusive cases were calculated. Afterwards, the theoretical expenses for evaluating incidentally discovered FLLs using CT or MRI as the first-line method were calculated. The results

were compared.

RESULTS

The total cost of the diagnostic process using CEUS for all enrolled patients with FLLs was 75884 USD. When the expenses for additional CT and MRI examinations performed in inconclusive cases were added, the total cost was 90540 US dollar (USD). If all patients had been examined by CT or MR as the first-line method, the costs would have been 78897 USD or 384235 USD, respectively. The difference between the cost of CT and CEUS was 3013 USD (4%) and that between MRI and CEUS was 308352 USD (406.3%). We correctly described 97.06% of benign or malignant lesions, with 96.99% sensitivity and 97.09% specificity. Positive predictive value was 94.16% and negative predictive value was 98.52%. In cases with 4 and more lesions, malignancy is significantly more frequent and inconclusive findings significantly less frequent (P < 0.001).

CONCLUSION

While the costs of CEUS and CT in evaluating FLLs are comparable, CEUS examination is far more cost-effective in comparison to MRI.

Key words: Contrast-enhanced ultrasonography; Focal liver lesion; Computed tomography; Magnetic resonance imaging; Economic analysis

Core tip: Diagnosing focal liver lesions (FLLs) is a part of everyday practice, and therefore the cost-effectiveness of their diagnosis is important. Our study compared the costs of contrast-enhanced ultrasonography (CEUS), computed tomography (CT) and magnetic resonance imaging (MRI) in assessing the biological nature of FLL. We have proven significant savings when using CEUS instead of MRI. The costs of CEUS and CT examinations can be considered comparable. There exist additional parameters which influence the efficacy of individual modalities.

Smajerova M, Petrasova H, Little J, Ovesna P, Andrasina T, Valek V, Nemcova E, Miklosova B. Contrast-enhanced ultrasonography in the evaluation of incidental focal liver lesions: A cost-effectiveness analysis. *World J Gastroenterol* 2016; 22(38): 8605-8614 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i38/8605.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i38.8605

INTRODUCTION

Incidental discovery of focal liver lesions (FLLs) occurs very commonly in everyday practice. Although the majority of these lesions are benign[1], non-contrast ultrasound cannot be counted upon reliably to distinguish between benign and malignant lesions. Therefore, the new ACG clinical guideline: the diagnosis and management of focal liver lesions[2] has been published and recommends further investigation of these lesions using CT or MRI. Contrast-enhanced ultrasound (CEUS) constitutes an alternative option for further evaluating FLLs. European Federation of Societies for Ultrasound in Medicine and Biology (EFSUMB) published guidelines and recommendations for application of CEUS in 2004[3]. These guidelines were revised in cooperation with the World Federation of Societies for Ultrasound in Medicine and Biology (WFUMB)[4,5] in 2008 and 2012.

CEUS is an ultrasound technique whereby a special intravenous contrast agent is administered. There are several registered products on the market worldwide: Definity (Lantheus Medical Imaging), Lumason (Bracco Diagnostics), Optison (GE Healthcare), SonoVue® (Bracco Diagnostics), and Sonazoid (GE Healthcare)[6]. SonoVue®) is the only product currently registered in the Czech Republic. This contrast agent is based on gas microbubbles of size 1-10 μm, stabilized by a phospholipid shell[7]. It is a strictly intravenous contrast agent enabling detailed evaluation of tissue perfusion in real time.

CEUS is not a time-consuming method and, unlike CT or MRI examinations, it can be performed in one session along with non-contrast ultrasound immediately upon finding a lesion. It benefits patients inasmuch as they are, in most cases, immediately informed of their diagnoses and are spared the stress of waiting for another examination. It is advantageous for the doctors, as well, because they can respond more flexibly and quickly to their findings. Considering how common liver lesions are, the cost of their diagnosis is also important.

The aim of this study was to assess the economic aspect and efficacy of CEUS in evaluating the biological nature of incidentally found FLLs in comparison with other standard modalities, namely CT or MRI.

MATERIALS AND METHODS

Patients

Between January 2010 and December 2015, CEUS of the liver was performed 3638 times in our department. Patients enrolled in the study were diagnosed with one or more FLLs incidentally discovered: (1) by abdominal ultrasound performed for another reason (abdominal pain, diarrhoea, vomitus, dyspepsia, anaemia, *etc.*); (2) during a staging ultrasound for a newly diagnosed malignancy; or (3) during a follow-up ultrasound in patients with an oncological disease (colorectal cancer, breast cancer, renal carcinoma, *etc.*).

Exclusion criteria were (1) FLL previously known and being followed up for some time; (2) solitary lesions ⩽ 1 cm with typical appearance of haemangioma or cyst in patients without a history of malignancy (no follow-up is needed according to the guidelines)[2]; (3) lesions

Table 1 Prices of individual examinations by year in USD

Modality	2010	2011	2012	2013	2014	2015	Average
Non-contrast ultrasound	31.89	37.98	37.98	38.09	38.31	38.42	37.11
Contrast-enhanced US	165.02	171.82	164.19	164.3	164.51	164.61	165.74
CT	139.14	142.99	132.52	133.12	133.22	130.62	135.27
MRI	856.79	850.51	825.08	793.17	763.98	778.23	811.29

The price of contrast agent is included when required. CT: Computed tomography; MRI: Magnetic resonance imaging.

found by another diagnostic imaging modality (CT, MRI) with an inconclusive result; (4) untargeted CEUS performed for the purpose of finding malignant lesions in the liver changed by cirrhosis or with heterogeneous appearance; (5) assessment of response to treatment of malignant FLL (*i.e.,* after radiofrequency ablation); and (6) CEUS results not confirmed by other diagnostic imaging modality or by ultrasound scan in 6 mo time or later.

System of public health care payments in the Czech Republic

The majority of health care payments in the Czech Republic are made by health care insurers, which are private or state-owned companies. The insurers are regulated by Act No. 48/1997 Coll. (known as the Public Health Care Insurance Act)[8]. There is a specific point value (issued annually as a Regulation of the Ministry of Health)[9-15] for every medical examination or treatment. The same regulation also announces the Czech crown (CZK) value of a point for the next year. In addition to the system of state regulation, every provider of health care (such as hospitals and general practitioners, among others) may negotiate slightly different conditions with all health insurers.

It is important also to note that some medical examinations are provided only by specialized departments. In our study, we disregarded possible differences which could ensue from negotiations with the various health care insurers and thus used the standard prices as issued by the Ministry of Health.

The presented costs are final (covering the costs of work by medical staff and consumption of material), and the providers of health care receive no other payments from either insurers or patients. The final prices of the examinations for each year are presented in Table 1.

Ultrasound

Upon discovering an FLL, a non-contrast ultrasound scan was made to assess its presence, number, size, and appearance in B mode. The cost of this examination also is a part of that for an FLL diagnosis. When calculating the total cost of CT and MRI strategies, the price of the non-contrast ultrasound scan also was included.

The price of a non-contrast abdominal ultrasound scan varied from 31.89 to 38.42 USD, depending on the year such examination was performed.

CEUS

When an FLL was incidentally discovered by non-contrast ultrasound scan during office hours, CEUS was subsequently performed by the same radiologist. That radiologist would not have been specialized in liver imaging. The radiologists performing the US and CEUS were either certified radiologists (5-15 years of experience), or residents (9 mo to 5 years of practice) under the supervision of a certified radiologist present at the US department. Consultation with another radiologist present at the US department was possible and common. A senior radiologist (9-14 years of experience) specialized in liver imaging was available for consultation in unclear cases.

The ultrasound machines used were Ultrasound System iU22 Vision (Philips, the Netherlands) and Ultrasound System EPIQ 7 (Philips, the Netherlands). When an FLL was found incidentally during a non-contrast ultrasound scan, an intravenous application of 1.5-4.8 mL (median 2.5 mL) of the contrast agent SonoVue® (Bracco, Italy) followed. The contrast-enhanced examination was performed in side by side mode (B mode and contrast mode in one picture) and lasted at least 3 min. Lesions were assessed and classified in accordance with the literature[5].

The cost of CEUS ranged between 164.19 USD and 171.82 USD, depending upon the year. The price of the non-contrast ultrasound examination and of the contrast agent was already included into those figures.

CT

CT examination was performed on a Brilliance 64 scanner (Philips, the Netherlands). Non-contrast scans were followed by contrast-enhanced CT according to a specific protocol for liver lesion evaluation which calls for administering 100-125 mL (median 125 mL) of an intravenous contrast agent in proportion to the patient's body weight. In our hospital, Iomeron 350 (Bracco, Germany) is mostly used.

The cost of CT was 130.62-142.99 USD, depending upon the year, including the cost of the contrast agent.

MRI

MRI of the liver was performed in supine position on an Achieva 1.5T MR system (Philips, the Netherlands) and using a SENSE SL Torso coil. Non-contrast images were first obtained and there followed intravenous application of the contrast agent Primovist 0.25 mmol/mL (Bayer, Germany). The amount of contrast medium

Table 2 Numbers of patients and contrast-enhanced ultrasonography findings by year *n*

	2010	2011	2012	2013	2014	2015	Total
Total sum	38	53	73	85	124	86	459
Benign	16	27	45	50	72	61	271
Malignant	15	21	19	27	39	16	137
Inconclusive	7	5	9	8	13	9	51

applied depended on the weight of the patient and was in the range 15-20 mL (median 15 mL; once opened the ampoule cannot be reused).

The price of MRI consists of the sum of the component procedures: basic sequences for liver evaluation, diffusion-weighted imaging (DWI) and apparent diffusion coefficient (ACD) maps, contrast-enhanced MRI. The price for 2 × 10 mL of Primovist is then added. The total cost of an MRI examination was in the range 763.98-856.79 USD, varying by year.

Statistical analysis

In accordance with the literature[5], the number, size, and biological nature (malignant, benign, indeterminate) of each FLL were evaluated with CEUS. When possible, benign lesions were subclassified as haemangioma, focal nodular hyperplasia, pseudolesion, focal steatosis, cyst, abscess, or haematoma). A χ^2 test was used to evaluate the relationship between the number and biological nature of the lesions.

The sensitivity and specificity of CEUS for our department were calculated.

Economic analysis

The total cost of CEUS examinations of all patients was calculated. We then worked out what the expenses would have been if the biological nature of the FLL had been assessed by CT or MRI as the first-line method in all patients. The price of the non-contrast ultrasound scan was added. The expenses were subsequently compared to establish theoretical savings or losses.

It is especially important for managing the patient to establish the biological nature of a lesion, meaning to determine if it is malignant or benign. Neither CEUS nor CT nor MRI is always reliable in defining the final diagnosis. Those cases in which the findings of CEUS were not clear formed the group "inconclusive". In these patients, further investigation was essential, either using another diagnostic imaging modality (CE-CT or CE-MRI) or with repeated ultrasonography scans. The cost of such additional examinations increased the expenses for diagnosing FLLs in cases of inconclusive CEUS findings. When CEUS results were conclusive, the expenses corresponded to the price of CEUS.

Given the sensitivity and specificity of CEUS in our study and considering data already published from other studies[16-20], we regarded the reliability of CEUS, CT, and MRI to be comparable. Therefore, the cost-minimization analysis was conducted.

In calculating the cost-effectiveness of CEUS, we counted the expenses for groups of patients in individual years and then the total sum for the period as a whole. We based our calculations on the prices of examinations mentioned above.

We established the costs for individual years and then for the period as a whole. All expenses are stated in USD, although all were paid in CZK. The average exchange rate for the period from 1 January 2010 through 31 December 2015 was used in the cost calculations, meaning 1 USD = 20.21 CZK[21].

The statistical methods of this study were reviewed by Petra Ovesna from the Institute of Biostatistics and Analyses, Masaryk University, Brno, Czech Republic.

RESULTS

The study included 459 patients, 212 (46.2%) of which were men and 247 (53.8%) of which were women. Ages ranged from 21 to 93 years (mean 63.4 years). Together, they accounted for 1335 FLLs discovered incidentally by non-contrast ultrasound. Benign lesions were discovered in 271 (59.1%) patients and malignant lesions in 137 patients (29.8%). In 51 (11.1%) patients (Figure 1), the CEUS result was inconclusive. All lesions were further evaluated with another diagnostic imaging modality or followed up with ultrasound. The findings during individual years are given in Table 2.

One lesion was found in 276 (60.1%) patients, 2 to 3 lesions in 101 (22%) patients, and 4 or more lesions in 82 (17.9%) patients.

As illustrated in Figure 2, it is clear from our results that in cases with 4 and more lesions malignancy is significantly more frequently seen and the proportion of inconclusive findings is significantly less ($P < 0.001$).

Of benign lesions, there were 136 (58.2%) haemangiomas (Figure 3), 23 (7.7%) focal nodular hyperplasia (Figure 4), 58 (24%) pseudolesions, 16 (3.1%) focal steatoses, 16 (4.01%) cysts, 6 (0.5%) liquid collections, and 5 other findings (haematoma, postoperative changes, dysplastic nodule in cirrhotic liver). In 26 (6.6%) patients, the lesions were not further subclassified but the nature of those lesions was described as benign. In 13 patients, two types of lesions occurred.

To be able to calculate the specificity and sensitivity of CEUS in our study, the findings had to be verified by another diagnostic imaging modality which is considered a gold standard or to be unchanged over an extended period of time. Lesions were subsequently

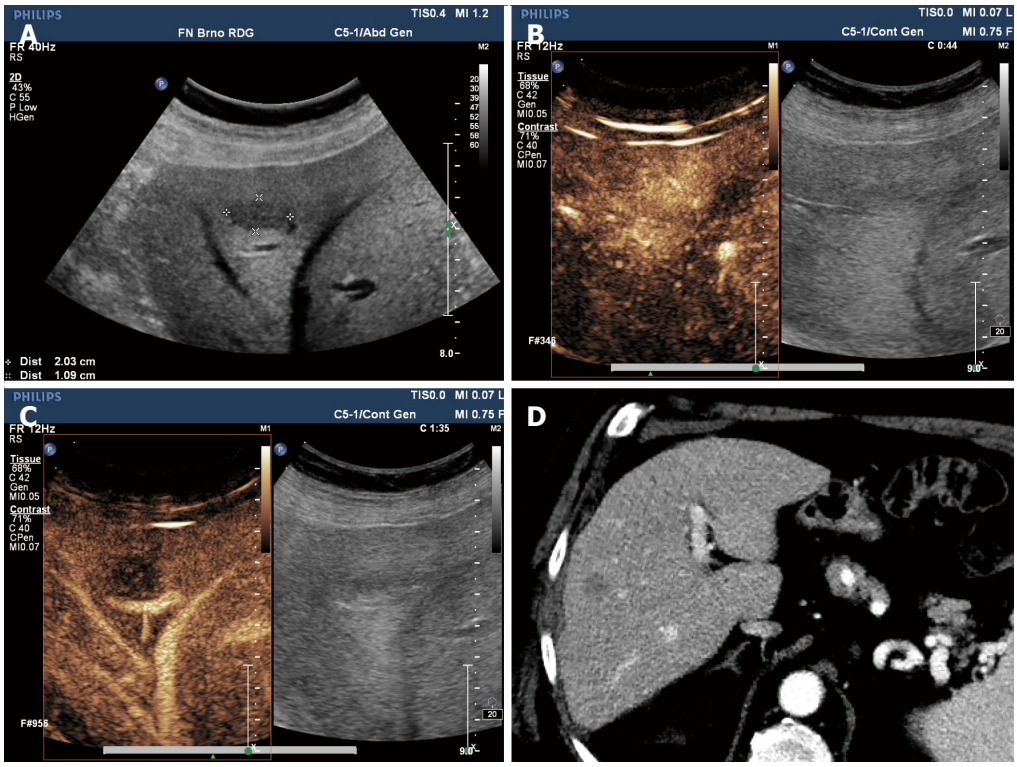

Figure 1 Liver metastasis of spinocellular carcinoma. Male, 75 years of age, with history of spinocellular carcinoma of the left lung. Emergency ultrasound performed for colicky abdominal pain identifies a hypoechoic lesion in segment S8 of the right liver lobe (A); Upon contrast-enhanced ultrasonography (CEUS) examination, the lesion shows increased enhancement in arterial phase compared to surrounding parenchyma (B); In following phases (C), there is detectable washout typical for malignant lesions. On CT performed for another reason there is a hypodense lesion on portal venous phase (D) corresponding to metastasis.

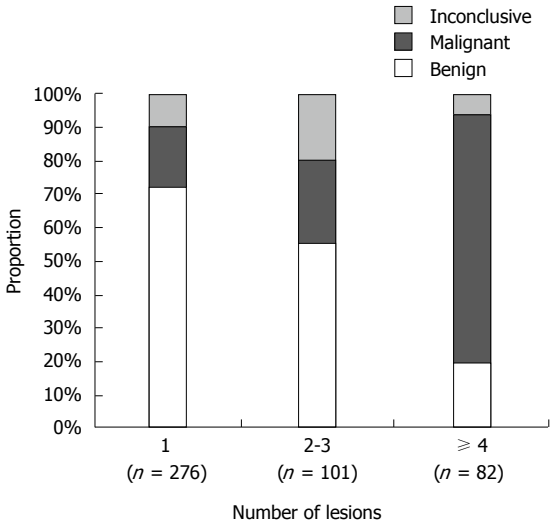

Figure 2 Correlation between number of lesions and diagnostic result of contrast-enhanced ultrasonography. When 4 and more lesions exist, the rate of malignancy is higher and the proportion of inconclusive results lower (P < 0.001).

evaluated by CT or MRI (either performed as a follow-up examination or as an assessment of a response to treatment) or they were observed by ultrasound for longer than 6 mo or verified by histology.

We described 97.06% of benign or malignant lesions correctly with 96.99% sensitivity and 97.09% specificity. Positive predictive value was 94.16% and negative predictive value was 98.52%.

The cost of CEUS for the 459 enrolled patients with incidentally found FLLs was 75884 USD, thus averaging 165.3 USD per patient.

In 51 patients with inconclusive CEUS findings, the costs were increased by additional CT or MRI examination or ultrasonography follow-up (CT was performed in 33 patients, MRI in 10 patients, and ultrasound follow-up in 8 patients; Table 3). The total cost of additional examinations was 14657 USD.

The total cost of diagnosing FLLs while pursuing a CEUS strategy was therefore 90540 USD, averaging 197.3 USD per patient.

Theoretically, if all FLLs incidentally found by non-contrast ultrasound scans would have been evaluated with CT, the expenses for 459 patients would have been 78897 USD for the whole period, meaning 171.9 USD per patient.

In case of using MRI after the initial non-contrast ultrasound scan, the cost for 459 patients would have been 384235 USD, meaning 837.1 USD per patient.

Inconclusive findings could be expected even when using CT or MRI, and such patients would have to be examined further (using MRI after CT, PET/CT, biopsy, CT or MRI follow-up). That would increase the total cost of these strategies. Considering that similar sensitivity and specificity are described in the literature for CEUS and CT[16-23], a similar incidence of inconclusive results could be expected. We could find

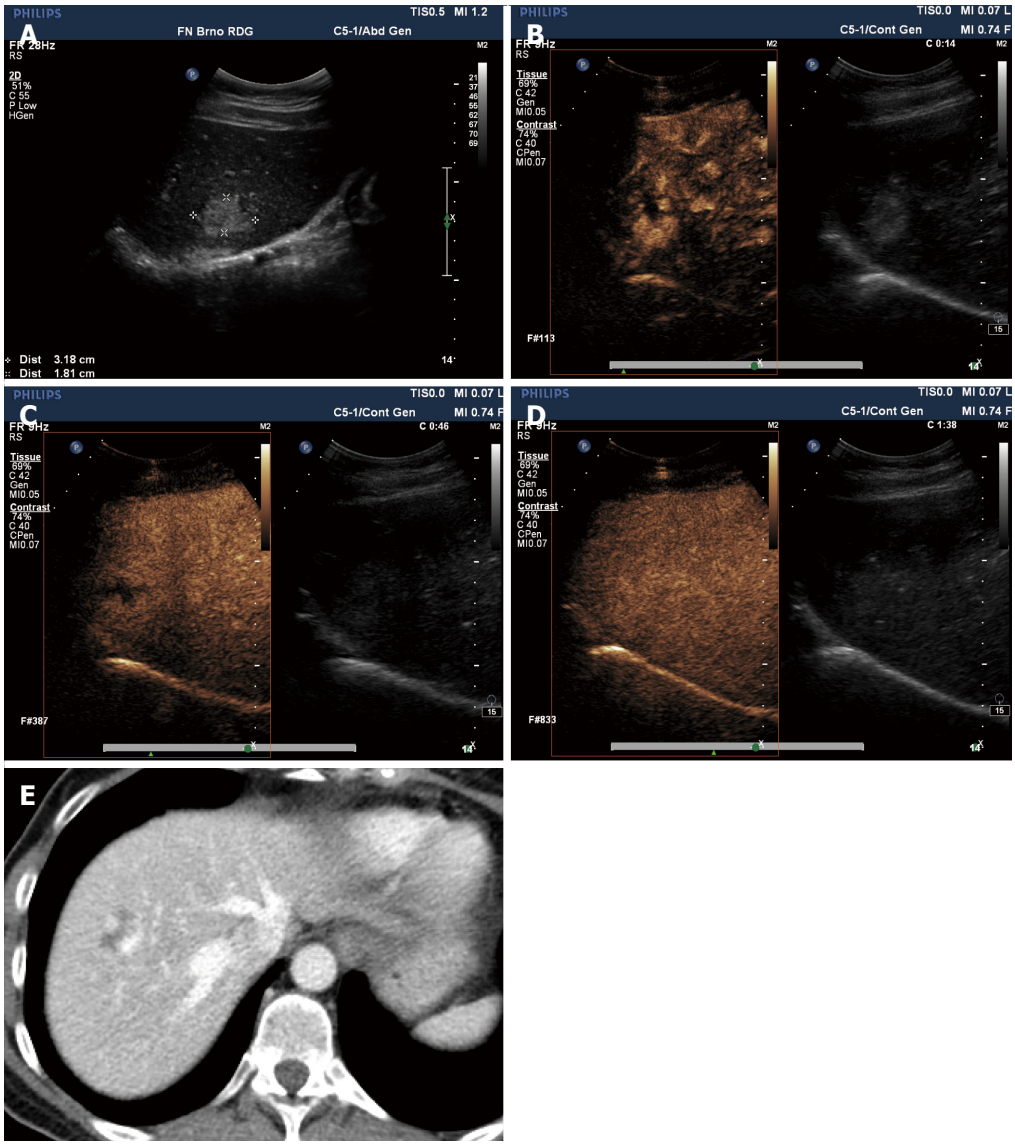

Figure 3 Haemangioma. Female, 57 years of age. Ultrasound scan performed for dyspepsia, a hyperechoic lesion found in segment S8 of the right liver lobe (A); Peripheral nodular enhancement after application of the contrast agent (B, C); In the late phase, the lesion blends in with the surrounding liver parenchyma (D); An identical pattern of enhancement could be seen in CT performed later from a different indication (E). Both findings suggest haemangioma.

Table 3 Number of patients with inconclusive contrast-enhanced ultrasonography findings by year and by second-line examination methods

	2010	2011	2012	2013	2014	2015	Total
Total	38	53	73	85	124	86	459
Methods used for examining inconclusive findings							
Ultrasound follow-up		1	3	1	2	1	8
CT	4	4	6	6	9	4	33
MRI	3			1	2	4	10

CT: Computed tomography; MRI: Magnetic resonance imaging.

no relevant data as to how many such results may occur, however, and therefore the cost of possible additional examinations could not be added.

Over the period of the study, the difference in total cost between CT and CEUS was 3013 USD (4%; *i.e.,* 6.6 USD per patient) and between MRI and CEUS it was 308352 USD (406.3%; *i.e.,* 471.8 USD per patient). Those also are the sums we hypothetically saved using CEUS as the method of choice instead of CT or MRI in evaluating the biological nature of incidentally found FLLs.

If the costs of further investigating inconclusive

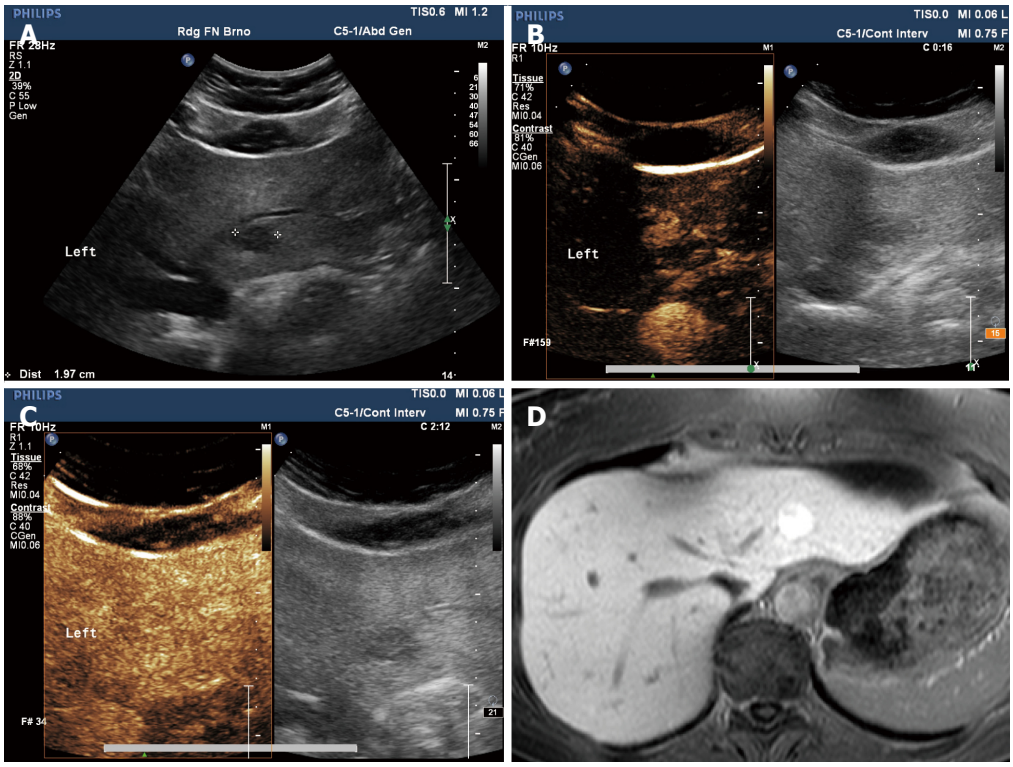

Figure 4 Focal nodular hyperplasia. Female, 52 years of age, with anaemia. Native ultrasound (A) shows 19 mm hypoechoic lesion in segment S2 of the left liver lobe. On subsequent contrast-enhanced ultrasonography (CEUS) examination, there is increased centrifugal perfusion in arterial phase (B); In the late phase (C), the lesion blends in with the surrounding liver parenchyma and there is no sign of washout. The CEUS diagnosis was FNH. Magnetic resonance imaging (MRI); (D) performed for another reason after three months verifies the diagnosis - lesion with high signal on hepatobiliary phase of contrast-enhanced MRI.

Table 4 Total costs of individual strategies in USD

	2010	2011	2012	2013	2014	2015	Total	Total per patient
CEUS								
Examination	6271	9107	11986	13965	20399	14156	75884	165.3
Additional examination	3350	800	1251	1935	3302	4020	14657	287.4
Total	9621	9906	13237	15900	23700	18176	90540	197.3
CT								
Examination	6499	9591	12447	14553	21269	14537	78897	171.9
MRI								
Examination	33770	47090	63004	70657	99483	70232	384235	837.1

Prices for computed tomography (CT) and MRI strategies do not include the expenses for investigating inconclusive cases. CEUS: Contrast-enhanced ultrasonography; MRI: Magnetic resonance imaging.

CEUS findings were included, then examination with CT would be less costly by 11643 USD (12.9%) for the period (*i.e.,* by 25.4 USD per patient). Evaluation with MRI would be still more costly by 293695 USD (324.4%) for the period (*i.e.,* 639.9 USD per patient). The prices for CT and MRI examinations do not include expenses for further evaluating possible inconclusive findings.

Table 4 summarizes the prices of individual examinations for the individual years and for the period as a whole.

Savings and losses for individual diagnostic strategies are summarized in Table 5.

DISCUSSION

The nature of all lesions of the patients enrolled in the study was evaluated by CEUS and subsequently verified. The nature of 97.06% of lesions was correctly determined by CEUS, with sensitivity 96.99% and specificity 97.09%.

Our results were comparable to those from other studies in other countries which pursued this topic[22-26]. The high sensitivity and specificity in our group may be attributed to the option to consult a specialized senior radiologist and to quantify the perfusion.

By comparison, a large German multicentre study

Table 5 Differences between costs of computed tomography and magnetic resonance imaging strategies and contrast-enhanced ultrasonography strategy (USD, %)

	Difference in USD	Difference per patient in USD	Difference (%)
CT			
CEUS *vs* CT	-3013	-6.6	-4.0
CEUS + additional *vs* CT	11643	25.4	12.9
MRI			
CEUS *vs* MRI	-308352	-671.8	-406.3
CEUS + additional *vs* MRI	-293695	-639.9	-324.4

Prices for computed tomography (CT) and MRI strategies do not include the expenses for investigating inconclusive cases. Negative values indicate magnitude of savings while positive values indicate level of higher costs. CEUS: Contrast-enhanced ultrasonography; MRI: Magnetic resonance imaging.

(the DEGUM study)[22] evaluated 1349 patients with liver lesions. Malignant and benign lesions were correctly differentiated in 95.7% of patients with sensitivity 95.8% and specificity 83.1%.

In a French multicentre study[23], 874 patients with 1034 FLLs were examined and CEUS results were compared to gold standard methods (CE-CT, CE-MR, or liver biopsy). In this case, 73% of benign and malignant FLLs were correctly evaluated, with sensitivity 79% and specificity 88%.

Finally, another German study[24] observed 317 patients, 89% of which were correctly diagnosed by CEUS, with sensitivity 90% and specificity 99%.

A number of other studies also address specificity and sensitivity of CEUS[27-30]. With regard to cost effectiveness, several published studies have reported significant savings when using CEUS[31-34].

An Italian multicentre prospective study[31] evaluated 485 patients with 575 lesions. Two diagnostic algorithms were compared: a standard approach (after finding a liver lesion with non-contrast ultrasound, CT or MR follows) at a total cost of 134576 EUR for diagnosing the nature of lesions and a new strategy (non-contrast ultrasound immediately followed by CEUS) at a cost of 55674 EUR. Using CEUS resulted in savings of 78902 EUR (*i.e.,* 162 EUR per patient).

A German study[32] also compared the economic aspect of using CEUS and CT in evaluating incidentally found FLLs. It concluded that CEUS was the more cost-effective method for all scenarios in which CEUS examinations were performed at specialized centres. The price for CEUS ranged between 122.18 and 184.53 EUR, and the price for multi-phase CT was 223.19 EUR. With approximately 40000 incidentally found FLLs in Germany per year, systematic implementation of CEUS would result in savings of around 4 million EUR/year. The cost of CEUS examination would be significantly higher, however, if performed also at non-specialized centres, where the price for one CEUS examination could average 407.87 EUR[32].

The data from a French multicentre study[33] assessed the costs of evaluating 149 liver lesions. The final savings were 128.5 EUR per lesion when using CEUS.

Finally, another Italian study[34] evaluated 398 patients with benign FLLs between 2002 and 2005. The cost of one CEUS examination was 101.51 EUR and that of CT was 211.48 EUR. The final cost saving was 47055 EUR when using CEUS as the first-line diagnostic imaging modality after non-contrast ultrasound.

In our prospective study, the difference between the total costs of CEUS and CT, at just 3013 USD (*i.e.,* + 4%) for the entire period, was relatively small compared to those from studies published previously. Adding in the cost for further investigation of inconclusive CEUS findings would bring that difference to 11643 USD (*i.e.,* +12.9%).

The high price of the contrast agent for CEUS in the Czech Republic increases the overall cost of a CEUS examination (average 165.74 USD) compared to the price of CT (average 135.27 USD) and contributes to the relatively small savings in comparison with other studies.

The difference between the cost of CEUS and MRI was significant even in our study, at 308352 USD (*i.e.* 406.3%). Adding in the cost for further investigation of inconclusive CEUS findings, that difference would have been 293695 USD (*i.e.,* +324.4%).

The hypothetical cost of diagnosing FLLs with CT and MRI does not include the expenses for further evaluation of inconclusive findings, which would certainly arise. Such patients would have to be followed up or examined using another diagnostic modality (MRI, PET/CT, biopsy or surgery, including the costs of hospitalization and possible complications). Accordingly, the total cost of these diagnostic scenarios would have increased and the cost-effectiveness of CEUS would have been even more pronounced. Unfortunately, we could find no published data which would indicate numbers of inconclusive CT and MRI findings in evaluating FLLs. Therefore, we could not include the hypothetical cost of possible additional examinations.

A disadvantage of CT is its necessity for intravenous application of iodinated contrast agent with accompanying risks of its having side effects and causing complications (allergic reaction, anaphylactic shock, *etc.*). The cost of treating such complications was also not included in the total expenses. These complications are rare, however, and the cost of their treatment would probably not significantly alter the result of this study. In cases of using MRI and CEUS examinations, as we did, these reactions are even rarer.

Additionally, patients with known or suspected allergy to iodinated contrast agent would have to be examined by MRI, which is more expensive. We did not take this into account, but it would certainly further raise the total cost of diagnosis while pursuing a CT strategy.

In the Czech Republic, MRI is still an expensive

examination and one that for many patients is poorly accessible due to a small number of available devices and their high workload. Therefore, CEUS is more advantageous not only in terms of cost-efficiency but also of accessibility and usage. Another significant impact of these disadvantages is that CT is used as the second-line examination method after inconclusive CEUS findings rather than MRI.

Important advantages of CEUS are the absence of ionizing radiation, good accessibility, short duration, and its possibility to be conducted immediately after the non-contrast ultrasound scan. Thus, the patient is examined in a single session and need not wait weeks for another examination and diagnosis.

Unfortunately, although health insurance covers CEUS examinations performed in our specialized centre, this is not the case for district hospitals in the Czech Republic.

In conclusion, the expenses for diagnosing FLLs with CEUS are comparable to those of CT. Important advantages of CEUS are the absence of radiation and the speed of diagnosis while maintaining diagnostic accuracy. Moreover, CEUS is significantly more cost-effective in comparison with MRI.

COMMENTS

Background

Incidental focal liver lesions are very common in daily practice at US departments. For the further management of a patient it is important to distinguish whether a lesion is benign or malignant. Computed tomography (CT) and magnetic resonance imaging (MRI) and contrast-enhanced ultrasonography (CEUS) are standard methods used for the investigation. Each has its advantages and disadvantages in terms of radiation exposure, availability, side effects, accuracy, price and others. How best to manage these diagnoses and their costs is much discussed today. Therefore, cost-effectiveness analysis of these methods is very pertinent.

Research frontiers

Numbers of focal liver lesions and their further investigation have a great financial impact on hospitals. Therefore, this study presents and compares costs of three strategies for evaluating the biological nature of incidentally found focal liver lesions.

Innovations and breakthroughs

This study shows that CEUS, as a first-line method, is significantly more cost-effective in comparison with MRI. Compared to other cost-effectiveness studies, this analysis is directed mainly to the comparative costs of CEUS and CT strategies because of the high price of CEUS contrast agent against the relatively low price of CT examination in the Czech Republic.

Applications

This study will enable knowledge regarding economic aspects of investigating focal liver lesions to be used in practice. It shows the advantages of using CEUS as a first-line method.

Terminology

CEUS: A sonographic method with use of contrast agents containing microbubbles of gas that allow evaluating perfusion of organs and lesions. Cost-effectiveness analysis: A form of economic analysis that compares the relative costs and outcomes (effects) of two or more courses of action.

Peer-review

The authors have performed a very good study. The manuscript is interesting.

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ORIGINAL ARTICLE

Prospective Study

Incidence, clinical features and para-clinical findings of achalasia in Algeria: Experience of 25 years

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Author contributions: Tebaibia A designed the study; Tebaibia A revised the manuscript for final submission; Tebaibia A, Boudjella MA, Boutarene D and Oumnia N recruited the patients; Tebaibia A, Boudjella MA and Benmediouni F interpreted the data and wrote the manuscript; Tebaibia A, Boudjella MA and Oumnia N performed endoscopy on all patients; Tebaibia A and Boutarene D collected the data; Tebaibia A, Boutarene D and Oumnia N performed the manometry; Brahimi H performed the statistical analysis.

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Abstract

AIM

To investigate the incidence of achalasia in Algeria and to determine its clinical and para-clinical profile. To evaluate the impact of continuing medical education (CME) on the incidence of this disease.

METHODS

From 1990 to 2014, 1256 patients with achalasia were enrolled in this prospective study. A campaign of CME on diagnosis involving different regions of the country was conducted between 1999 and 2003. Annual incidence and prevalence were calculated by relating the number of diagnosed cases to 10^5 inhabitants. Each patient completed a standardized questionnaire, and underwent upper endoscopy, barium swallow and esophageal manometry. We systematically looked for Allgrove syndrome and familial achalasia.

RESULTS

The mean annual incidence raised from 0.04 (95%CI: 0.028-0.052) during the 1990s to 0.27/10^5 inhabitants/year (95%CI: 0.215-0.321) during the 2000s. The incidence of the disease was two and half times higher in the north and the center compared to the south of the country. One-hundred-and-twenty-nine (10%) were

children and 97 (7.7%) had Allgrove syndrome. Familial achalasia was noted in 18 different families. Patients had dysphagia (99%), regurgitation (83%), chest pain (51%), heartburn 24.5% and weight loss (70%). The lower esophageal sphincter was hypertensive in 53% and hypotensive in 0.6%.

CONCLUSION

The mean incidence of achalasia in Algeria is at least $0.27/10^5$ inhabitants. A good impact on the incidence of CME was noted. A gradient of incidence between different regions of the country was found. This variability is probably related to genetic and environmental factors. The discovery of an infantile achalasia must lead to looking for Allgrove syndrome and similar cases in the family.

Key words: Achalasia; Incidence; Allgrove syndrome; Manometry; Continuing medical education

Core tip: The exact incidence of achalasia is unknown. Few epidemiological studies around the world have been devoted to it. The impact of continuing medical education (CME) on incidence of achalasia has not been evaluated. This study showed that CME has a positive effect on the incidence of this disease. In fact, the mean incidence raised from 0.04 (95%CI: 0.028-0.052) during the 1990s to $0.27/10^5$ inhabitants/year (95%CI: 0.215-0.321) during the 2000s. This incidence was two and half times higher in the north and the center compared to the south of the country. This variability is probably related to genetic and environmental factors.

Tebaibia A, Boudjella MA, Boutarene D, Benmediouni F, Brahimi H, Oumnia N. Incidence, clinical features and para-clinical findings of achalasia in Algeria: Experience of 25 years. *World J Gastroenterol* 2016; 22(38): 8615-8623 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i38/8615.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i38.8615

INTRODUCTION

Achalasia is a primary esophageal motility disorder characterized by esophageal aperistalsis and failure of the lower esophageal sphincter (LES) to relax normally with swallowing. It is self-secondary to a degeneration of the myenteric plexus[1,2]. Its etiology remains, to date, unknown. However, the existence of family cases, most often falling under Allgrove syndrome, suggests the existence of genetic factors predisposing to this condition. Infectious and auto-immune processes have also been postulated[3,4].

Achalasia is a motility disorder that can occur at any age, from newborns to the elderly. The revealing symptoms are aspecific, such as dysphagia, chest pain, regurgitation, weight loss, and rarely pulmonary complications. The diagnosis is based on esophageal manometry (EM) results, noticed during swallowing[5]. Few epidemiological studies around the world have been devoted to it. Most of them were carried out in Western countries and showed that it is a rare condition. The mean prevalence and incidence worldwide have been estimated as $10/10^5$ and $1/10^5$ inhabitants respectively[6]. No study assessing the characteristics of achalasia in Algeria, an African and Mediterranean country, has been published so far.

This work aims to evaluate the incidence and prevalence of achalasia, and to study its clinical and para-clinical features in this country.

MATERIALS AND METHODS

Patients and methods

In this prospective study, 1256 patients [653 (52%) females and 603 (48%) males; mean age: 43.3 ±18.7 years (3 mo-86 years)] with achalasia were enrolled over a period of 25 years, from 1990 to 2014, from among the 106795 patients investigated by upper endoscopy for various purposes. The patients were grouped based on their geographical origin, among three regions (Figure 1). Region 1 included coastal and center cities, characterized by a Mediterranean climate and a diet based on fruits, vegetables and cereals (14 cities; number of inhabitants: 12689307). Region 2 included the highlands cities, characterized by a dry climate and a diet based on cereals and vegetables (22 cities; number of inhabitants: 15501671). Region 3 included the Southern cities adjacent to the sub-Saharan Africa, characterized by hot climate and a diet based on dates, meat and cereals (12 cities; number of inhabitants: 3870697). This work was funded by the Ministries of Health and of Higher Education. Continuing medical education (CME) campaigns were conducted between 1999 and 2003 in different regions of the country (Figures 1 and 2). It focused on the clinical manifestations of the disease, particularly in the interest of EM to confirm diagnosis and provide information to both general practitioners and specialists that our department is the reference center in the management of achalasia, as well.

This study was carried out following two steps at our institution, which is the national reference center in Algeria regarding the management of esophageal motility disorders. Along the first step, from 1990 to 1998, the disease was largely unknown in Algeria and during the second step, from 1999 to 2014, the disease was more familiar to practitioners through CME campaigns.

Clinical evaluation

A standardized questionnaire clarified the geographical

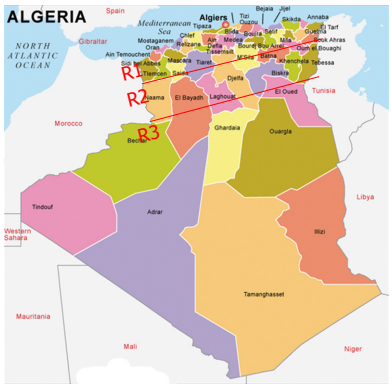

Figure 1 Map of Algeria. R: Region.

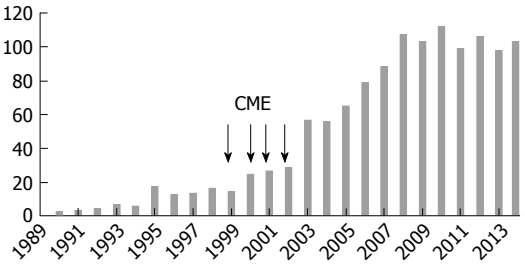

Figure 2 Number of cases of achalasia diagnosed each year and continuing medical education campaign. CME: Continuing medical education.

origin, the number of consultations before achalasia was diagnosed, the history of symptoms and their nature, as well as their severity. Dysphagia, regurgitation and chest pain were scored from 0 to 3, based on their frequency, but weight loss was scored based on its severity from 0 to 3, as well. We also systematically looked for heartburn and respiratory events (*i.e*. cough, dyspnea, asthma), familial achalasia and Allgrove syndrome.

Esophageal barium swallow: The diagnosis of achalasia was suspected in the presence of at least one of the following radiological signs: regular narrowing of the gastroesophageal junction (GEJ), slightly extended in "bird's beak" and/or dilatation of the esophageal body (EB) with absence of contractions. This dilatation was classified into four stages defined according to the diameter of the esophagus.

Esophagogastroduodenoscopy: Endoscopy was performed by four senior doctors aiming to look for signs in favor of achalasia and complications such as esophagitis, esophageal candidiasis or an overcardial diverticulum. It was also intended to eliminate other causes of stenosis of GEJ (*i.e.*, neoplasm, peptic stenosis).

EM: EM was carried out using an Arndorfer pneumohydraulic perfusion system with a low compliance capillary infusion, connected to a polygraph. Patients had to fast for at least 12 h. Stopping all drugs that can act on esophageal motility was systematically recommended.

The probe was nasally introduced until the side holes of the 4 sensors were placed in the stomach. The probe was then progressively removed from the stomach, cm by cm, with identification of the LES on the recorded graph at each of the 4 channels in order to calculate the length and the mean basal pressure of the LES. The esophageal motility was studied by performing 10 water swallows spaced at least 30 s apart. Classic achalasia diagnosis was made when a total aperistalsis was noticed (100% of non-peristaltic waves) associated or not with an increased LES tone (> 34 mm Hg) and/or a LES failure to relax (< 80% of the basal pressure), with absence of any organic barrier at the GEJ. The diagnosis of vigorous achalasia was made when aperistalsis was associated to an amplitude contraction wave $\geqslant$ 40 mm Hg.

Diagnosis criteria of Allgrove syndrome and familial achalasia: Allgrove syndrome diagnosis was made when at least 2 out of the 3 following signs were present: achalasia, Alacrima, adrenal insufficiency.

Familial achalasia diagnosis was established when at least 2 members of one family had achalasia, whether it was isolated (sporadic achalasia) or fell under Allgrove syndrome (syndromic achalasia). Consanguinity, defined as first- or second-cousin marriages, was systematically looked for.

Exclusion criteria: Other esophageal motility disorders, collagen diseases, prior esogastric surgeries, eosinophilic esophagitis and tumors of GEJ were excluded.

Statistical analysis

Statistical analysis was performed using the SPSS software version 17.0. Annual incidence of achalasia was calculated by relating the number of diagnosed cases to 10^5 inhabitants, based on a population reported annually by the National Office of Statistics in Algeria (ONS; web: http://www.ons.dz). Quantitative variables were expressed in gross figures, proportions and rates. Rates of different results have been calculated with their 95%CI. Differences between proportions were calculated using χ^2 and the *P* value was considered significant when it was lower than 0.05.

Comparison of the achalasia different means incidence, delay diagnosis means and age at diagnosis corresponding to the three periods of 1990-1997, 1998-2005 and 2006-2014 was performed through a variance analysis. Comparison of the achalasia different means incidence corresponding to the different regions in Algeria (north, highlands and south) was made, as well.

Figure 3 Number of cases of achalasia in the different regions according to time.

Table 1 Mean incidence of achalasia between 1990-2014

Year interval	Mid-period population	Cases, *n*	Mean incidence, (cases/10^5 inhabitants/yr)	95%CI
1990-1997	28385621	87	0.04	0.028-0.025
1998-2005	32370618	274	0.108	0.072-0.144
2006-2014	37128618	895	0.268	0.215-0.321

Significance degree $P < 0.001$.

Table 2 Mean Incidence of achalasia according to different region of country

Regions of country	Mid-period population 1990 to 2014 (2002)	Cases, *n*	Cases/10^5 habitants/yr	95%CI
R1 North	12689307	693	0.2184	0.1811-0.2501
R2 High plains	15501671	488	0.1256	0.0921-0.1536
R3 South	3870607	75	0.0772	0.0432-0.1121

Mean incidence of cases: $P < 0.001$.

RESULTS

Our study included 1256 patients from the 48 Wilayas (provinces) of the country (Figure 1). The average age at diagnosis was 45 years (95%CI: 38.0-52.0) during the first period, 40.7 years (95%CI: 38.6-42.9) during the second one and 40.3 (95%CI: 39.0-41.6) between 2006 and 2014, but the difference was not statistically significant ($P > 0.05$).

The number of cases diagnosed each year is shown in Figures 2 and 3. The average number of new cases of achalasia diagnosed per year during periods from 1990 to 1997, 1998 to 2005 and 2006 to 2014 was respectively 10, 34.3 and 99.4 cases. The mean annual incidence from 1990 to 1997 was of 0.04 patients/year/10^5 inhabitants. From 1998 to 2005, it increased to 0.1 patients/year/10^5 inhabitants to reach a rate of 0.27 patients/year/10^5 inhabitants from 2006 to 2014 ($P < 0.001$) (Table 1). The prevalence of achalasia in Algeria during 2014 has been estimated to 3.2 patients/10^5 inhabitants (1256/39928947). More interesting, the incidence in regions 1 and 2 were three times more important when compared to region 3 (Table 2): 0.21 patients/year/10^5 inhabitants *vs* 0.12 *vs* 0.07 ($P < 0.001$).

The average number of consultations that preceded the diagnosis of achalasia was 6 ± 8.7 (range: 3 to 21 consultations).

The mean delay diagnosis of achalasia from 1990 to 1997 was 75.78 mo (95%CI: 50.93-100.63). From 1998 to 2005, it dropped to 54.68 mo (95%CI: 45.39-63.97) to reach a mean of 48.76 mo (95%CI: 43.22-54.30) from 2006 to 2014. However, this difference was not significant between the three periods ($P > 0.05$). Allgrove syndrome was noted in 97/1256 (7.7%) patients [female (53%); mean age: 16.23 ± 10.4; range: 8 mo-41 years]. It was a 3A syndrome in 46.4% (45/97) of cases, a 2A syndrome in 25.7% (25/97) of cases and a 4A syndrome in 27.8% (27/97) of cases. Consanguinity was found in 63% (61/97) of cases. It always included a neonatal alacrima while respective rates of achalasia, adrenal insufficiency, neurological abnormalities were 100%

Table 3 Clinical profile of 1256 patients with achalasia *n* (%)

Female/male	653 (52)/603 (48)
Age	43.3 ± 18.7 yr (3 mo-86 yr)
Adult/children	1127 (89)/129 (10.3)
Non-syndromic achalasia/ syndromic achalasia	1153/97
Familial achalasia (18)	41
Syndromic achalasia	34
Sporadic (isolated) achalasia	7
Duration of symptoms (mo)	59.5 (2-480)
Dysphagia	1243 (99)
Regurgitation	1042 (83)
Chest pain	690 (55)
Weight loss	879 (70)
Mean weight loss (kg)	7 ± 5.9 (1-40)
Heartburn	308 (24.5)
Respiratory manifestations	280 (22.3)

Values are expressed as number of patients (%) or mean ± SD.

Table 4 Esophageal barium swallow results in 1193 (95) patients with achalasia *n* (%)

Bird's beak aspect	1037 (87)
Mean esophageal diameter (cm), mean ± SD	5.8 ± 4.3
⩽ 2 cm (normal)	60 (5)
2-4	334 (28)
4-6	513 (43)
6-9	286 (24)
Hiatal hernia	6
Epiphrenic diverticula	2

(97/97), 46% (45/97) and 52.6% (51/97). There was a familial achalasia in 41 patients belonging to 18 different families. It was a syndromic achalasia in 34/41 belonging to 15/18 families. Achalasia was sporadic in 7/41 (17%) other patients from 3/18 remaining families. The average number of subjects affected by family was 2.26 (range: 2-4). All familial achalasia cases were observed in siblings (brothers and sisters). No case of parent/child achalasia has been recorded. Among children, classic and familial syndromic achalasia were noted in respectively 55% (71/129) and 45% (58/129) of cases.

Clinical results

Dysphagia was observed in 1243/1256 (98.9%) patients and regurgitation in 1042/1256 (83%) patients. Retro-sternal, epigastric or inter-scapulars pain were noted in 640/1243 (51%) patients. They preceded dysphagia of several months [average: 23 ± 14 mo (6-36) mo] in 2.5% (32/1256) of patients. Weight loss was present in 879 (70%) patients. The mean weight loss was 9.5 ± 7 kg (range: 1-40 kg). Heartburn was noticed in 308/1256 patients (24.5%) and respiratory symptoms were present in 280/1256 (22.3%) patients. Nocturnal cough, effort dyspnea and recurrent bronchial infections were noted in respectively 14.5% (182/1256), 5% (63/1256) and 2% (25/1256) of cases. More rarely, it was a genuine bronchial asthma in 12/1256 (0.9%). Pulmonary banal germ abscess, pulmonary tuberculosis and a mediastinal syndrome were noted in respectively 0.3% (4/1256), 0.16% (2/1256) and 0.3% (4/1256) of cases (Table 3).

Para-clinical data

Esophageal barium swallow was performed in 95% (1193/1256) of patients. It allowed note of a typical aspect of bird's beak stenosis in 87% (1037/1193) of cases and a dilatation of the EB in 95% (1193/1256) of cases. The mean esophageal diameter was 5.8 ± 4.3 cm (95%CI: 1.9-8.5). The respective rates of stages 0, Ⅰ, Ⅱ and Ⅲ of dilatation were 6%, 28%, 42% and 24%.

Endoscopy was pathological in 85% (1067/1256) of cases. A popping effect, a fluid stasis and the EB dilation aspect were observed respectively in 47% and 53% of cases. The popping effect was isolated in 414/1256 (33%) patients, whereas the fluid stasis was always associated to an EB dilation aspect. Esophageal candidiasis and stasis esophagitis were noted respectively in 6% (75/1256) and 0.4% (5/1256) of cases.

Manometry was performed in 1186 patients and the LES pressure (LESP) was specified in 76% (954/1256) cases. A failure of the probe passage through the cardia, due to an EB tortuosity or an absence of LES opening, was observed in 24% (302/1256) of patients. The mean LESP was 32.17 ± 15 mmHg. The LES was normotensive in 47% (448/954), hypertensive in 53% (505/954) of patients and hypotensive in 0.6% (6/954) of cases.

Incomplete or absent LES relaxation was noted in 98% (934/954) of cases. Relaxation rate ranged from 0% to 25% in 383/954 (40%) patients, from 26% to 50% in 327/954 patients (34%) and from 52 to 80% in 196/954 (20.5%) patients. Esophageal peristalsis was evaluated in 94.4% (1186/1256) of patients. An extended aperistalsis to the whole EB was constant. Vigorous achalasia was noted in 26% (308/1186) of cases. The average amplitude of the contractile wave was 20.78 ± 18.67 (95%CI: 0-37) mmHg in the classic achalasia group and 57 ± 19 (range: 40-134 mmHg) in the vigorous achalasia group. The esophageal basal pressure was studied in 277 patients. It was always positive with a mean value of 4.7 ± 5 mmHg (range: 0-18) (Tables 4 and 5).

DISCUSSION

Data concerning the epidemiology of achalasia in Africa are lacking, and the epidemiology of the disease in Algeria has never been studied. To our knowledge, this is the first study of achalasia in our country. This prospective study focused on a large series of 1256

Table 5 Manometric results in 1186 (94.5) *n* (%)

LES pressure (mmHg)	954 (76); mean: 32 ± 15 (10-87)
12-24	257 (27)
24-34	192 (20)
> 34	504 (53.5)
< 12	2 (0.6)
LES relaxation	1130 (90)
Absent	46%
Incomplete	56%
EAC mean (mmHg)	1186 (94.5) 26.78 ± 19.67 (0-134)
Classic achalasia (mmHg)	20.78 ± 18.76 (0-37)
Vigorous achalasia	57 ± 19 (40-134)

LES: Lower esophageal sphincter; EAC: Esophageal amplitude contraction.

patients with achalasia, from the 48 provinces of Algeria. This is a homogenous and representative sample of Algerian population, from which it was possible to estimate, for a period of 25 years, the incidence of this disease in Algeria and to study its clinical and para-clinical features. Our work has shown that the average annual incidence of achalasia has followed an upward curve during the different periods of the study. Indeed, it increased from $0.04/10^5$ inhabitants/year during 1990-1997 to $0.27/10^5$ inhabitants/year from 2008 to 2014.

The few studies[6-12] that have investigated the epidemiology of this disease worldwide, mostly carried out in Western countries, showed that the annual incidence of achalasia varies between 0.55 and $1.63/10^5$ inhabitants, 2 to 5 times higher rates compared to those noted in our study and those of the two Asian series[11,12]. The same trend was noted with the prevalence results, their values ranged from $7.1/10^5$ inhabitants to $13.4/10^5$ inhabitants in Western countries[10] and between $1.77/10^5$ and $6.29/10^5$ inhabitants in Asia and Algeria (3.14 patients/10^5 inhabitants).

All these studies conclude that achalasia is a rare condition; the prevalence is probably underestimated because many physicians are inadequately familiar with the clinical picture of achalasia. This approach is supported probably by the very likely impact that the awareness campaign had on the increase of the annual incidence of achalasia in Algeria. This condition is even rarer in black African patients or those living in the tropics. Actually, both series published on black patients - the SILBER series in South Africa[13], which enrolled 26 cases in 10 years, and the STEIN[14] series in Zimbabwe, which reported 25 cases between 1974 and 1983 - suggest that the incidence of achalasia in sub-Saharan Africa is $0.003/10^5$ inhabitants/year. Thus, this motility disorder is 10 times less common in these countries compared to Western countries.

However, the low number of works that have been devoted to it and the lack of diagnostic facilities in these regions of the world suggest that the exact frequency of this disorder is probably underestimated in these regions.

In our study, we found a gradient between different regions of the country. The incidence of the disease was two and half times higher in the north and the center compared to the south (0.196 *vs* 0.116 *vs* 0.062). These regions have ethnic (predominance of black patients at the south of the country), climatic and food differences. We believe that the incidence of achalasia is underestimated in Algeria, especially in the south, because of many factors, one of them being the limited access to our institution due to distance and possibly to socio-economic status and the quality of medical coverage. In the United States, various epidemiological studies carried out in the country showed no achalasia incidence differences between black people and those with white skin[15,16]. Thus, this variability in incidence between races, different regions in the world and different regions of a same country, implies, as has been suggested by some authors, the existence of environmental and genetic factors responsible for the occurrence of the disease[17-19]. Achalasia affects both sexes with equal rate[20] as shown in our study. The mean age of diagnosis of this motility disorder which affects adults in most cases in Western series, varies between 46-years-old and 66-years-old, with two peaks of incidence in the third and seventh decade of life [15,18,21].

In our study, patients were relatively younger; whereas the mean diagnosis period was 59.7 mo, ranging from 2 mo to 480 mo. This diagnosis latency is probably due to aspecific symptoms that may delay the condition diagnosis investigation[22]. To our knowledge, the diagnosis period (mean delay diagnosis) and age at diagnosis have never been studied on such an important series. In this work, we noticed that both of them have been improved during the three periods. This trend is probably related to the awareness campaign, conducted regularly from 1999 as a CME, in different regions of the country. Further studies are required in other countries with a larger population to confirm these results.

The frequency of clinical signs varies depending on the studied symptom and series[5,16].

Dysphagia is almost constant (82%-100%), while a large variability between series characterizes regurgitation (56%-97%), weight loss (30%-91%), chest pain (17%-95%) and heartburn (27%-42%). The same results were found in our series. One of the most interesting clinical aspects of this study is to have shown that chest pain preceded dysphagia by several months (mean: 23 ± 14 mo) in 5% of patients. This misleading clinical presentation of this affection in our series is probably one of the causes of delayed diagnosis, since patients were initially oriented to cardiology looking for a coronary disease. Broncho-pulmonary signs, noted in 11% to 46% of cases,

may also be the cause of delayed diagnosis because they usually lead patients to pneumology consultation first[23].

The diagnosis of achalasia relies on EM[24,25]. However, this procedure, which is available only in reference centers in low-incomes countries, is not performed as first-line. It is preceded, due to the nature of revealing signs as dysphagia and regurgitation, by barium swallow and upper endoscopy to fully exclude inflammatory or structural lesions.

The benefit of manometry is particularly important when the barium swallow and endoscopy do not reveal abnormalities[26]. EB aperistalsis, constant in our study, is the only manometric cardinal feature that leads to achalasia diagnosis. It is characterized in most cases by simultaneous or undefined esophageal contractions of very low amplitude, thus realizing the aspect of classical manometric achalasia.

Achalasia is considered vigorous when the mean amplitude threshold is more than 37 mmHg[27]. This form, which still subject of controversy as to its individualization as a separate entity from classic achalasia[28], would in fact correspond to an early disease stage characterized by a high amplitude contractions of the EB with intense retro-sternal pain. In our experience, vigorous achalasia remains a rare situation that presents no clinical or manometric feature compared to classic achalasia.

For a long time, LES hypertonia was considered as essential for achalasia diagnosis, while LES hypotonia was described as incompatible with the diagnosis of untreated achalasia[25,29]; its presence should instead lead to scleroderma diagnosis or gastroesophageal reflux disease.

In fact, some authors recently reported that LES hypertonia was present in only 42% of patients while hypotonia was noted in 25% of them[30]. Our work has also shown that LES hypertonia was inconstant, being observed in only 53% of patients while hypotonia was very rare. More constant is the LES relaxation defect, which was found in 98% of patients, the association with esophageal aperistalsis is highly suggestive of this condition's diagnosis. If LES is manometrically normal, the diagnosis of achalasia should rely on the confrontation of manometric, clinical, radiological and endoscopic data.

The few studies that have been devoted to children show that achalasia is even infrequent in this age group compared to adults. Pediatric achalasia represents only 4% to 5% of all achalasia diagnosed worldwide and 10% of those included in our study. The incidence in this group age is estimated between 0.1-0.18/10^5 children/year[31,32].

At the family level, our study showed that, infantile achalasia can take many forms. It may be a non-familial isolated classic achalasia, a sporadic Allgrove syndrome, a familial achalasia without Allgrove syndrome or a familial Allgrove syndrome. In this work, the respective prevalence of familial and non-familial forms in children achalasia was 45% and 55%.

These results, which show a high rate of family forms, are most probably related to the widespread tradition of consanguineous marriage in Algeria. Infantile achalasia must systematically lead to seeking of similar cases in the family and other signs of Allgrove syndrome. This syndrome is a very rare genetic autosomal recessive disorder, and its occurrence is favored by consanguineous marriages.

The AAAS gene responsible for this disease is carried by the long arm of chromosome 12 (12q13) and contains 16 exons. Mutations of this gene which were currently implicated are the IVS14, most common mutation, and EVS9. These mutations were identified for the first time in 2000 by Tullio-Pelet *et al*[33] in a study that gathered 7 of the 18 Algerian families included in this study, 3 Tunisian families, and a sing Turkish, French and Spanish family each. The few studies that have tackled familial achalasia reported that 1/3 of children were from consanguineous marriages, whereas parent/children transmission was very rare or exceptional[34,35].

In summary, achalasia is a rare disorder, the worldwide frequency of which, most probably underestimated, varies depending on countries. This variability is probably related to genetic and environmental factors. In Algeria, the incidence of the disease is at least 0.27/10^5 inhabitants. This motility disorder sometimes raises the difficulty of its delayed diagnosis due to its aspecific revealing signs. However, in this study there is suggestion that CME improved (reduced) the delay diagnosis, age at diagnosis and increased the incidence of achalasia. The diagnosis is evident when EM shows EB aperistalsis associated to LES hypertonia. When hypertonia is absent, achalasia diagnosis should rely on a confrontation of manometry, barium swallow and upper endoscopy data. Finally, the discovery of an infantile achalasia must systematically lead to looking for Allgrove syndrome and similar cases in the family.

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We are indebted to the patients for their invaluable cooperation and we recognize our master, the deceased Professor Brahim Touchene.

COMMENTS

Background

There is a lack of epidemiological data of achalasia and Allgrove syndrome in Algeria. The impact of continuing medical education (CME) on incidence of achalasia has not been evaluated. The current study was designed to investigate the incidence of achalasia and to evaluate the impact of CME on the incidence of this disease.

Research frontiers

For some authors, the incidence of achalasia is increasing and environmental factors have been implicated. But, few epidemiological studies around the world

have been devoted to it.

Innovation and breakthroughs

This large prospective study has shown that the incidence in Algeria is at last 0.27/10^5 inhabitants/year. The incidence of achalasia is increasing and there is a variability according to different regions in the same country. This variability is probably due to genetic and environmental factors. There is positive impact of CME on the incidence of achalasia.

Applications

This study serves as additional evidence supporting the investigation of the potential role of the environmental factors in increasing of the incidence of achalasia. The potential of CME in increasing incidence and possibly reducing the delay and age at diagnosis of achalasia is paramount.

Peer-review

This paper reports a very large experience, including very pertinent, meticulously collected clinical information over a period of 25 years, and is well written overall.

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CASE REPORT

Limited, local, extracolonic spread of mucinous appendiceal adenocarcinoma after perforation with formation of a malignant appendix-to-sigmoid fistula: Case report and literature review

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Abstract

A 68-year-old man presented with progressive right lower quadrant abdominal pain and tenderness without rebound tenderness, and with constipation during the prior 9 mo. Abdomino-pelvic computed tomography and magnetic resonance imaging demonstrated a dilated appendix forming a fistula to the sigmoid colon. Open laparotomy revealed a bulky abdominal tumor involving appendix, cecum, and sigmoid, and extending up to adjacent viscera, without ascites or peritoneal implants. The abdominal mass was removed en bloc, including resection of sigmoid colon, cecum (with preservation of ileocecal valve), appendix, right vas deferens, testicular vessels, and minimal amounts of anterior abdominal wall; and shaving off of small parts of the walls of the urinary bladder and small bowel. Gross and microscopic pathologic examination revealed an appendix-to-sigmoid malignant fistula secondary to perforation of mucinous adenocarcinoma of the appendix with minimal local spread (stage T4). However, the surgical margins were

clear, all 13 resected lymph nodes were cancer-free, and pseudomyxoma peritonei or peritoneal implants were not present. The patient did well during 1 year of follow-up with no clinical or radiologic evidence of local recurrence, metastases, or pseudomyxoma peritonei despite presenting with extensive stage T4 cancer that was debulked without administering chemotherapy, and despite presenting with malignant appendiceal perforation. This case illustrates the non-aggressive biologic behavior of this low-grade malignancy. The fistula may have prevented free spillage of cancerous cells and consequent distant metastases by containing the appendiceal contents largely within the colon.

Key words: Mucinous adenocarcinoma; Appendicitis; Appendix; Malignant fistula; Pseudomyxoma peritonei; Colon cancer; Metastases

Core tip: A patient with mucinous appendiceal adenocarcinoma had appendiceal perforation that was locally contained by a malignant appendix-to-sigmoid fistula. The patient presented with right lower quadrant pain and tenderness and constipation. Abdomino-pelvic computed tomography and magnetic resonance imaging revealed a bulky peri-appendiceal mass containing an appendix-to-sigmoid-fistula. Pathologic analysis after debulking surgery revealed a locally extensive cancer involving appendix, sigmoid, and cecum and extending up to adjacent viscera with clear surgical margins and benign lymph nodes. The patient remained free of local recurrence/metastases during 1 year of follow-up despite not receiving chemotherapy/radiotherapy. This apparently favorable outcome is due to this cancer's nonaggressive biology, and the fistula which likely largely contained cancer cell spillage within the colon and prevented free cancer cell spillage.

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INTRODUCTION

Malignant colonic perforation entails a poor prognosis because of presentation with acute sepsis/peritonitis and subsequent development of gross metastases from intraperitoneal seeding of malignant cells from the perforation[1]. A case is reported of mucinous appendiceal adenocarcinoma (MAA) presenting as a bulky mass due to appendiceal perforation and fistulization, treated by debulking surgery; and presenting initially without sepsis; and subsequently at 1 year follow-up had no evident local or distant metastases despite the prior malignant appendiceal perforation. The pathophysiology of this clinical presentation and course is explained by the appendix-to-sigmoid fistula containing spillage of cancerous cells within the colon and preventing free spillage, and by the low grade, nonaggressive biology of MAA[2].

The literature was systematically reviewed using the medical subject headings/key words of: "mucinous adenocarcinoma" or "pseudomyxoma peritonei" or "appendiceal neoplasm" or "appendiceal adenocarcinoma". Two authors independently reviewed the literature and decided by consensus which articles to incorporate in the study. This case report received exemption/approval from the William Beaumont Hospital IRB on June 16, 2016.

CASE REPORT

A 68-year-old man with past medical history of hypertension, hyperlipidemia, and colonic diverticulosis presented with progressive right lower quadrant abdominal pain and constipation during the prior 9 mo. Colonoscopy with good cecal visualization, performed 2 years earlier for routine colon cancer screening, had revealed a normal colon and normal appendiceal orifice. Physical examination revealed normal vital signs, soft abdomen, minimal right lower quadrant tenderness, no rebound tenderness, and no palpable abdominal mass. Laboratory analysis revealed hemoglobin = 13.1 gm/dL, leukocyte count = 12000/mL, and serum bicarbonate = 28 mmol/L. Serum electrolytes, serum parameters of liver function, serum parameters of renal function, and serum lactate level were within normal limits. Abdomino-pelvic computed tomography (CT) revealed a dilated, heterogeneous, appendix with an 8-cm-long, ovoid, periappendiceal mass containing a fistula to sigmoid colon (Figure 1A), and revealed no findings suggestive of pseudomyxoma peritonei or peritoneal implants, including intraperitoneal fluid, peritoneal calcifications, or scalloping of the liver. Abdomino-pelvic magnetic resonant imaging (MRI) showed on coronal view a dilated, 8-cm-long, appendix fistulizing to the sigmoid (Figure 1B and C); and showed on axial view an abnormally thick, enhancing, appendiceal wall without significant peri-appendiceal inflammation (Figure 1D). Open laparotomy revealed an extensive mass involving appendix, cecum, sigmoid colon, anterior abdominal wall, and urinary bladder (Figure 2A); no peritoneal implants, and no pseudomyxoma peritonei. The abdominal mass was removed en-bloc, including resection of sigmoid colon, cecum (with preservation of ileocecal valve), appendix, right vas deferens, testicular vessels, and minimal amounts of anterior abdominal wall; and shaving off of small parts of the walls of the urinary bladder and small bowel. Gross pathological examination of the resected mass

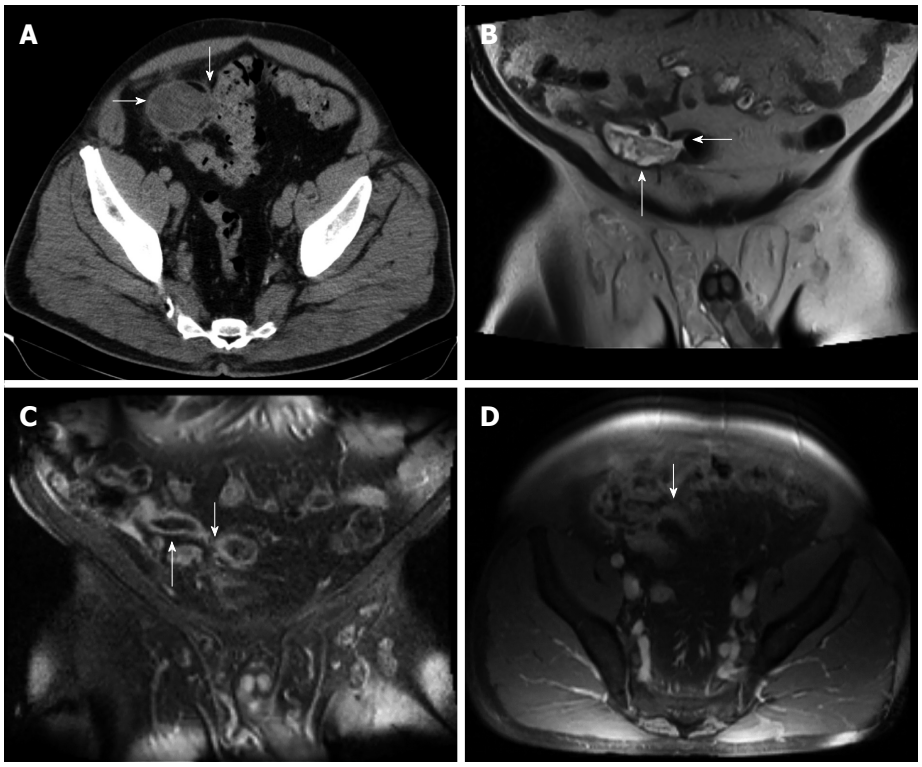

Figure 1 Abdomino-pelvic computed tomography. A: Axial computed tomography (CT) image of the pelvis, enhanced with iodinated contrast, demonstrates a dilated, heterogeneous appendix (horizontal arrow) forming a fistula with sigmoid colon (fistulous connection at vertical arrow); B: Coronal T2-weighted fluid-sensitive MRI sequence depicts dilated, fluid-filled, appendix (vertical arrow), extending and forming a fistula with the sigmoid colon (horizontal arrow); C: Coronal post-gadolinium contrast enhanced T1-weighted MRI image demonstrates dilated appendix with non-enhanced, central fluid and enhanced wall (upward arrow) that extends towards sigmoid colon (downward arrow); D: Axial post-gadolinium contrast enhanced T1-weighted MRI image demonstrates abnormally thick and enhanced appendiceal wall (arrow) without significant surrounding inflammatory changes.

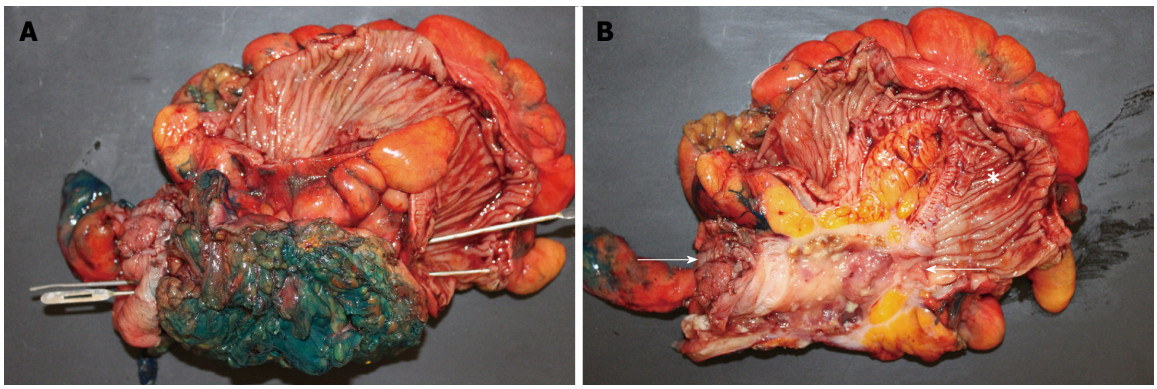

Figure 2 Open laparotomy revealed an extensive mass involving appendix, cecum, sigmoid colon, anterior abdominal wall, and urinary bladder. A: Gross pathologic photograph shows entire surgically resected specimen (including appendix, cecum, sigmoid colon, part of bladder, and anterior abdominal wall) after bisecting the sigmoid colon that exposes the haustral folds within sigmoid, but before bisecting the centro-inferior tumor (note green ink identifying surgical margins of tumor). Insertion of two parallel probes (one inserted from left to right and the other inserted from right to left) demonstrate a fistula traveling from the cecum, on the bottom left, to the sigmoid, on the bottom right. Bisection of the centro-inferior tumor (B) reveals that the appendix forms the malignant fistula; B: Gross pathologic specimen after bisection of centro-inferior tumor (seen before bisection in A) exposes an irregular, cystically dilated, malignant appendix that forms the malignant fistula. It is lined by a variably thick wall composed of the mucinous adenocarcinoma and filled with viscous, mucoid, content. Sigmoid (star). Cecum is at left bottom of photograph immediately adjacent to arrow.

revealed an appendix-to-sigmoid fistula, as confirmed by a probe, from prior perforation of a promontoric (preileal/postileal appendix traveling from cecal base towards the sigmoid in the pelvis) appendix (Figure 2B). Microscopic pathology showed well-differentiated, invasive, mucinous, adenocarcinoma diffusely involving the appendix, sigmoid, and cecum through the serosa (Figure 3). Histopathology showed no invasion of adjacent organs, such as the bladder wall or anterior abdominal wall. Lymphovascular invasion and satellite

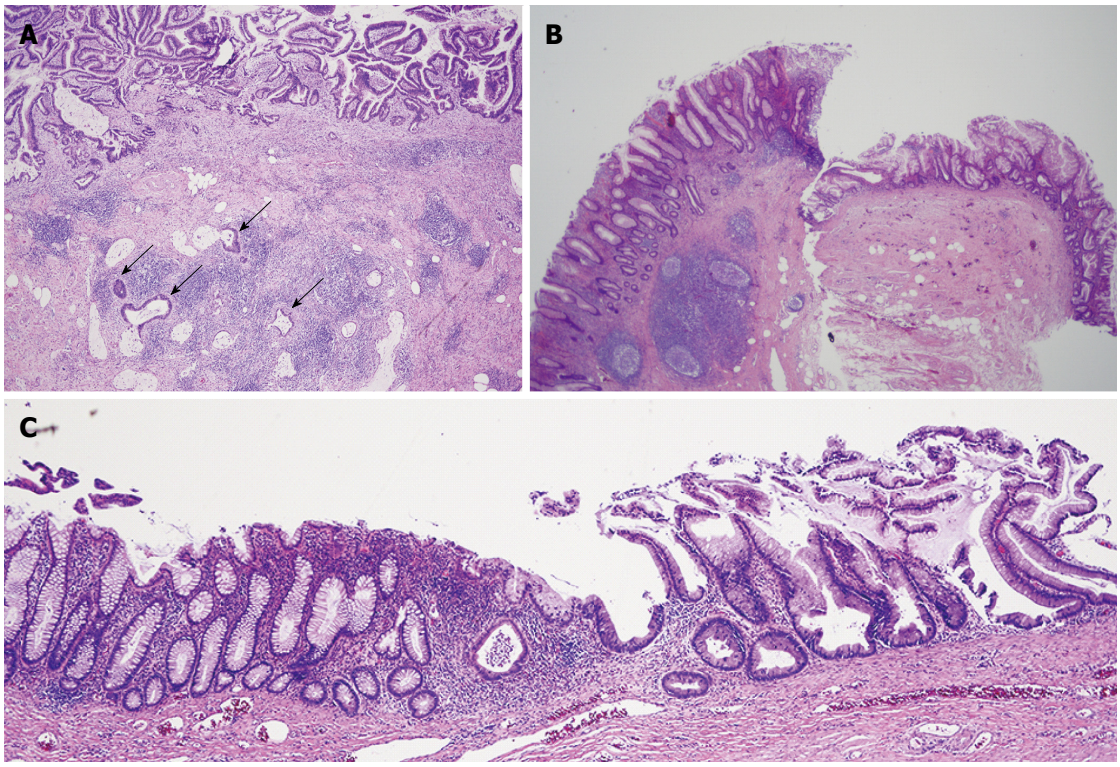

Figure 3 Microscopic pathology showed well-differentiated, invasive, mucinous, adenocarcinoma diffusely involving the appendix, sigmoid, and cecum through the serosa. A: Low power photomicrograph of mucinous appendiceal adenocarcinoma (MAA) within fistulous (appendiceal) wall showing villoglandular adenomatous epithelium containing basophilic cells, within bizarre mucosal glands (upper area), and infiltration of cancerous mucinous glands deep in the wall (arrows). The fistulous wall also shows extensive fibrosis and chronic inflammation [hematoxylin-eosin staining (HE)]; B: Low power photomicrograph shows mucinous appendiceal adenocarcinoma with serrated glands, on the right, contiguous with normal cecal mucosa, with normal cecal glands, on the left (HE); C: Low power photomicrograph shows junction of mucinous appendiceal adenocarcinoma with serrated glands on the right, contiguous with normal sigmoid mucosa with normal glands, on the left (HE).

peritumoral nodules were not present. All surgical margins and all 13 resected lymph nodes were devoid of cancer (Stage pT4b N0). The patient developed postoperative ileus from which he recovered, and was discharged 11 d postoperatively. No postoperative chemotherapy or radiotherapy was administered. The patient was doing well 1 year later, with no ascites, pseudomyxoma peritonei, or cancer recurrence, as observed clinically by an oncologist and by repeat abdomino-pelvic CT examination.

DISCUSSION

Primary adenocarcinoma of the appendix is rare. It accounts for 0.12 cases per 1000000 patients per annum, 0.05%-0.2% of all appendectomies, about 0.2% of all gastrointestinal neoplasms, and only 6% of appendiceal cancers[3]. Appendiceal adenocarcinoma is divided into colonic, mucinous (MAA), goblet cell, and signet ring cell types[4,5]. MAA usually presents with nonspecific findings. It frequently presents with acute abdominal pain resembling acute appendicitis; sometimes presents as an abdominal mass detected by palpation or abdominal imaging, as occurred in the currently reported case; and rarely presents with nausea, vomiting, ascites, or involuntary weight loss. Due to its nonspecific presentation, it is rarely diagnosed preoperatively and usually diagnosed postoperatively by histopathological examination of the resected specimen after exploratory surgery for other suspected disease[3,6,7].

MAA, a low-grade, relatively noninvasive, cancer, rarely produces distant metastases, except when signet ring cells occur in addition to mucin (denoted separately as signet ring cell cancer) that indicates high-grade mucinous adenocarcinoma[8]. No cases of distant lymphatic or hematogenous metastases were reported by Nitecki *et al*[2] among 52 patients with MAA, even in patients with severe intraperitoneal disease, but one patient had pulmonary metastases from MAA in a case report by Gourgiotis *et al*[3]. Local nodal involvement is also uncommon when the cancer involves only the mucosa or submucosa[9], but the incidence increases to 20%-25% when the primary cancer more deeply invades the appendiceal wall[10,11]. Ovarian involvement from MAA is, however, common in females; among 23 female patients undergoing oophorectomy reported by Niteck[2], 13 had ovarian involvement. Pseudomyxoma peritonei (PMP) occurs when mucin escapes intraperitoneally, often secondary to appendiceal perforation (disseminated peritoneal adenomucinosis), or uncommonly from malignant peritoneal seeding of mucin-producing cancerous cells (disseminated peritoneal carcinomatosis)[10,12].

MAA frequently causes appendiceal perforation, as occurred in this case, attributed to the mucinous gel obstructing the lumen and the narrow appendiceal lumen. For example, in a comprehensive literature review encompassing 316 cases of appendiceal adenocarcinomas, 55% of patients presented with appendiceal perforation[13,14].

Data on therapy are sparse because MAA is rare. Therapy depends upon pathologic stage. Right hemicolectomy is recommended after an initial appendectomy when pathologic examination of the resected specimen reveals MAA localized to the appendix. Nitecki *et al*[2] reported in a study of 29 patients with clinically localized MAA, a 73% 5-year-survival after right hemicolectomy versus a 44% 5-year-survival after appendectomy alone; patients undergoing right hemicolectomy after initial appendectomy have residual cancer detected in up to 27% of right hemicolectomy specimens[10]. Abdomino-pelvic CT and colonoscopy should be performed before undergoing the right hemicolectomy after initial appendectomy to detect synchronous colon cancers which occur in up to 18%-27% of patients[2,7]. Women should standardly undergo oophorectomy because of frequent ovarian metastases[2,7].

Presence of peritoneal spread requires surgical debulking by primary tumor excision, right hemicolectomy, lymph node dissection, omentectomy, peritoneotomy, and removal of malignant ascites[7]. Patients with intraperitoneal lymphadenopathy or PMP might theoretically benefit from adjuvant intraperitoneal chemotherapy, using 5-flurouracil and levamisol, but this chemotherapy is not of proven benefit and therefore controversial[7,10,15]. However, intraperitoneal chemotherapy is generally recommended if peritoneal implants are identified[16-18]. Frequent surveillance colonoscopy is recommended after surgery because of an approximately 17% incidence of metachronous colon cancer[2,7].

The current case illustrates the nonaggressive biologic behavior of this low-grade malignancy. The patient presented with abdominal pain and constipation for 9 mo; laparotomy revealed a bulky tumor involving appendix, cecum, sigmoid colon, and adjacent viscera. The promontoric appendiceal location (appendix located in pelvis and traveling towards the sigmoid, as observed intraoperatively), an appendiceal anatomic variant that occurs in several percent of patients[19,20], may have promoted sigmoid fistulization. The adhesiveness of mucin may also have promoted fistulization. Rapid fistulization presumably contained the leakage of mucin and cancerous cells within the fistula and sigmoid colon, and prevented free intraperitoneal spillage, as would have been expected from free appendiceal perforation. Despite appendiceal rupture, malignant fistulization, and pathologic T4 stage, no lymph nodes were involved at presentation (stage N0), and no recurrent tumor or PMP was detected, by clinical examination or repeat abdomino-pelvic CT, 1 year after the initial surgery without chemotherapy. The patient had undergone colonoscopy 2 years before the surgery which had not detected MAA; appendiceal cancer is commonly missed at colonoscopy because the cancer is located deep in the appendix and not visible from the cecal lumen[21].

This study is limited because it reports only one case, and surgical cure cannot be definitively claimed because of only 1 year of follow-up. However, a favorable prognosis is indicated by the clear surgical margins, absence of lymph node involvement, absence of the aggressive signet ring cell histology, and absence of PMP or distant metastases detected at surgery or 1 year thereafter. In conclusion, this patient had no evident local, peritoneal, or distant metastasis 1 year after undergoing debulking surgery without chemotherapy, despite appendicular perforation and malignant fistulization from MAA. This phenomenon is explained by the appendix-to-sigmoid fistula containing the spillage of cancerous cells within the colon and preventing free spillage, by the low grade, nonaggressive nature of MAA[2], and perhaps by the adhesiveness of mucin which might promote containment of appendiceal spillage.

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COMMENTS

Case characteristics

A 68-year-old man presented with progressive constipation and right lower quadrant (RLQ) abdominal pain for the prior 9 months and with right lower quadrant tenderness, without rebound tenderness, on physical examination. Colonoscopy with good cecal visualization, performed 2 years earlier for routine colon cancer screening, had revealed a normal colon and normal appendiceal orifice.

Clinical diagnosis/ differential diagnosis

The differential diagnosis based on patient history of RLQ abdominal pain and constipation for 9 mo, and physical finding of RLQ tenderness without rebound tenderness is very broad. This mild clinical presentation contrasts with the severe final pathologic diagnosis of mucinous appendiceal adenocarcinoma with perforation/fistulization. This discrepancy is explained by the relatively non-aggressive biology of this cancer, prevention of acute peritonitis and distant cancer spread from containment of the appendiceal perforation by fistulization, and perhaps the adhesiveness of the mucin produced by this cancer which may promote fistulization.

Laboratory diagnosis

All routine blood tests were within normal limits, except for very mild leukocytosis. This mild presentation contrasts with the severe final pathologic diagnosis of mucinous appendiceal adenocarcinoma with perforation/fistulization. This discrepancy is explained by the pathological finding that the cancer at perforation formed a malignant appendiceal-to-sigmoid fistula that

most likely contained the inflammation and prevented acute appendicitis after perforation, and helped locally contain the malignancy without distant malignant seeding and consequent development of distant metastases.

Imaging diagnosis

Abdomino-pelvic computed tomography revealed a dilated, heterogeneous, appendix with an 8-cm-long, ovoid, periappendiceal mass containing an appendix-to-sigmoid fistula, without evident pseudomyxoma peritonei or peritoneal spread. Abdomino-pelvic MRI showed on coronal view a dilated, 8-cm-long, fluid-filled, appendix fistulizing to the sigmoid; and showed on axial view an abnormally thick, enhancing, appendiceal wall without significant peri-appendiceal inflammation. These imaging tests, however, did not reveal the cause of the appendiceal fistulization. The differential diagnosis of the radiologic findings included most likely benign perforating appendicitis, and unlikely appendiceal malignancy with fistulization, including the rare malignancy of mucinous appendiceal adenocarcinoma.

Treatment

Open laparotomy revealed an extensive mass involving appendix, cecum, sigmoid colon, anterior abdominal wall, and urinary bladder; no peritoneal implants, and no pseudomyxoma peritonei. The abdominal mass was removed en-bloc including resection of sigmoid colon, cecum (with preservation of ileocecal valve), appendix, right vas deferens, testicular vessels, and minimal amounts of anterior abdominal wall; and shaving off of small parts of the wall of the urinary bladder and small bowel. No chemotherapy or radiotherapy was administered postoperatively because pathologic examination revealed a bulky primary tumor with surgical margins clear of cancer, no involved lymph nodes, and no distant metastases or pseudomyxoma peritonei.

Pathological diagnosis

Gross pathological examination of the resected mass showed an appendix-to-sigmoid fistula, as confirmed by a probe, from prior perforation of a promontoric (preileal/postileal appendix traveling from cecal base towards the sigmoid in the pelvis). Microscopic pathology showed well-differentiated, invasive, mucinous, adenocarcinoma diffusely involving the appendix and adjacent mass. All surgical margins and all 13 lymph nodes in the resected specimen were devoid of cancer (Stage pT4b N0).

Related reports

Mucinous appendiceal adenocarcinoma is a low-grade cancer that rarely metastasizes to distant organs, except for the ovaries in females. The relatively benign clinical presentation and clinical course during 1 year of follow-up is explained by the well-described indolent, nonaggressive, nature of this cancer, and the sigmoid fistulization after malignant perforation that likely prevented the acute clinical presentation of sepsis normally expected after free appendiceal perforation, and likely contained the spillage of cancer cells and prevented subsequent distant metastases. The finding of mucinous appendiceal carcinoma despite a normal appearing appendiceal orifice at colonoscopy 2 years earlier is pathophysiologically reasonable. Colonoscopy performed soon before the pathologic diagnosis of mucinous appendiceal adenocarcinoma usually does not reveal the cancer because only the appendiceal orifice is visualized at colonoscopy and cancer deep in the appendix is not visualized.

Experiences and lessons

The key finding is the favorable clinical course and apparent cancer cure with negative surgical margins, no involved lymph nodes, and no pseudomyxoma peritonei at surgery and at 1-year follow-up, despite the malignant perforation and bulky primary cancer. This phenomenon is explained by the appendix-to-sigmoid fistula containing the spillage of cancerous cells within the colon and preventing free spillage and consequent distant metastases, by the low grade, nonaggressive nature of MAA, and perhaps by the adhesiveness of mucin which might have promoted fistulization.

Peer-review

This is an interesting presentation of a Case report with literature review in which is evaluated limited, local, extracolonic spread of mucinous appendiceal adenocarcinoma after perforation with formation of a malignant appendix-to-sigmoid fistula.

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CASE REPORT

Anaplastic carcinoma of the pancreas: Case report and literature review of reported cases in Japan

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Abstract

We report a case of a 64-year-old woman with anaplastic carcinoma of the pancreas (ACP) with cyst formation and review 60 ACP cases reported in Japan. In 20% of cases, laboratory tests revealed severe anemia (hemoglobin level < 10.0 g/dL) and elevated leucocyte counts (> 12000/mm^3), which were likely attributable to rapid tumor growth, intratumoral hemorrhage, and necrosis. Elevated serum CA19-9 levels were observed in 55% of cases. Cyst-like structures were observed on imaging in 47% of cases, and this finding appears to reflect subsequent cystic degeneration in the lesion. Macroscopically, hemorrhagic necrosis was observed in 77% of cases, and cyst formation was observed in 33% of cases. ACP should be considered when diagnosing pancreatic tumors with a cyst-like appearance, especially in the presence of severe anemia, elevated leucocyte counts, or elevated serum CA19-9 levels.

Key words: Anaplastic carcinoma; Pancreatic cancer; Pleomorphic type; Prognosis; Undifferentiated carcinoma

Core tip: Anaplastic carcinoma of the pancreas (ACP), an uncommon histologic subtype of pancreatic cancer, is well known to be associated with more aggressive tumor behavior and less favorable prognosis than conventional pancreatic ductal adenocarcinoma. How-

ever, the literature on ACP has been very limited, and its clinicopathological features, therapeutic management, and clinical outcome remain uncertain. We report a case of ACP showing cyst formation and review 60 cases of ACP reported in Japan to elucidate the clinical and radiological features of ACP. This literature review describes the greatest number cases of ACP of any report to date.

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INTRODUCTION

Anaplastic (undifferentiated) carcinoma of the pancreas (ACP), an uncommon histologic subtype of pancreatic cancer that accounts for 0.8% to 5.7% of all pancreatic exocrine neoplasms[1-4], is well known to be associated with more aggressive tumor behavior and a less favorable prognosis than conventional pancreatic ductal adenocarcinoma (PDAC)[1]. Different subtypes of ACP have been described using various terms, including spindle cell, giant cell, pleomorphic giant cell, and round cell[5]. On one hand, according to the World Health Organization (WHO) classification of tumors of the digestive system[6], ACP, as a synonym for undifferentiated carcinoma of the pancreas, has been defined as a malignant epithelial neoplasm in which a significant component of the neoplasm does not show a definitive direction of differentiation. However, undifferentiated carcinoma with osteoclast-like giant cells has been classified as a distinct entity from other subtypes of ACP. On the other hand, the sixth edition of the General Rules for the Study of Pancreatic Cancer by the Japan Pancreas Society (JPS) has defined ACP as a variant of pancreatic ductal carcinoma, which consists of a minor ductal carcinoma component along with a predominantly undifferentiated carcinoma component. In addition, the JPS has classified ACP into the following four subtypes: giant cell type, pleomorphic type, spindle cell type, and giant cell carcinoma of osteoclastoid (GCCO) type. In this classification system, undifferentiated carcinoma that shows no directionality of differentiation into any lineage has been distinguished from ACP[7]. Because the literature on ACP has been very limited, mostly represented by single case reports or analyses of small case series, large studies describing the clinicopathological features, therapeutic management, and clinical outcome of ACP are lacking. Additionally, accurate preoperative diagnosis of ACP is difficult because of a lack of proposed imaging features that can distinguish this malignancy from PDAC. Here, we report a case of pleomorphic ACP, according to the JPS classification; this case showed extensive intratumoral hemorrhage and necrosis, leading to cyst formation. Additionally, we review 60 cases of ACP, including 59 previously published cases in Japan and the present case, to elucidate the clinical, morphological, and radiological features of this malignancy. These findings enable a greater understanding of ACP, improve the accuracy of preoperative diagnosis and provide a more effective treatment strategy for this aggressive and uncommon subtype of pancreatic cancer.

CASE REPORT

A 64-year-old woman with upper abdominal pain and back pain was referred to our hospital for examination of a pancreatic mass that had been identified by the previous doctor on abdominal ultrasonography (US). Laboratory examinations revealed anemia (hemoglobin level of 10.3 g/dL) and a slightly elevated carbohydrate antigen (CA) 19-9 level (65.1 U/mL). Liver, biliary, and pancreatic enzyme activity levels were normal. Abdominal US revealed a heterogeneous hypoechoic mass with a cystic component in the pancreatic body. Contrast-enhanced abdominal computed tomography (CT) revealed a well-demarcated low-density mass measuring 35 mm in diameter, with an enhancement of the peripheral portion of the lesion in the pancreatic body during the delayed phase (Figure 1A). Magnetic resonance imaging (MRI) revealed a tumor showing mixed signal intensity and a fluid-fluid level on T2-weighted images, suggestive of intratumoral hemorrhage (Figure 1B). Endoscopic retrograde pancreatography (ERP) revealed compression of the main pancreatic duct (MPD) in the pancreatic body without any strictures or upstream dilatation of the MPD. Although the appearance on these images was not typical of conventional pancreatic cancer, cytology of the pancreatic juice samples collected during ERP was suggestive of adenocarcinoma. Based on a preoperative diagnosis of PDAC, surgery was performed. On laparotomy, the tumor had rapidly increased in size and involved the common hepatic artery. Therefore, distal pancreatectomy and splenectomy with en bloc celiac axis resection were conducted. On gross examination, a poorly demarcated, whitish tumor measuring 60 mm in diameter was observed (Figure 2A). In the central portion of the tumor, a large cystic lesion filled with hemorrhagic material was present. Histologically, the major part of the tumor exhibited a sarcomatous appearance and was composed of non-cohesive, pleomorphic tumor cells (Figure 2B). In the minor part of the tumor, a moderately differentiated tubular adenocarcinoma component was detected (Figure 2C). The central part of the tumor formed a cystic lesion, which contained hemorrhagic and/or fluidic material and was surrounded by necrotic area of the sarcomatous tumor (Figure 2D). Immunohistochemical analyses revealed that the sarcomatous component

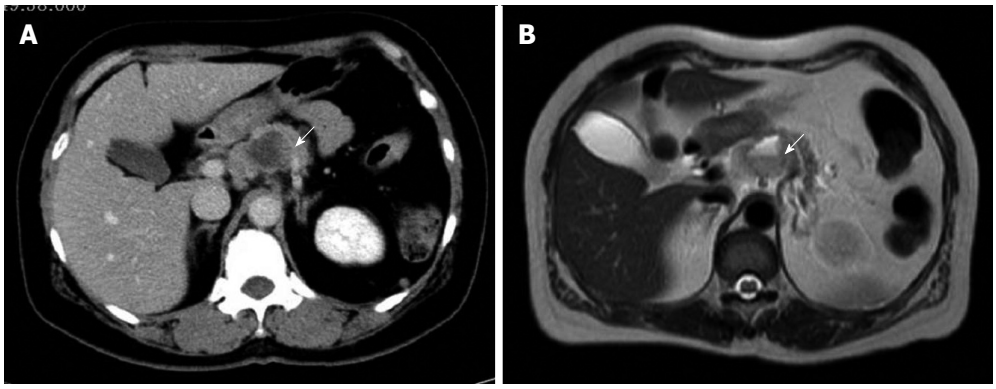

Figure 1 Imaging studies. A: Abdominal computed tomography revealing a well-demarcated cystic tumor with slight enhancement of the peripheral portion of the lesion in the pancreatic body (arrow); B: Magnetic resonance imaging revealing mixed signal intensity of the tumor and a fluid-fluid level on T2-weighted imaging (arrow).

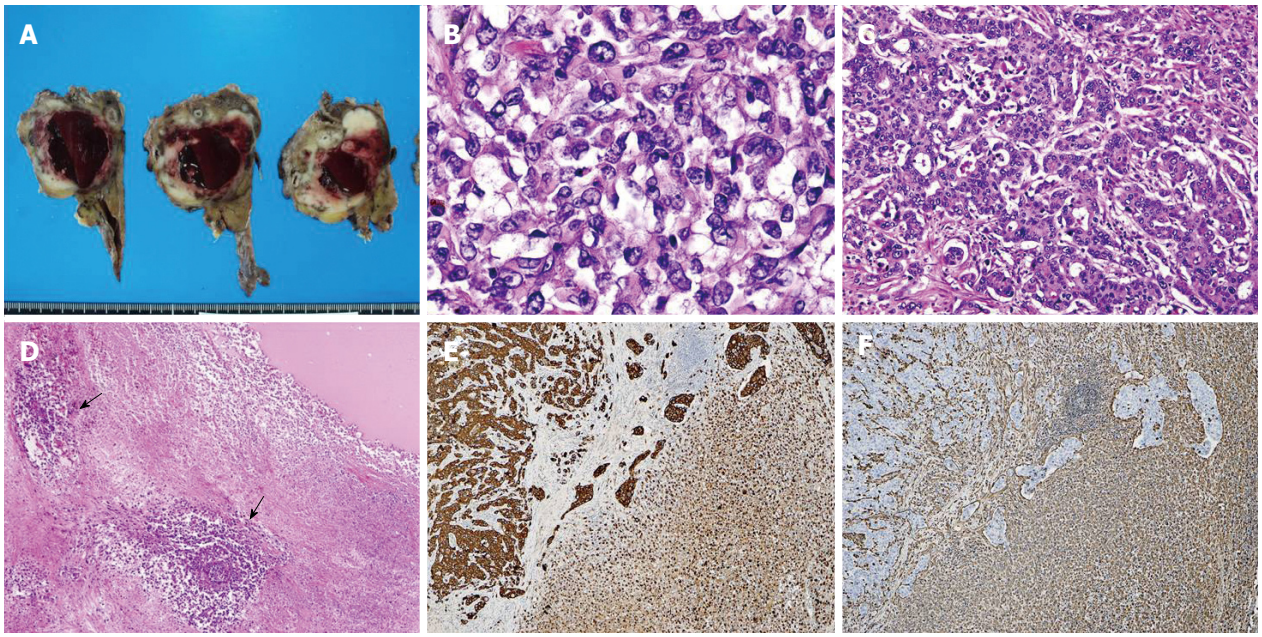

Figure 2 Pathology of the pancreatic tumor. A: Macroscopic appearance of the resected specimen. The tumor forms a poorly defined, whitish mass lesion measuring 60 mm in diameter. A large cyst filled with hemorrhagic material is present in the central portion of the tumor; B-C: Histology of the tumor. The major part of the tumor is composed of non-cohesive, pleomorphic tumor cells showing a sarcomatous appearance (B), whereas the minor part of the tumor is composed of irregularly shaped fused glandular structures, exhibiting the histology of moderately differentiated adenocarcinoma (C) [hematoxylin and eosin (HE) staining; original magnification, B: × 40, C: × 10]; D: Central part of the tumor. A cystic lesion filled with fluidic material (upper right) is surrounded by necrotic tumor tissue focally containing viable anaplastic carcinoma cells around the blood vessels (lower left/arrow) (HE staining; original magnification, × 4); E, F: Immunohistochemistry. Positive staining for pancytokeratin (AE1/AE3) is observed in both the adenocarcinoma component (E; left) and the sarcomatoid component (E; right), whereas positive vimentin staining is observed in the sarcomatoid component (F; right) and normal stroma but not in the adenocarcinoma component (F; left) (original magnification × 4).

was positive for both pancytokeratin (AE1/AE3) (Figure 2E), an epithelial marker, and vimentin, a mesenchymal marker (Figure 2F). In contrast, the adenocarcinoma component was positive for only pancytokeratin (Figure 2E) but was negative for vimentin (Figure 2F). Based on these pathological findings, the tumor was diagnosed as pleomorphic ACP according to the JPS classification and was classified as T3N1M0, Stage IIB, according to the UICC TNM staging system. Despite receiving gemcitabine-based postoperative chemotherapy, the patient died of local recurrence and multiple liver and lung metastases five months after surgery.

Clinicopathological and radiological features of 60 reported cases of ACP in Japan

We conducted a comprehensive search of Ichushi-Web (a domestic medical literature database service provided by the NPO Japan Medical Abstracts Society) from 1995 to 2014 using the term "anaplastic carcinoma of the pancreas", and we retrieved 59 previously published case reports of patients with ACP who underwent surgical resection and for whom clearly presented data were available in the Japanese language literature (Supplementary Table 1). The clinicopathological data and radiological findings described for these 60 cases, including the present case, were evaluated. The

Table 1 Characteristics of patients with anaplastic carcinoma of the pancreas reported in Japan

Variables		*n* (%)
Sex	Male : Female	38 (63) : 22 (37)
Tumor location	Ph	32 (53)
	Pb and/or Pt	25 (42)
	Entire pancreas	3 (5)
Anemia (hemoglobin level < 10.0 g/dL)		12 (20)
Elevated leucocyte count (> 12000/mm^3)		12 (20)
Elevated serum CA19-9 level (> 37 U/mL)		33 (55)
Radiological findings	Tumor/rim enhancement	49 (82)
	Cyst-like appearance	28 (47)
Macroscopic findings	Hemorrhagic necrosis	46 (77)
	Cyst formation	20 (33)
Lymph node metastases (*n* = 45 with relevant data)		18 (45)
UICC TNM stage	Ⅰ A	1 (2)
(*n* = 48 with relevant data)	Ⅰ B	6 (13)
	Ⅱ A	19 (40)
	Ⅱ B	16 (33)
	Ⅳ	6 (12)
Histological subtype		
Giant cell type		11 (18)
Pleomorphic type		18 (30)
Spindle cell type		10 (17)
GCOC type		21 (35)
Recurrence site	Liver	23 (68)
(*n* = 34 with relevant data)	Local site	10 (29)
	Peritoneum	8 (23)
	Lymph node	7 (21)
< 1-yr survivor (*n* = 57 with relevant data)		29 (57)
Giant cell type (*n* = 11)		7 (64)
Pleomorphic type (*n* = 18)		13 (72)
Spindle cell type (*n* = 10)		7 (70)
GCOC type (*n* = 18)		2 (11)
5-yr survivor (*n* = 57 with relevant data)		7 (12)
Giant cell type (*n* = 11)		0 (0)
Pleomorphic type (*n* = 18)		4 (22)
Spindle cell type (*n* = 10)		1 (10)
GCCO type (*n* = 18)		2 (11)

CA19-9: Carbohydrate antigen 19-9; GCCO: Giant cell carcinoma of osteoclastoid; Ph: Pancreatic head; Pb: Pancreatic body; Pt: Pancreatic tail.

tumor stages of each case were assigned according to the UICC classification system based on the surgical and pathological findings reported in the literature unless the descriptions of the extent of the tumor were insufficient. The prognostic outcome of each case was also obtained from the published data. The clinicopathological, radiological, and morphological characteristics of the 60 cases are summarized in Table 1. The average age at diagnosis was 61.5 years (age range, 32 to 85 years), and 38 of the 60 cases (63%) were men. Most patients presented with symptoms such as abdominal pain (*n* = 29, 48%), back pain (*n* = 10, 17%), fatigue (*n* = 8, 13%), fever (*n* = 6, 10%), jaundice (*n* = 6, 10%), body weight loss (*n* = 6, 10%), and abdominal discomfort (*n* = 6, 10%). In four cases (7%), pancreatic tumors were diagnosed incidentally. The tumors were located in the pancreatic head in 32 cases (53%), the body and/or tail in 25 (42%), and the entire pancreas in three (5%). The tumor size ranged from 1.5 to 24.0 cm, with a median of 6.0 cm. Laboratory studies revealed severe anemia (hemoglobin level of < 10.0 g/dL) in 12 cases (20%) and a markedly elevated leucocyte count (> 12000/mm^3) in 12 cases (20%). Elevation of serum CA19-9 levels (> 37 U/mL) was observed in 33 cases (55%). Tumor or rim enhancement on abdominal CT was observed in 49 cases (82%). On imaging studies including abdominal CT, US, and MRI, a cyst-like appearance of the lesion was observed in 28 cases (47%). Regarding surgical procedures, pancreaticoduodenectomy (PD), including pylorus-preserving PD and subtotal stomach-preserving PD, was performed in 32 cases (53%), distal pancreatectomy in 24 (40%), total pancreatectomy in two (3%), and duodenum-preserving pancreatic head resection and tumorectomy in one (2%) each. Combined resection of adjacent organs, including the stomach, colon, jejunum, or left adrenal gland, was required in 14 cases (23%). Vascular involvement, including involvement of the common hepatic artery, celiac artery, or portal vein, were observed in 7 cases (12%). In the resected specimens, macroscopically visible hemorrhagic necrosis was observed in 46 cases (77%). Cyst formation was observed macroscopically in 20 cases (33%). According to the sixth edition of the General Rules for the Study of Pancreatic Cancer by the JPS[7], the 60 cases were histopathologically classified as giant cell type (*n* = 11), pleomorphic type (*n* = 18), spindle cell type (*n* = 10), or GCCO type (*n* = 21). Lymph node metastases were found in 18 of 45 cases for which relevant data were available (40%). Recurrence was reported in 34 cases at one or more sites. The representative sites of recurrence were the liver (*n* = 23, 68%), a local site (*n* = 10, 29%), the peritoneum (*n* = 8, 23%), or lymph nodes (*n* = 7, 21%). Only two cases (6%) presented with lung metastasis. Regarding clinical outcome, 29 of 57 patients (51%) for whom relevant data were available died of their disease within 12 mo (< 1-year survivors). In contrast, seven patients (12%) were reported to be 5-year survivors. According to histological subtype, the group of < 1-year survivors comprised seven of 11 patients with giant cell type (64%), 13 of 18 with pleomorphic type (72%), seven of 10 with spindle cell type (70%), and two of 18 with GCCO type ACP (11%). Furthermore, the group of 5-year survivors comprised 0 (0%), 4 (22%), 1 (10%), and 2 patients (11%) with giant cell type, pleomorphic type, spindle cell type, and GCCO type ACP, respectively.

DISCUSSION

Patients with ACP are frequently diagnosed at an advanced stage with a bulky tumor and adjacent organ involvement. According to the Pancreatic Cancer

Registry in Japan[8], in which 27335 patients were recorded from 2001 to 2004, ACP represented only 0.1% (n = 38) of all pancreatic exocrine neoplasms. The recorded patients with ACP (n = 20) had a median survival duration of 3.3 months and 1- and 2-year overall survival (OS) rates of 14.4% and 0%, respectively[8].

This review demonstrated that ACP tends to present in men (63%) and be located at the pancreatic head (53%). Severe anemia and an elevated leucocyte count were very frequently observed in ACP patients. These findings might be attributed to rapid tumor growth and subsequent intratumoral hemorrhage and necrosis. Moreover, some ACP patients have been reported to produce granulocyte-colony stimulating factor (G-CSF), resulting in leucocytosis[9-11]. Kitade *et al*[9] have noted that the prognosis of patients with G-CSF-producing pancreatic cancer was very poor, and three of 6 reported cases of pancreatic cancer with G-CSF production were histologically diagnosed as ACP. In fact, we identified three cases (5%) with G-CSF production in the present review (one case each of giant cell type, pleomorphic type, and GCCO type ACP), and all of these cases survived fewer than nine months after surgery. Elevated serum CA19-9 levels were observed in more than half of the reviewed patients, and this finding is inconsistent with an earlier report in which patients with ACP presented with elevated serum CA19-9 levels less frequently than patients with PDAC[4]. This discrepancy may be due to the difference in the classification of ACP between the UICC and the JPS systems, the latter of which has defined ACP as exhibiting at least a small degree of ductal differentiation and has distinguished ACP from undifferentiated carcinoma that shows no directionality of differentiation into any lineage[7].

In the present review, tumor or rim enhancement on abdominal CT was observed in 82% of patients with ACP. This finding is in contrast to the typical findings of hypodensity in PDAC patients but extremely similar to an earlier report (83%)[4]. Appearance of a cyst-like structure in the lesion on imaging studies was frequently observed in this series (47%), and this result is also concordant with an earlier report (50%)[4]. Based on this finding, a preoperative diagnosis of a pancreatic cystic neoplasm, including intraductal papillary neoplasm, mucinous cystic neoplasm, or serous cystic neoplasm, was made in nine cases (15%). Moreover, 17 cases (28%) were preoperatively diagnosed as neuroendocrine neoplasm, acinar cell carcinoma, or solid-pseudopapillary neoplasm, which occasionally accompanied hemorrhage, necrosis, and subsequent cystic degeneration in the lesion.

The role of endoscopic-ultrasound-guided fine-needle aspiration (EUS-FNA) in the diagnosis of solid pancreatic tumors has been evaluated in many studies. Two recent meta-analyses examining the validity of diagnosis of malignancy based on cytology reported pooled sensitivities of 85% and 89% and pooled specificities of 98% and 99%[12,13]. However, the accuracy of EUS-FNA for ACP remains unclear. Khashab *et al*[2] reported that four of 5 patients with ACP who underwent EUS-FNA were cytologically diagnosed with ACP. In our series, preoperative endoscopic biopsy or EUS-FNA identified ACP in only three cases (5%). Therefore, we suggest that EUS-FNA is an indispensable diagnostic modality in combination with comprehensive data including characteristic imaging and serological findings for differentiating ACP from other pancreatic neoplasms.

In the resected specimens for the cases in the present review, cyst formation was observed in 20 cases (33%), presumably due to rapid tumor growth, intratumoral hemorrhage, necrosis, and subsequent cystic degeneration. This finding is similar to that in a previous report of a large series of ACP cases, which demonstrated that cyst formation occurred in nine of 35 ACP cases (26%)[5]. On the other hand, PDAC with cystic features has been reported to account for 7%-11% of all PDAC cases[14,15]. Kosmahl *et al*[16] reported that 38 of 483 (8%) cases of PDAC and its variants, including adenosquamous carcinomas and ACPs, had cystic features. According to the nature of cyst formation, the authors classified the cysts into four categories: large-gland features that were lined by atypical cuboidal to flat epithelial cells; intratumoral degenerative cystic changes; retention cysts; and attached pseudocysts[16]. All 24 cases with large-gland features were PDAC, whereas five of the eight cases with intratumoral degenerative cystic changes were ACP or undifferentiated carcinoma with osteoclast-like giant cells[16]. In the present review, the wall of the formed cysts was composed of tumor cells that showed hemorrhagic necrosis at the inner surface of the cyst in most previously reported cases as well as in the presently reported case, indicating degenerative cystic changes. Only three case reports described that the cysts in the tumors were lined by normal or atypical epithelial cells and formed a single cell layer. In addition, some authors have reported cases of ACP associated with mucinous cystic neoplasms[17-19], although the occurrence of histogenesis of ACP coincident with mucinous cystic neoplasm remains uncertain. These cases appear to display similar imaging findings to those of other forms of ACP with extensive cystic degeneration; however, in the present review, there were no reported cases of ACP coincident with mucinous cystic neoplasm in the lesion.

Conflicting survival data were presented in previous reports; the incidence of ACP is rare, and the number of reported cases, especially cases for which data are available, is small. Due to these limitations, the survival benefit of surgery for ACP remains uncertain and no therapeutic strategies for ACP have been established. Clark *et al*[1] reported a study of a large series of 35 patients with ACP using a population-based registry and demonstrated that OS was significantly shorter among patients with ACP than among patients with

PDAC; however, the 1-, 2-, and 5-year OS rates of patients with resected ACP were 59.1%, 30.7%, and 12.2%, respectively, which was comparable to those of PDAC patients. These results suggest that radical resection provides a similar survival advantage between ACP and PDAC. In contrast, Strobel *et al*[4] demonstrated that the median survival duration of ACP patients after curative resection was shorter than that of PDAC patients (not significant). In the present review, 29 of 57 patients (51%) for whom survival data were available were < 1-year survivors, and only seven patients (12%) were reported to be 5-year survivors despite aggressive surgical resection. Regarding histological subtypes, Clark *et al*[1] reported longer OS in patients with undifferentiated carcinoma with osteoclast-like giant cells than in those with all other subtypes of ACP, although this survival benefit was not observed when the analysis was limited to resected patients. However, according to their data, pancreatic resection was performed on 10 of 11 patients with undifferentiated carcinoma with osteoclast-like giant cells (91%) but only 71 of 342 patients with other subtypes of ACP (21%). These results suggest that undifferentiated carcinoma with osteoclast-like giant cells is more resectable. In the present review, the group of < 1-year survivors included only two patients with GCCO type ACP (11%) but more than 60% of patients with other subtypes of ACP, suggesting that the GCCO type may not progress as rapidly as other subtypes of ACP.

The present review had several limitations. First, the present review is based on reported cases diagnosed as ACP according to the JPS classification system, and cases that showed no directionality of differentiation into any lineage were excluded. Therefore, our results may not accurately reflect the population of ACP patients diagnosed according to the WHO classification system. Second, based on this review of reported ACP cases, survival analysis of patients with ACP is limited. Nevertheless, this is the first literature review comprehensively analyzing various clinical parameters, including radiological and morphological findings, in a large number of ACP cases. This review suggests that ACP should be considered when diagnosing pancreatic tumors with a cyst-like appearance, especially in the presence of severe anemia, elevated leucocyte counts, or elevated serum CA19-9 levels. Further investigation including the performance of multi-institutional studies or the examination of data from a nationwide database will be required to determine the clinical outcome and the appropriate surgical indication for this malignancy.

COMMENTS

Case characteristics

A 64-year-old woman with no significant medical history presented with upper abdominal pain and back pain.

Clinical diagnosis

A cystic mass in the pancreatic body was identified by the previous doctor on abdominal ultrasonography.

Differential diagnosis

Pancreatic ductal adenocarcinoma or pancreatic cystic neoplasm.

Laboratory diagnosis

Laboratory examinations revealed anemia (hemoglobin level of 10.3 g/dL) and a slightly elevated carbohydrate antigen (CA) 19-9 level (65.1 U/mL).

Imaging diagnosis

Contrast-enhanced abdominal computed tomography revealed a low-density mass measuring 35 mm in diameter, with cystic component in the pancreatic body.

Pathological diagnosis

The major part of the tumor exhibited a sarcomatous appearance and was composed of non-cohesive, pleomorphic tumor cells, which was diagnosed as pleomorphic anaplastic carcinoma of the pancreas according to the JPS classification.

Treatment

Distal pancreatectomy and splenectomy with en bloc celiac axis resection were conducted.

Related reports

In the resected specimens for the present case, a cyst formation was observed, presumably due to rapid tumor growth, intratumoral hemorrhage, necrosis, and subsequent cystic degeneration. Macroscopically visible hemorrhagic necrosis and cyst formation were observed with high frequency in ACP cases of the present review.

Term explanation

Anaplastic carcinoma of the pancreas (ACP) is an uncommon histologic subtype of pancreatic cancer, which has been defined as a malignant epithelial neoplasm in which a significant component of the neoplasm does not show a definitive direction of differentiation, and well known to be associated with a less favorable prognosis than conventional pancreatic ductal adenocarcinoma.

Experiences and lessons

ACP should be considered when diagnosing pancreatic tumors with a cyst-like appearance, especially in the presence of severe anemia, elevated leucocyte counts, or elevated serum CA19-9 levels.

Peer-review

This case report and literature review enables a greater understanding of the clinical, radiological, and morphological features of ACP. However, survival analysis based on this review of reported ACP cases is limited because of considerable publication bias.

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Role of concomitant therapy for *Helicobacter pylori* eradication: A technical note

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Abstract

We read with interest the recent meta-analysis by Lin *et al* who evaluated the effectiveness of concomitant regimen for *Helicobacter pylori* (*H. pylori*) in Chinese regions. They found that 7-d concomitant regimen is undoubtedly superior to 7-d triple therapy (91.2% *vs* 77.9%, $P < 0.0001$). However, it is a common belief that a triple therapy lasting 7 d should be definitively removed from the clinical practice for its ineffectiveness. Only its prolongation to 14 d may give satisfactory success rate. Thus, the assessment of an old and outdated treatment versus a more recent and successful one does not seem to bring novel and useful information. Moreover, a 7-d duration has not been ascertained for concomitant regimen, as main guidelines recommend a 10-d schedule for this scheme. Therefore, only studies comparing 10-d concomitant versus 14-d triple seem to be appropriate according to current Guidelines and would clarify which regimen is the most suitable worldwide. Additionally, in this meta-analysis concomitant and sequential therapy showed similar performances, despite it is common opinion that sequential is more prone than concomitant therapy to fail when metronidazole resistance occurs, and China is characterized by high rate of resistance to this antibiotic. None of the included studies evaluated *a priori* antibiotic resistances, and the lack of this detail hampers the unveiling of this apparent contradiction. In conclusion, the lack of the evaluation of the quality of included trials as well as their high heterogeneity constitute a burdensome limit to draw solid conclusions in this meta-analysis. On the bases of these considerations and the low number of examined trials, we believe that further studies and the knowledge of antibiotic resistances will support with high quality evidence which is the best regimen and its optimal duration.

Key words: *Helicobacter pylori*; Eradication; Sequential; Concomitant; Triple therapy; Antibiotic resistances

Core tip: Concomitant therapy is one of the most effective first line regimen for *Helicobacter pylori* eradication. The comparison with other regimens (sequential or triple) in a selected geographical region (China in this case) implies several issues. The low number of included studies, the lack of quality evaluation and the high heterogeneity may undermine the strength of a meta-analysis. Therefore, further studies are needed to prove which is the best first line eradication treatment in China, according to the geographical differences in antibiotic resistances.

Losurdo G, Giorgio F, Iannone A, Principi M, Barone M, Di Leo A, Ierardi E. Role of concomitant therapy for *Helicobacter pylori* eradication: A technical note. *World J Gastroenterol* 2016; 22(38): 8638-8640 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i38/8638.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i38.8638

TO THE EDITOR

We read with interest the recent meta-analysis by Lin *et al*[1], who evaluated the effectiveness of concomitant regimen for *Helicobacter pylori* (*H. pylori*) in Chinese regions in a first line context. The choice of deal with this eradication treatment in a selected geographical area is absolutely appropriate, since, as we have already demonstrated[2], a single therapeutic approach is not fitting worldwide. Indeed, geographical variations in antibiotic resistance rates strongly influence the outcome, therefore the most proper regimen should be considered on the basis of the antibiotic resistance pattern in each area.

However, the results of this meta-analysis deserve special considerations. First, three studies compared 7-d concomitant versus 7-d conventional triple therapy, demonstrating that concomitant regimen is significantly superior to 7-d triple therapy (91.2% *vs* 77.9%, $P < 0.0001$). However, it is a common belief that a triple therapy lasting 7 d should be definitively abandoned, due to the disappointingly low success rate. Currently, only the prolongation of triple therapy to 14 d can be advised to overcome this limit[3,4]. Moreover, the outcome of a meta-analysis should be the comparison of two regimens that may have a similar effectiveness; the assessment of an old and outdated treatment versus a more recent and successful one could not bring novel and useful information. The search strategy of this systematic review did not retrieve trials evaluating concomitant versus 14-d triple therapy in China, but only this comparison could state whether concomitant is really more effective than triple therapy. Furthermore, the duration of 7 d for concomitant therapy is not considered optimal, since Guidelines recommend a 10-d regimen[3,5]. Additionally, the comparison between 10-d concomitant and 10-d triple therapy showed a similar success, even if this analysis was based only on one study and this aspect could cause to judge the result questionable.

Another issue involves the comparison of concomitant versus sequential regimen. Herein, it has been demonstrated a similar eradication rate (86.9% and 86% respectively, $P = 0.69$). This finding is in agreement with other meta-analyses[6,7] and previous experiences in Western countries[8,9]. However, it is common opinion that sequential therapy is more prone to fail when metronidazole resistance occurs[10]. Indeed, Georgopoulos *et al*[11] showed that the success rate of sequential regimen decreased from 89.8% to 70%, while a reduction of only 2% occurred for concomitant regimen in the presence of metronidazole resistance. This observation does not seem to agree with the results of this meta-analysis, since China and Far East are considered as high metronidazole resistance areas[2]. Therefore, concomitant therapy would be expected to achieve a higher success rate than sequential therapy. Furthermore, in order to support this consideration, a recent trial in Korea showed eradication rates of 77.8% for concomitant and 70.6% for sequential regimens at intention-to-treat analysis[12]. This controversial aspect could be explained only by the analysis of metronidazole resistance in treated patients. On the other hand, Authors themselves observed that this issue was not investigated in included trials.

Finally, we believe that the lack of quality assessment and the high heterogeneity of included studies constitute a relevant limit to draw solid conclusions in this meta-analysis. Therefore, further studies and the knowledge of antibiotic resistance pattern will support with high quality evidence the assessment of the best regimen and its optimal duration[13-17].

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