reported with promise as tissue- or pancreatic juice-based markers. Hypomethylation of normally methylated genes, which was reported to be identified in serum from patients with testicular cancer, has been recognized in genes including claudin 4, lipocalin 2, 14-3-3 sigma, trefoil factor 2, S100A4, and other, from pancreatic cancer cells or tissues [25, 26, 27, 28].

Proteomics, which is the mass spectrometry-based direct analysis of unknown protein in clinical specimens including serum, has also shown promise in the identification of new biomarkers. Among several technologies for proteomics researches, surface-enhanced laser desorption/ionization (SELDI)-mass spectrometry is considered to be the most useful tools available for the analysis of serum and plasma. A recent study has demonstrated a set of four mass peaks in plasma as most accurately discriminating pancreatic cancer patients from healthy controls in a training cohort with a sensitivity of 97.2% and a specificity of 94.4% and in the validation cohort with a sensitivity of 90.9% and a specificity of 91.1% [29]. The introduction of this technology has enlarged the possibility of identifying novel markers with the potential to overtake and replace CA 19-9.

A bewildering number of investigations to identify useful tumor markers for pancreatic cancer have been conducted, whereas in the vast majority of research studies over the past two decades, CA19-9 alone has been applied as the 'gold standard'. The recent accumulation of knowledge in the molecular biology of pancreatic cancer and rapid advances in technology in this field has enhanced the promising confirmation of novel serum markers with a diagnostic accuracy higher than CA 19-9. The most important obligations for these markers are higher sensitivity to detect early-stage pancreatic cancer and an almost perfect specificity in the screening for this malignancy. The enthusiasm to develop effective molecular targeted agents and other cytotoxic drugs for pancreatic cancer has been increasing rapidly after the introduction of gemcitabine and the recent FDA's approval of erlotinib. These circumstances are also highlighting the need to find the markers in serum and other biological specimens which are able to predict the response to and toxicity of the treatments.

Keywords Biological Markers; CA-19-9 Antigen; Pancreatic Neoplasms

Abbreviations MIC-1: macrophage inhibitory cytokine-1; SELDI: surface-enhanced laser desorption/ionization

Acknowledgment We wish to thank Ms. K Kondo for her help with the manuscript preparation

Correspondence
Takuji Okusaka
Hepatobiliary and Pancreatic Oncology Division
National Cancer Center Hospital
5-1-1 Tsukiji, Chuo-ku
Tokyo, 104-0045
Japan
Phone: +81-3.3542.2511
Fax: +81-3.3542.3815
E-mail: tokusaka@ncc.go.jp

References

1. Rosewicz S, Wiedenmann B. Pancreatic carcinoma. Lancet 1997; 349:485-9. [PMID 9040589]

2. Statistics and Information Department, Minister's Secretariat, Ministry of Health, Labour and Welfare. Vital Statistics of Japan 2003 (in Japanese). Ministry of Health, Labour and Welfare, Tokyo.

3. Ajiki W, Tsukuma H, Oshima A; Research Group for Population-based Cancer Registration in Japan. Cancer incidence and incidence rates in Japan in 1999: estimates based on data from 11 population-based cancer registries. Jpn J Clin Oncol 2004; 34:352-6. [PMID 15333689]

4. Nazli O, Bozdag AD, Tansug T, Kir R, Kaymak E. The diagnostic importance of CEA and CA 19-9 for the early diagnosis of pancreatic carcinoma. Hepatogastroenterology 2000; 47:1750-2. [PMID 11149048]

5. Slesak B, Harlozinska-Szmyrka A, Knast W, Sedlaczek P, van Dalen A, Einarsson R. Tissue polypeptide specific antigen (TPS), a marker for differentiation between pancreatic carcinoma and

chronic pancreatitis. A comparative study with CA 19-9. Cancer 2000; 89:83-8. [PMID 10897004]

6. Hayakawa T, Kondo T, Shibata T, Hamano H, Kitagawa M, Sakai Y, et al. Sensitive serum markers for detecting pancreatic cancer. Cancer 1988; 61:1827-31. [PMID 2451556]

7. Pasquali C, Sperti C, D'Andrea AA, Bonadimani B, Del Favero G, Petrin P, et al. Evaluation of carbohydrate antigens 19-9 and 12-5 in patients with pancreatic cancer. Pancreas 1987; 2:34-7. [PMID 3472197]

8. Del Villano BC, Brennan S, Brock P, Bucher C, Liu V, McClure M, et al. Radioimmunometric assay for a monoclonal antibody-defined tumor marker, CA 19-9. Clin Chem 1983; 29:549-52. [PMID 6825270]

9. Egawa S, Takeda K, Fukuyama S, Motoi F, Sunamura M, Matsuno S. Clinicopathological aspects of small pancreatic cancer. Pancreas 2004; 28:235-40. [PMID 15084963]

10. Tanaka M, Chari S, Adsay V, Fernandez-Del Castillo C, Falconi M, Shimizu M, et al. International Consensus Guidelines for Management of Intraductal Papillary Mucinous Neoplasms and Mucinous Cystic Neoplasms of the Pancreas. Pancreatology 2005; 29: 17-32. [PMID 16327281]

11. Maisey NR, Norman AR, Hill A, Massey A, Oates J, Cunningham D. CA19-9 as a prognostic factor in inoperable pancreatic cancer: the implication for clinical trials. Br J Cancer 2005; 93:740-3. [PMID 16175188]

12. Saad ED, Machado MC, Wajsbrot D, Abramoff R, Hoff PM, Tabacof J, et al. Pretreatment CA 19-9 level as a prognostic factor in patients with advanced pancreatic cancer treated with gemcitabine. Int J Gastrointest Cancer 2002; 32:35-41. [PMID 12630768]

13. Safi F, Schlosser W, Falkenreck S, Beger HG. Prognostic value of CA 19-9 serum course in pancreatic cancer. Hepatogastroenterology 1998; 45:253-9. [PMID 9496523]

14. Lundin J, Roberts PJ, Kuusela P, Haglund C. The prognostic value of preoperative serum levels of CA 19-9 and CEA in patients with pancreatic cancer. Br J Cancer 1994; 69:515-9. [PMID 7510116]

15. Ueno H, Okada S, Okusaka T, Ikeda M. Prognostic factors in patients with metastatic pancreatic adenocarcinoma receiving systemic chemotherapy. Oncology 2000; 59:296-301. [PMID 11096341]

16. Tian F, Appert HE, Myles J, Howard JM. Prognostic value of serum CA 19-9 levels in pancreatic adenocarcinoma. Ann Surg 1992; 215:350-5. [PMID 1348409]

17. Aoki K, Okada S, Moriyama N, Ishii H, Nose H, Yoshimori M, et al. Accuracy of computed tomography in determining pancreatic cancer tumor size. Jpn J Clin Oncol 1994; 24:85-7. [PMID 8158861]

18. Goggins MG. The molecular diagnosis of pancreatic cancer. In: Von Hoff DD, Evans DB, Hruban RH. Eds. Pancreatic Cancer. 1st ed. Sudbury: Jones and Bartlett Publishers, 2005:251-264.

19. Koopmann J, Rosenzweig CN, Zhang Z, Canto MI, Brown DA, Hunter M, et al. Serum markers in patients with resectable pancreatic adenocarcinoma: macrophage inhibitory cytokine 1 versus CA19-9. Clin Cancer Res 2006; 12:442-6. [PMID 16428484]

20. Narimatsu H, Iwasaki H, Nakayama F, Ikehara Y, Kudo T, Nishihara S, et al. Lewis and secretor gene dosages affect CA19-9 and DU-PAN-2 serum levels in normal individuals and colorectal cancer patients. Cancer Res 1998; 58:512-8. [PMID 9458099]

21. Li Z, Sclabas GM, Peng B, Hess KR, Abbruzzese JL, Evans DB, et al. Overexpression of synuclein-gamma in pancreatic adenocarcinoma. Cancer 2004; 101:58-65. [PMID 15221989]

22. Koopmann J, Fedarko NS, Jain A, Maitra A, Iacobuzio-Donahue C, Rahman A, et al. Evaluation of osteopontin as biomarker for pancreatic adenocarcinoma. Cancer Epidemiol Biomarkers Prev 2004; 13:487-91. [PMID 15006928]

23. Ueki T, Toyota M, Sohn T, Yeo CJ, Issa JP, Hruban RH, et al. Hypermethylation of multiple genes in pancreatic adenocarcinoma. Cancer Res 2000; 60:1835-9. [PMID 10766168]

24. Hustinx SR, Leoni LM, Yeo CJ, Brown PN, Goggins M, Kern SE, et al. Concordant loss of MTAP and p16/CDKN2A expression in pancreatic intraepithelial neoplasia: evidence of homozygous deletion in a noninvasive precursor lesion. Mod Pathol 2005; 18:959-63. [PMID 15832197]

25. Sato N, Maitra A, Fukushima N, van Heek NT, Matsubayashi H, Iacobuzio-Donahue CA, et al. Frequent hypomethylation of multiple genes overexpressed in pancreatic ductal adenocarcinoma. Cancer Res 2003; 63:4158-66. [PMID 12874021]

26. Guweidhi A, Kleeff J, Giese N, Fitori JE, Ketterer K, Giese T, et al. Enhanced expression of 14-3-3sigma in pancreatic cancer and its role in cell cycle regulation and apoptosis. Carcinogenesis 2004; 25:1575-85. [PMID 15073049]

27. Rosty C, Ueki T, Argani P, Jansen M, Yeo CJ, Cameron JL, et al. Overexpression of S100A4 in pancreatic ductal adenocarcinomas is associated with poor differentiation and DNA hypomethylation. Am J Pathol 2002; 60:45-50. [PMID 11786397]

28. Argani P, Rosty C, Reiter RE, Wilentz RE, Murugesan SR, Leach SD, et al. Discovery of new markers of cancer through serial analysis of gene expression: prostate stem cell antigen is overexpressed

in pancreatic adenocarcinoma. Cancer Res 2001; 61:4320-4. [PMID 11389052]

29. Honda K, Hayashida Y, Umaki T, Okusaka T, Kosuge T, Kikuchi S, et al. Possible detection of pancreatic cancer by plasma protein profiling. Cancer Res 2005; 65:10613-22. [PMID 16288055]

J Gastroenterol 2007; 42[Suppl XVII]:131–134
DOI 10.1007/s00535-006-1936-5

Journal of Gastroenterology

Alcoholic pancreatopathy: a proposed new diagnostic category representing the preclinical stage of alcoholic pancreatic injury

Naohiro Sata, Masaru Koizumi, and Hideo Nagai

Department of Surgery, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke 329-0498, Japan

Alcoholic chronic pancreatitis (CP) is not usually diagnosed until the end stage of the disease, and hence enormous medical and social resources are consumed in the treatment of established alcoholic CP. With the aim of early diagnosis and prevention of alcoholic CP, we here propose "alcoholic pancreatopathy" as a new category of pancreatic disorder induced by alcohol intake. In addition to a history of excessive alcohol intake (>80 g/day), the presence of at least one of the following conditions establishes the diagnosis of alcoholic pancreatopathy:

1. History of alcoholic acute pancreatitis.
2. Recurrent abdominal pain or gastrointestinal symptoms induced by alcohol intake.
3. Hyperamylasemia or a high serum level of any other pancreatic enzymes.
4. Abnormal findings in the pancreas by routine abdominal ultrasonography.

Alcoholic pancreatopathy is a comprehensive concept that includes the early stage of pancreatic injury induced by alcohol, and is useful for detecting the preclinical stage of pancreatic injury induced by alcohol and hence for treating the early stage of the disease. Further assessments and well-designed studies for investigating the early stage of alcoholic CP are necessary, in which alcoholic pancreatopathy could play a key role.

Key words: alcoholic pancreatopathy, chronic pancreatitis, acute pancreatitis, alcohol intake

Introduction

Chronic pancreatitis (CP) is defined as a continuing inflammatory disease of the pancreas characterized by progressive and irreversible morphologic changes that typically cause severe abdominal pain and permanent loss of function.[1–4] Alcohol intake is a leading cause of CP, with alcohol use preceding the disease in 55%–80% of patients with CP.[5–8] However, most cases of alcoholic CP are not diagnosed until the end stage of the disease, and hence enormous medical and social resources are consumed in the treatment of established alcoholic CP.[7] Early diagnosis and prevention of alcoholic CP would therefore be beneficial, with temperance currently being the only effective preventive method.

The early stage of alcoholic CP is not yet clearly defined. The first international meeting on the classification of pancreatitis was held in Marseille, France, in 1963,[9] and four subsequent international meetings aimed to improve the classification of pancreatitis.[10–14] None of the resulting international classifications has provided significant information about early-stage CP. The Zurich classification (Table 1), which targets the alcoholic form of CP, defines recurrent attacks of clinical alcoholic acute pancreatitis as early-stage CP, and it is the only international classification system to provide such a clinical definition.[14,15] The Japan Pancreas Society classification for CP defines two stages of CP, probable and definite, but even the former relies on well-established histological changes and does not represent the preclinical stage of alcoholic pancreatic injury.[3,16] The inadequacy of these established classifications in preventing alcoholic CP underlies the necessity of a new diagnostic category representing the preclinical stage of alcoholic CP or pancreatic injury.

Standard quantity of alcohol intake

Alcoholic CP is a common disorder induced by alcohol, but the standard quantity of alcohol intake for inducing alcoholic CP has not been established. Although the risk of CP increases with alcohol consumption, there is

Reprint requests to: N. Sata

Table 1. Zurich classification of alcoholic chronic pancreatitis (CP)[14]

A. Definite alcoholic CP[a]
In addition to a typical history of excessive alcohol intake (>80 g/day), one or more of the following criteria establish the diagnosis:
- Calcification of the pancreas
- Moderate to marked ductal lesions (Cambridge criteria)
- Marked exocrine insufficiency defined as steatorrhea (>7 g fat/24 h) normalized or markedly reduced by enzyme supplementation
- Typical histology of an adequate surgical specimen

B. Probable alcoholic CP
In addition to a typical history of excessive alcohol intake (>80 g/day), the diagnosis of probable CP should be made if one or more of the following criteria are present:
- Mild ductal alterations (Cambridge criteria)
- Recurrent or persistent pseudocysts
- Pathological secretin test
- Endocrine insufficiency

Etiological factors
- Alcoholic CP
- Nonalcoholic CP
  - Tropical (nutritional) CP
  - Hereditary CP
  - Metabolic (hypercalcemic, hyperglyceridemic) CP
  - Idiopathic (early and late onset) CP
  - Autoimmune CP
  - CP due to miscellaneous causes; e.g., radiation, injury, phenacetin abuse
  - CP associated with anatomic abnormalities (anatomic CP: periampullary duodenal wall cysts, pancreas divisum, obstructive pancreatitis, posttraumatic pancreatic duct scars)

Clinical staging
- Early stage: recurrent attacks of clinical alcoholic acute pancreatitis (with or without local complications) without evidence of CP abnormalities.
- Late stage: any evidence of probable or definite CP

[a] These diagnostic definitions may also be used for nonalcoholic CP

Table 2. Definition of alcoholic pancreatopathy

In addition to a history of excessive alcohol intake (>80 g/day), the presence of at least one of the following conditions establishes the diagnosis of alcoholic pancreatopathy:
1. History of alcoholic acute pancreatitis
2. Recurrent abdominal pain or gastrointestinal symptoms induced by alcohol intake
3. Hyperamylasemia or a high serum level of any other pancreatic enzymes
4. Abnormal findings in the pancreas on routine abdominal ultrasonography

no absolute toxicity threshold. Additionally, only about 10% of heavy alcohol drinkers ever suffer from clinically recognized pancreatitis,[5,17,18] and more than 30% of patients in whom alcohol is considered to be the contributing cause of CP have either "social" or uncertain levels of alcohol intake.[19] The relationship between alcohol consumption and CP is weak compared with the association between alcohol consumption and liver cirrhosis and other common alcohol-related problems.

In the established classifications for CP, only the Zurich classification defines excessive alcohol intake (as 80 g ethanol/day).[14] The Japanese liver group also has a standard of alcohol intake for alcoholic liver injury. It defines moderate drinkers as those with a daily consumption of 80 g of ethanol over 5 years and heavy drinkers as those with a daily consumption of 140 g over 10 years.[20] Eighty grams of ethanol corresponds to about three 500-ml bottles of beer or three units of Japanese liquor, which seems a moderate consumption amount for regular drinkers and is a suitable definition for a standard quantity of alcohol intake.

Alcoholic pancreatopathy

Here we propose using the term "alcoholic pancreatopathy" (APP) to signify a new category of pancreatic disorder induced by alcohol intake (Table 2). APP is a comprehensive concept that includes the early stage of pancreatic injury induced by alcohol, and it is useful for detecting the preclinical stage of alcoholic CP (Fig. 1). APP includes patients who have recovered from alcoholic acute pancreatitis and those before the onset of alcoholic CP. Ammann et al.[21] reported that 78% of 140 patients with recurrent acute alcohol pancreatitis developed CP. Alcoholic acute pancreatitis is a precursor to some cases of alcoholic CP[22,23] (Fig. 2). Other criteria have been selected by their accessibility. As patients with disease in the APP category would seldom visit a hospital for invasive examinations, such as endoscopic retrograde cholangiopancreatography or endoscopic ultrasonography, the criteria used for the diagnosis of APP should involve simple, noninvasive, and inexpensive tests. We selected recurrent abdominal pain as a clinical manifestation, hyperamylasemia in laboratory data, and any abnormal findings in the pancreas by routine ultrasonography. Additionally, any gastrointestinal symptoms and a high level of any pancreatic enzymes were included as criteria, because steatorrhea and simple diarrhea are common disorders in heavy drinkers, and serum lipase, trypsin, and other enzymes are reportedly more specific for the diagnosis of pancreatitis.[24–27]

Discussion

APP is a new diagnostic category designed for cases that may progress to CP in the near future. The natural course of alcoholic CP and the precise role of alcohol in this disease are not known. Indeed, alcohol consumption is more weakly related to CP than to liver cirrhosis

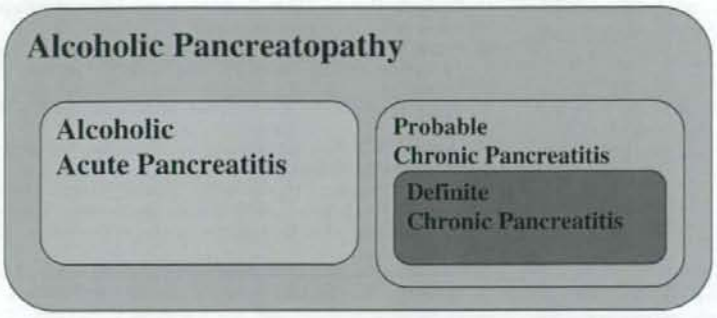

Fig. 1. The concept of pancreatic injury induced by alcohol. Alcoholic pancreatopathy is a comprehensive concept that includes cases after recovery from alcoholic acute pancreatitis and those before the onset of alcoholic chronic pancreatitis

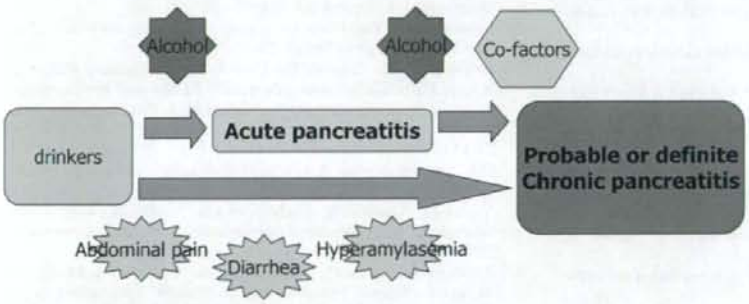

Fig. 2. Pathway to alcoholic chronic pancreatitis (CP). Some cases of CP develop via alcoholic acute pancreatitis. The onset of CP is preceded by abdominal pain, diarrhea, hyperamylasemia, and other subtle clinical manifestations

and other common alcohol-related problems. High alcohol consumption can play an important role in the development of CP. The initial changes associated with CP that are induced by alcohol occur in the pancreatic parenchyma, with changes affecting the pancreatic ductal system being irreversible.[1,3] The histological changes associated with APP, whether reversible or irreversible, are not known. The most important role of defining APP is in preventing alcoholic CP, since it provides a way of determining candidates for alcoholic CP. In studies focusing on early events of alcoholic pancreatic injury, selecting an APP cohort would produce significant information.

Recent advances in the research of alcoholic CP have yielded new insight into the onset of the disease. Alcohol abuse is not solely responsible for the development of CP, but rather alcoholic CP is the outcome of the interaction of several cofactors, such as cigarette smoking, a high-lipid diet, gene mutations, and infections.[1,2,6,28–30] It is likely that multiple genetic and environmental cofactors interact to produce expression of the disease. The TIGER-O classification of CP proposes risk modifiers that may interact to produce pancreatic injury (Table 3). Further assessments and well-designed stud-

Table 3. TIGER-O classification system of etiologies of CP[1]

Toxic-metabolic	Alcohol
	Tobacco
	Hypercalcemia
	Chronic renal failure
Idiopathic	Early onset
	Late onset
	Tropical
Genetic	Hereditary pancreatitis (cationic trypsinogen mutation)
	CFTR mutation
	SPINK1 mutation
	Alpha-1 antitrypsin deficiency
Autoimmune	Isolated autoimmune CP
	Syndromic autoimmune CP (PSC, Sjögren's-associated pancreatitis, etc.)
Recurrent and severe AP	Postnecrotic
	Recurrent acute pancreatitis
	Ischemic/vascular
Obstructive	Pancreas divisum
	Intrapapillary mucinous tumor
	Ductal adenocarcinoma

AP, acute pancreatitis; PSC, pancreatic stellate cells

ies on the interaction of alcohol and cofactors could reveal the entire landscape of alcoholic CP, in which APP is expected to play a key role.

Acknowledgment. This study was supported by the Research Committee on Intractable Diseases of the Pancreas (Chairman, M. Otsuki), under the auspices of the Ministry of Health, Labour and Welfare of Japan.

References

1. Stevens T, Conwell DL, Zuccaro G. Pathogenesis of chronic pancreatitis: an evidenced-based review of past theories and recent developments. Am J Gastroenterol 2004;99:2256–70.
2. Etemad B, Whitcomb DC. Chronic pancreatitis: diagnosis, classification, and new genetic developments. Gastroenterol 2001;120: 682–707.
3. Otsuki M. Chronic pancreatitis. Pancreatology 2004;4:28–41.
4. Lankisch PG. Natural course of chronic pancreatitis. Pancreatology 2001;1:3–14.
5. Ammann RW. The natural history of alcoholic chronic pancreatitis. Intern Med 2001;40:368–75.
6. Saluja AK, Bhagat L. Pathophysiology of alcohol-induced pancreatic injury. Pancreas 2003;27:327–31.
7. Dufour MC, Adamson MD. The epidemiology of alcohol-induced pancreatitis. Pancreas 2003;27:286–90.
8. Magliori M, Pezzilli R, Tomassetti P, Gullo L. Exocrine pancreatic function after alcoholic or biliary acute pancreatitis. Pancreas 2004;28:359–63.
9. Sarles H. Pancreatitis symposium, Marseille 1963. Basel: S. Karger; 1965.
10. Sarner M, Cotton PB. Classification of pancreatitis. Gut 1984; 25:756–9.
11. Gry K, Singer MV, Sarles H. Pancreatitis: concepts and classifications. Proceedings of the second international symposium of the classification of pancreatitis in Marseille, France, March 28–30, 1984. Amsterdam: Excepta Medica; 1984.
12. Singer MV, Gry K, Sarles H. Revised classification of pancreatitis: report of the second international symposium on the classification of pancreatitis in Marseille, France, March 28–30, 1984. Gastroenterology 1985;89:683–90.
13. Sarles H, Adler G, Dani R. The classification of pancreatitis and definition of pancreatic disease. Digestion 1989;43:234–6.
14. Ammann RW. A clinical based classification system for alcoholic chronic pancreatitis: summary of an international workshop on chronic pancreatitis. Pancreas 1997;14:215–21.
15. Uomo G. How far are we from the most accurate classification system for chronic pancreatitis. JOP 2002;3:62–5.
16. Homma T, Harada H, Koizumi M. Diagnostic criteria for chronic pancreatitis by the Japan Pancreas Society. Pancreas 1997; 15:14–5.
17. Bisceglie AM, Segal I. Cirrhosis and chronic pancreatitis in alcoholics. J Clin Gastroenterol 1984;6:199–200.
18. Gumaste VV. Alcoholic pancreatitis: unraveling the mystery. Gastroenterol 1995;108:297–9.
19. Layer P, Yamamoto H, Kalthoff L, Clain JE, Bakken LJ, DiMagno EP. The different courses of early- and late-onset idiopathic and alcoholic chronic pancreatitis. Gastroenterology 1994; 107:1481–7.
20. Takada A, Tsutsumi M. National survey of alcoholic liver disease in Japan (1968–91). J Gastroenterol Hepatol 1995;10:509–16.
21. Ammann RW, Muellhaupt B, Meyerberger C, Heitz PU. Alcoholic nonprogressive chronic pancreatitis: prospective long-term study of a large cohort with alcoholic acute pancreatitis (1976–1992). Pancreas 1994;9:365–73.
22. Strate T, Yekebas E, Knoefel WT, Bloechle C, Izbicki J. Pathogenesis and the natural course of chronic pancreatitis. Euro J Gastroenterol Hepatol 2002;14:929–34.
23. Lankisch PG. Progression from acute to chronic pancreatitis. Surg Clin North Am 1999;79:815–27.
24. Grandval P, De Caro A, De Caro J, Sias B, Carriere F, Verger R, et al. Critical evaluation of a specific ELISA and two enzymatic assays of pancreatic lipases in human sera. Pancreatology 2004; 4:495–503.
25. Le Moine O, Devaster JM, Deviere J, Thiry P, Cremer M, Ooms HA. Trypsin activity. A new marker of acute alcoholic pancreatitis. Dig Dis Sci 1994;39:2634–8.
26. Yadav D, Agarwal N, Pitchumoni CS. A critical evaluation of laboratory tests in acute pancreatitis. Am J Gastroenterol 2002;97:1309–18.
27. Printz H, Siegmund H, Wojte C, Schafer C, Hesse H, Rothmund M, et al. "Human pancreas-specific protein" (procarboxypeptidase B): a valuable marker in pancreatitis? Pancreas 1995;10: 222–30.
28. Chari ST, Singer MV. The problem of classification and staging of chronic pancreatitis: proposals based on current knowledge of its natural history. Scand J Gastroenterol 1994;29:949–60.
29. Gang PK, Tandon RK. Survey on chronic pancreatitis in the Asia-Pacific region. J Gastroenterol Hepatol 2004;19:998–1004.
30. Maisonneuve P, Lowenfels AB, Muellhaupt B, Cavallini G, Lankish PG, Anderson JR, et al. Cigarette smoking accelerates progression of alcoholic chronic pancreatitis. Gut 2005;54:510–4.

HPB, 2007; 9: 150–155

informa
healthcare

ORIGINAL ARTICLE

Gene expression analysis for predicting gemcitabine sensitivity in pancreatic cancer patients

JIANFENG BAI[1,2], NAOHIRO SATA[1] & HIDEO NAGAI[1]

[1]*Department of Surgery, Jichi Medical School, Tochigi, Japan and* [2]*Department of Surgery, First Affiliated Hospital of Nanjing Medical University, Nanjing, China*

Abstract

Background/aims: Current *in vitro* drug sensitivity tests have limitations and disadvantages. This study investigated the use of gene expression data to predict the sensitivity of pancreatic cancers to gemcitabine. *Materials and methods:* Cancer cells isolated from 14 pancreatic cancer patients were tested *in vitro* for gemcitabine sensitivity using the collagen droplet drug sensitivity test (CD-DST). On the basis of this test, 9 of the 14 cancers were identified as either gemcitabine-sensitive or gemcitabine-resistant. Total RNA was extracted from each of those nine cancers and used as a template to synthesize Cy3-labeled cDNA. Pancreatic RNA extracted from six normal individuals was used as a control. Labeled probes were hybridized to an Atlas Glass Human 1.0 Microarray chip, after which the chips were washed and scanned, and the data were analyzed using Microsoft Excel-embedded software. The expression profiles of selected genes were confirmed using real-time PCR analysis. *Results:* Statistical analysis of the microarray data showed that four genes were differentially expressed in gemcitabine-sensitive cancers: microsomal glutathione S-transferase 1 (GSTT1), topoisomerase II alpha (TOP2A), caspase 3, and ATP-binding cassette and subfamily C member 2 (ABCC2). More than 20 other genes were additionally identified as possible candidate genes associated with drug resistance. *Conclusions:* Expression of drug resistance-related genes appeared to predict whether a cancer was gemcitabine-sensitive or -resistant. Further study will enable a drug resistance scoring system to be established on the basis of gene expression. Such a system will allow more efficient application of chemotherapy.

Key Words: *Gemcitabine, pancreatic cancer, microarray, drug resistance*

Introduction

Treatment of carcinoma of the exocrine pancreas is a major problem, with approximately 80% of patients presenting with unresectable disease due to metastases and/or local invasion [1]. Despite many advances in solid tumor therapies over recent decades, unresectable pancreatic cancer continues to have a median survival time of only 3–6 months. The development of gemcitabine, a deoxycytidine analog related to cytarabine, has prompted renewed interest in developing cytotoxic therapies for pancreatic cancer. Although gemcitabine is a well-tolerated drug and ideal for palliation of symptomatic cancer, the efficacy rates remain at only 20–30%. However, in some cases tumors respond well to this treatment and patients experience long survival times.

Since the characteristics of pancreatic cancer can vary between individuals, chemotherapy should ideally be tailored to each patient based on the nature of their particular disease. The detection of potentially chemo-sensitive tumors would significantly improve response rates and facilitate the selection of effective individualized regimens. Developing a method of assessing the likely effectiveness of anticancer drugs using resected or biopsied materials before treatment is likely to avoid unnecessary treatment.

A number of tests to determine the chemosensitivity of cancers to particular drugs have been developed, including the nude mouse method, subrenal capsule assay (SRC), human tumor clonogenic assay (HTCA), thymidine incorporation assay (TIA), succinic dehydrogenase inhibition test (SDI test), and the MTT assay [2]. However, none of these

Correspondence: Naohiro Sata, MD, PhD, Department of Surgery, Jichi Medical School, 3311-1 Yakushiji, Minamikawachi, Tochigi 329-0498, Japan. Tel: +81 285 58 7371. Fax: +81 285 44 3234. E-mail: sata@jichi.ac.jp

(Received 26 August 2006; accepted 13 December 2006)

ISSN 1365-182X print/ISSN 1477-2574 online
DOI: 10.1080/13651820601175918

methods has been widely adopted in clinical practice for various reasons, including low success rates for primary culture, a large number of cells being required for testing, difficulty in ruling out the effect of contaminating fibroblasts, assays taking more than a week, and skilled technicians being required. Although Kobayashi et al. developed a collagen-gel droplet embedded culture drug sensitivity test (CD-DST) which avoids some of these shortcomings [3], the method still requires significant quantities of fresh tissue, usually more than 0.5 cm^3.

Pancreatic cancers contain a high proportion of fibroblasts and connective tissue, making it difficult to obtain sufficient cancer tissue for primary culture testing. The success rate of such cultures remains at about 60–80%. However, the development of micro-array technology has made it possible to evaluate pancreatic cancers using less tissue than required for CD-DST, and with a higher success rate. Assersohn et al. showed breast cancer gene expression profiles in 15% of patients using tissue from fine needle aspirates and cDNA microarrays [4]. This microarray approach is likely to become more common with increasingly sensitive scanning techniques and validated amplification techniques.

The present study investigated the use of gene expression microarray technology for predicting the chemo-sensitivity of pancreatic cancers.

Materials and methods

The study involved 14 patients with biopsy-proven ductal adenocarcinoma of the pancreas admitted to the Jichi Medical School Hospital (Tochigi, Japan) between January 2001 and December 2003 (Table I). We obtained approval from the ethics committee in Jichi Medical University, and documented informed consents from all patients. A laparotomy was performed to obtain 250–1000 mg of fresh pancreatic cancer tissue.

CD-DST chemo-sensitivity tests were performed using a human tumor cell primary culture system kit (Primaster, Nitta Gelatin, Osaka, Japan) [5]. Briefly, fresh surgical specimens from pancreatic cancers were cut into small pieces aseptically and suspended in Hanks's balanced saline solution (HBSS). Cells were dispersed by incubating tissue at 37°C for 1–3 h in a 0.1% cell dispersion enzyme solution (EZ, Nitta Gelatin). Cells were then centrifuged at 900 *g* for 3 min and the pellet was resuspended in PCM-1 medium (Nitta Gelatin), and the suspension filtered through an 80 μm pore nylon mesh. After preliminary culture in a collagen gel-coated flask in a CO_2 incubator at 37°C for 24–48 h, 3×10^3 cells were added to a 30 μl collagen gel droplet. Cells were cultured in DF medium (Nissui Pharmaceutical Co., Tokyo, Japan) containing 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA) with or without 0.4 mg/ml gemcitabine for 24 h. Quantification of the total volume of a cell colony, utilizing differences in the growth morphologies of tumor cells and fibroblasts, was determined using an image analysis method [6]. The effect of gemcitabine was determined by calculating the ratio of the total colony volume of cells with (T) and without (C) gemcitabine. Cells with a T/C ratio <50% were considered gemcitabine-sensitive, while those with a ratio >50% were considered gemcitabine-resistant.

Gene expression profiles were evaluated using microarray techniques. Briefly, purified total RNA from frozen samples was isolated using Atlas Glass Total RNA Isolation Kits (Clontech, Palo Alto, USA) according to the manufacturer's protocols. cDNA was synthesized using BD Atlas PowerScript Fluorescent Labeling Kits, and the resultant Cy3-labeled (Amersham Pharmacia Biotech, Bucks, UK) double-stranded cDNA was purified using QIAquick PCR

Table I. Profiles and chemo-sensitivities of the 14 pancreatic cancer patients.

Case	Age (years) /gender	Location	Operation	T/C ratio	Sensitive /resistant	Outcome	Duration (M)	Chemotherapy (M)
1	69/F	Head	PpPD	20.8	Sensitive	Dead	15.9	7
2	62/M	Head	Unresectable	None	Unknown	Dead	6.8	2
3	75/M	Head	Unresectable	74.4	Resistant	Dead	14.3	8
4	61/M	Head	Unresectable	89.4	Resistant	Dead	8.1	4
5	57/M	Head	Unresectable	76.2	Resistant	Dead	15.5	7
6	59/F	Head	PpPD	64.6	Resistant	Dead	15.6	11
7	70/F	Body	DP	None	Unknown	Dead	11.3	2
8	78/F	Head	PpPD	None	Unknown	Alive	41.2	12
9	72/F	Head	PpPD	76.2	Resistant	Dead	10.1	5
10	75/F	Tail	DP	None	Unknown	Dead	12.3	7
11	77/F	Head	PpPD	30	Sensitive	Alive	42.0	12
12	64/M	Head	Unresectable	33.3	Sensitive	Dead	16.1	11
13	65/M	Body	DP	None	Unknown	Dead	24.4	12
14	74/M	Head	Unresectable	38.7	Sensitive	Dead	16.6	10

PpPD, pylorus-preserving pancreatoduodenectomy; DP, distal pancreatectomy.

Purification Kits (QIAGEN Valencia). Cy3-labeled cDNA synthesized from a pool of normal pancreatic RNA (BioChain Institute, Hay ward) was used as a control. Cy3-labeled cDNA was hybridized to a BD Atlas Glass Human 1.0 Microarray (Clontech) in a water bath at 50°C for 16 h. Chips were then washed in four high-volume wash chambers (Clontech). Using a GMS 418 Array Scanner (Takara, Tokyo) and accompanying software, fluorescence intensities for dyes Cy3 were determined and subtraction of local background values for individual spots was performed. The data were exported to Microsoft Excel spreadsheets for analysis. To normalize for the amount of total RNA on each chip, the sample/control ratio for the expression of each gene was adjusted so that the averaged Cy3:Cy3 ratio of seven housekeeping genes was given the value of 1.0, and the data then underwent log2 transformation. To identify genes that were differentially expressed between drug-sensitive and drug-resistant cancers, the Excel-embedded statistical software 'Analyse-it' was used to calculate the U and p values for the Mann–Whitney analysis of each gene. A difference in gene expression was considered significant if the p value was <0.05.

Differential expression of genes identified by microarray analysis was confirmed using real-time PCR analysis and specific primers (Table II). Total RNA used for the microarray analysis was also used for the real-time PCR analysis. Primers were designed for the genes of interest using GENETYX-WIN software (Software Development Corporation, Tokyo, Japan), and then PCR conditions were optimized for each pair of primers (QuantiTec SYBR Green PCR Kit, Qiagen KK, Tokyo, Japan). First strand cDNA was then synthesized from 2 μg total RNA (Superscript First Strand cDNA Synthesis Kit), and 1 μl RT-PCR product was used in real-time PCR assays under optimized reaction conditions. The 50 μl reaction mixture comprised 25 μl SYBR Green PCR Master Mix, 1 μl sense primer, 1 μl antisense primer, 1 μl cDNA, 0.5 μl uracil-N-glycosylase, and 21.5 μl RNase-free water. The real-time cycler conditions were 50°C for 2 min, 95°C for 10 min, 94°C for 15 s, optimized annealing temperature for 30 s, 72°C for 30 s, 50 cycles. β-Actin expression was used as a control for normalizing the amounts of cDNA used. Reaction products were analyzed using 2% agarose gel electrophoresis to confirm that the signals detected by the GeneAmp PCR system 7700 (Perkin-Elmer Corporation, Foster City, USA) were from the expected products. Three independent experiments were performed.

Results

Using CD-DST, valid T/C ratios were obtained in 9 (64.3%) of the 14 cancers. A T/C ratio of 50% or less was regarded as indicating that cells were gemcitabine-sensitive *in vitro* (Table III). On this basis, four cancers were classified as gemcitabine-sensitive and five as gemcitabine-resistant. However, an arbitrarily assigned growth inhibition rate may not always reflect clinical response because clinical response needs to be based on log killed cells.

The log2 transformed Cy3/Cy3 signal data from microarray analyses are shown in Table III (original data are available to readers upon request by e-mail). In all 1081 human genes contained in the Atlas Glass Human 1.0 microarray, statistical analysis of the microarray data identified 4 genes that were differentially expressed between gemcitabine-sensitive and -resistant cancers: microsomal glutathione S-transferase 1 (GSTT1), topoisomerase II alpha (TOP2A), caspase 3, and ATP-binding cassette subfamily C member 2 (ABCC2). Real-time PCR analyses confirmed the differential expression of these genes (Figure 1). Paired Student's t test showed no difference between the results of the two methods ($p > 0.05$). The fluctuations in mRNA expression between the nine patients were found to be similar using either analytical method.

For a further 22 genes, while statistical analysis indicated that the difference in their expression between the two tissues was not significant, the p values were close to 0.05. These genes were associated with gemcitabine resistance and included cyclin-dependent kinase inhibitor 1A, tumor protein p53 binding protein 2, activated p21cdc42Hs kinase, v-akt 2, insulin-like growth factor 1 receptor, BCL2-interacting killer, BCL2-like 2, BCL2-like 1, BCL2-related protein A1, BCL2-interacting killer, topoisomerase I, APEX nuclease, transforming growth factor beta receptor II, interleukin 6 receptor, cytochrome P450 subfamily 1 (dioxin-inducible), polypeptide 1, glutathione S-transferase M1, transforming growth factor beta 1, interleukin 8, insulin-like growth factor 1, nuclear

Table II. Sequences of primers used for PCR.

Gene	Sense	Antisense
β-Actin	AATCTGGCACCACACCTTCTAC	GCTTCTCCTTAATGTCACGCAC
GSTT1	GCATAAGGTGATGTTCCCTGTGT	CGGTGCAAGGGTGAGGTTTC
ABCC2	GACATCTATCTTCTAGATGACC	TAGATGGAGAACTTCACCTT
TOP2A	GGGTAGCAATAATCTAAACCTC	CCAGTTCTTCAATAGTACCCT
Caspase 3	TGAAGCTACCTCAAACTTCC	CAGCATCACTGTAACTTGCT

Table III. The log2 transformed Cy3/Cy3 signals of selected genes.

Sensitivity	Case no.	GSTT1	ABCC2	TOP2A	Caspase 3
Sensitive	Case 1	0.18	−0.24	1.10	0.30
	Case 11	1.25	−1.50	1.37	−1.25
	Case 12	−0.19	0.38	−0.18	1.26
	Case 14	0.16	−0.22	0.19	0.09
Resistant	Case 3	1.20	0.29	−0.25	−2.54
	Case 4	2.80	1.20	−2.33	−1.92
	Case 5	1.72	1.87	−0.32	−1.78
	Case 6	2.05	1.07	0.11	−0.52
	Case 9	3.58	2.45	−1.78	−1.77
	p value	<0.05	<0.05	<0.05	<0.05

Expressions of GSTT1, ABCC2, TOP2A, and caspase 3 between the gemcitabine-sensitive group and gemcitabine-resistant group are significantly different ($p < 0.05$).

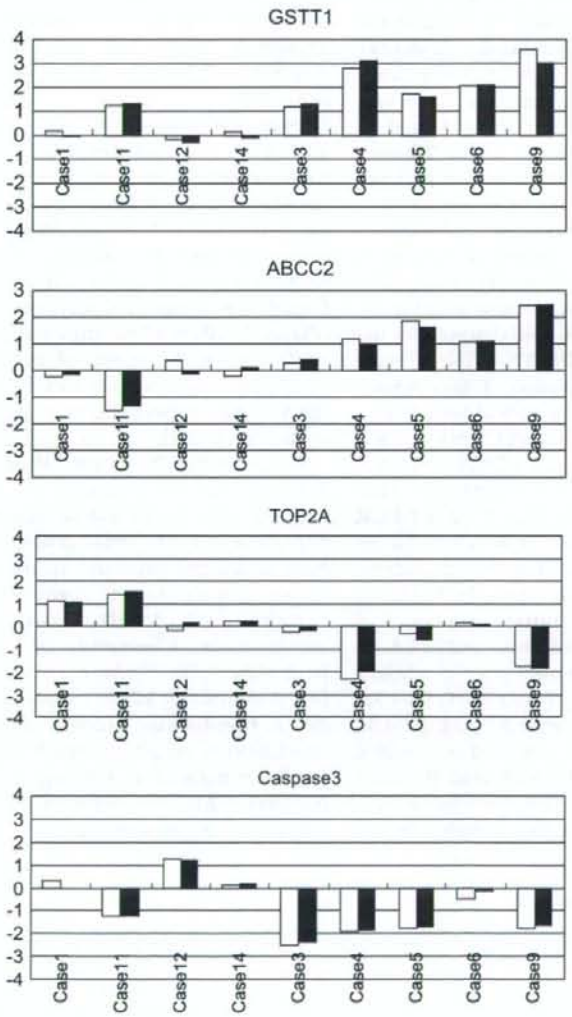

Figure 1. Real-time PCR analyses (■) for confirmation of microarray data (□). The same RNA source was used for both microarray and real-time PCR analyses. The Y-axis indicates the log2 transformed ratio of mRNA expression. Paired Student's t test showed no difference between the results of two methods ($p > 0.05$).

factor of kappa light polypeptide gene enhancer in B-cells 2, ligase III and ligase I.

Discussion

Gemcitabine is a nucleoside analog which exhibits metabolic characteristics that distinguish it from related compounds and may explain its activity against solid tumors. The active nucleotide effectively accumulates at high concentrations in cells due to both efficient phosphorylation and relatively slow elimination. The diphosphate is a potent inhibitor of ribonucleotide reductase, resulting in reduced deoxynucleotide pools. Decreased cellular concentrations of deoxycytidine triphosphate permit more rapid phosphorylation of gemcitabine and decrease the metabolic clearance of gemcitabine nucleotides by deoxycytidine monophosphate deaminase. Most importantly, increasing the ratio of the cellular concentration of gemcitabine triphosphate to deoxycytidine triphosphate increases analog incorporation into DNA, which is strongly associated with loss of cell viability [7]. Gemcitabine alone or in combination with other anticancer drugs has become a popular regimen in pancreatic cancer.

Analysis of gene expression using cDNA chips showed that four genes were differentially expressed according to cells being either gemcitabine-sensitive or -resistant. The four genes were identified as GSTT1, TOP2A, caspase 3, and ABCC2. For each gene, expression was associated with drug resistance. These findings are in agreement with a report by Scherf et al. indicating that gene expression profiles may reflect drug sensitivity in cancer cells [8].

The cellular glutathione system (GSH) is a critical component of the cytostatic detoxification pathway in cells. GSTT1 is a member of a protein superfamily that catalyzes the conjugation of reduced glutathione to a variety of electrophilic and hydrophobic compounds. The conjugate is less active and more water-soluble, and it is excluded from the cell with the participation of GS-X transporter proteins. GSTT1 is claimed to play an important role in human carcinogenesis [9,10]. Inhibitors of glutathione transferases have been shown to enhance the cytotoxicity of alkylating chemotherapeutic drugs in cultured cancer cells otherwise resistant to this class of agent [11].

ABCC2 (MRP2) is a member of the ATP-binding cassette (ABC) transporter superfamily, which is involved in biliary, renal, and intestinal secretion of numerous organic anions, including endogenous compounds such as bilirubin and exogenous compounds such as drugs and toxic chemicals [12]. This protein is a member of the MRP subfamily that is involved in multi-drug resistance [13].

TOP2A functions as the target for several anticancer agents, and a variety of mutations in this gene have been associated with drug resistance [14]. The activity or quantity of this enzyme was lower in cell lines resistant to topoisomerase II-inhibiting drugs. In those lines, topoisomerase gene mutations were found which were presumed to be the bases for the drug resistance [15,16].

Caspase 3 cleaves and activates caspases 6, 7, and 9, is processed by caspases 8, 9, and 10, and plays an important role in apoptosis. A broad spectrum of anticancer drugs used in the clinic has been shown to activate apoptosis *in vitro* and *in vivo* [17,18]. Low expression of caspase 3 has been shown to inhibit chemotherapy-induced apoptosis [19].

In the present study, differences in the expression of a further 22 genes almost reached statistical significance. Many of these candidate genes have been previously linked to drug resistance or carcinogenesis. Some of the expressed sequence tags (ESTs) identified may represent genes that might be future targets for novel anticancer drugs. Identification of further differentially expressed genes will enable development of an accurate drug response system (DRS) for predicting the suitability of a particular cancer patient for gemcitabine therapy [20].

Conclusions

Microarray evaluation has a number of distinct advantages compared with the CD-DST method, such as the requirements for less tissue and less time (3 days), and the ease with which experiments can be repeated if required. These advantages are of particular importance in pancreatic cancer analysis where it is difficult to obtain large amounts of cancer tissue. We believe that clinical application of such a DRS will prevent cancer patients from undergoing ineffective adjuvant chemotherapy.

Acknowledgements

This work appeared in the Poster Prize Session at the 7th World Congress of the International Hepato-Pancreato-Biliary Association in Edinburgh, September 2006.

References

[1] DeVita VT, Hellman S, Rosenberg SA, eds. Cancer: principles and practice of oncology, 6th edn. Philadelphia, PA: Lippincott Williams and Wilkins, 2001:1126–61.

[2] Do JK, Kubota T, Ura HT, Yamaue H, Akiyama S, Maehara Y, et al. Cumulative results of chemosensitivity tests for antitumor agents in Japan. Anticancer Res 2000;20:2389–92.

[3] Kobayshi H, Tanisaka K, Kondo N, Mito Y, Koezuka M, Yokouchi H, et al. Development of new *in vitro* chemosensitivity test using collagen gel droplet embedded culture and its clinical usefulness. Gan To Kagaku Ryoho 1995 Nov;22(13): 1933–9. Japanese.

[4] Assersohn L, Gangi L, Zhao Y, Dowsett M, Simon R, Powles TJ, et al. The feasibility of using fine needle aspiration from primary breast cancer for cDNA microarray analyses. Clin Cancer Res 2002;8:794–801.

[5] Yasuda H, Takada T, Wada K, Amano H, Isaka T, Yoshida M, et al. A new in-vitro drug sensitivity test (collagen-gel droplet embedded-culture drug sensitivity test) in carcinomas of pancreas and biliary tract: possible clinical utility. J Hepato-biliary Pancreat Surg 1998;5:261–8.

[6] Kobayashi H. Collagen gel droplet culture method to examine *in vitro* chemosensitivity. Methods Mol Med 2005;110:59–67.

[7] Plunkett W, Huang P, Searcy CE, Gandhi V. Gemcitabine: preclinical pharmacology and mechanisms of action. Semin Oncol 1996;23(5 Suppl 10):3–15.

[8] Scherf U, Ross DT, Waltham M, Smith LH, Lee JK, Tanabe L, et al. A gene expression database for the molecular pharmacology of cancer. Nat Genet 2000;24:236–44.

[9] Trizna Z, Clayman GL, Spitz MR, Briggs KL, Goefert H. Glutathione s-transferase genotypes as risk factors for head and neck cancer. Am J Surg 1995;170:499–501.

[10] Strange RC, Lear JT, Fryer AA. Glutathione s-transferase polymorphisms: influence on susceptibility to cancer. Chem Biol Interact 1998 Apr 24;111–112:351–64.

[11] Awasthi S, Srivastava SK, Ahmad F, Ahmad H, Ansari GA. Interactions of glutathione s-transferase-pi with ethacrynic acid and its glutathione conjugate. Biochim Biophys Acta 1993;1164:173–8.

[12] Payen L, Sparfel L, Courtois A, Vernhet L, Guillouzo A, Fardel O. The drug efflux pump MRP2: regulation of expression in physiopathological situations and by endogenous and exogenous compounds. Cell Biol Toxicol 2002;18:221–3.

[13] Suzuki H, Sugiyama Y. Single nucleotide polymorphisms in multidrug resistance associated protein 2 (MRP2/ABCC2): its impact on drug disposition. Adv Drug Deliv Rev 2002;54: 1311–31.

[14] Holden JK. Human deoxyribonucleic acid topoisomerase: molecular targets of anticancer drugs. Ann Clin Lab Sci 1997;27:402–12.

[15] Pommier Y, Leteurte F, Fesen MR, Fujimori A, Bertrand R, Solary E, et al. Cellular determinants of sensitivity and resistance to DNA topoisomerase inhibitors. Cancer Invest 1994;12:530–42.

[16] Matsumoto Y, Takano H, Kunishio K, Nagao S, Fojo T. Hypophosphorylation of topoisomerase II alpha in etoposide (VP-16)-resistant human carcinoma cell lines associated with carboxy-terminal truncation. Jpn J Cancer Res 2001;92:799–805.

[17] Dive C, Hickman JA. Drug-target interactions: only the first step in the commitment to a programmed cell death? Br J Cancer 1991;64:192–6.

[18] Basu A, Castle VP, Bouziane M, Bhalla K, Haldar S. Crosstalk between extrinsic and intrinsic cell death pathways in pancreatic cancer: synergistic action of estrogen metabolite and ligands of death receptor family. Cancer Res 2006;66:4309–18.

[19] Yang XH, Sladek TL, Liu X, Butler BR, Froelich CJ, Thor AD. Reconstitution of caspase sensitizes MCF-7 breast cancer cells to doxorubicin- and etoposide-induced apoptosis. Cancer Res 2001;61:348–54.

[20] Kihara C, Tsunoda T, Tanaka T, Yamana H, Furukawa Y, Ono K, et al. Prediction of sensitivity of esophageal tumors to adjuvant chemotherapy by cDNA microarray analysis of gene-expression profiles. Cancer Res 2001;61:6474–9.

ELSEVIER

Journal of Hepatology 46 (2007) 77–88

Journal of Hepatology

www.elsevier.com/locate/jhep

Role of the Fas/FasL pathway in combination therapy with interferon-α and fluorouracil against hepatocellular carcinoma in vitro ☆,☆☆

Masato Nakamura, Hiroaki Nagano*, Masato Sakon, Tameyoshi Yamamoto, Hideo Ota, Hiroshi Wada, Bazarragchaa Damdinsuren, Takehiro Noda, Shigeru Marubashi, Atsushi Miyamoto, Yutaka Takeda, Koji Umeshita, Shoji Nakamori, Keizo Dono, Morito Monden

Department of Surgery, Graduate School of Medicine, Osaka University, Osaka, Japan

***Background/Aims*: Several studies have reported the efficacy of combination therapy of interferon (IFN) α and 5-fluorouracil (5-FU) for hepatocellular carcinoma (HCC). However, the mechanism underlying the clinical anti-tumor effects of this treatment is not well understood. The aim of this study was to determine the role of Fas/FasL signaling in the anti-tumor effect of this combination therapy.**

***Methods and Results*: We used six human hepatoma cell lines, three of which are known Fas-expressing cells. Growth of Fas-positive hepatoma cell lines was inhibited by an agonistic anti-Fas antibody in a dose-dependent manner, and these effects were enhanced by IFNα or 5-FU alone, but even more so by combination therapy using both agents. Annexin-V assay implicated apoptosis as the main mechanism underlying these growth inhibitory effects, although changes in Fas expression regulated by IFNα and/or 5-FU did not correlate with increased apoptosis. Caspase-3 activation was exclusively increased by IFNα/5-FU combination treatment, which was compatible with enhancement of the synergistic apoptotic effect, and other caspases and apoptotic factors (FLIP, BCL-xl, and Bax) were also regulated by IFNα/5-FU. ^{51}Cr-release assay revealed that pretreatment with IFN activated cytotoxicity of peripheral blood mononuclear cells (PBMCs) against HCC cells. The largest interaction was observed when both PBMC and HCC cells were pretreated with the combination of IFNα/5-FU. These cytotoxicities were markedly inhibited by a neutralizing anti-Fas antibody.**

***Conclusions*: Our results indicated that IFNα/5-FU combination treatment enhances the induction of apoptosis and the cytotoxic effect of PBMCs via the Fas/FasL pathway. The Fas/FasL pathway seems, at least in part, to contribute to the anti-tumor effects of IFNα/5-FU against HCCs.**

***Keywords*: Hepatocellular carcinoma; Combination therapy; Interferon-α; 5-Fluorouracil; Apoptosis; Fas/FasL pathway; Caspase-3**

Received 21 February 2006; received in revised form 7 June 2006; accepted 5 July 2006; available online 25 September 2006

☆ This work was supported by a Grant-in-Aid for cancer research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

☆☆ The authors who have taken part in this study declared that they do not have a relationship with the manufacturers of the drugs involved either in the past or present and did not receive funding from the manufacturers to carry out their research.

* Corresponding author. Tel.: +81 6 6879 3251; fax: +81 6 6879 3259.
E-mail address: hnagano@surg2.med.osaka-u.ac.jp (H. Nagano).

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common solid tumors [1]. The prognosis for patients with HCC remains poor and most die within several months after diagnosis, particularly in advanced cases with tumor thrombosis in the major portal vein (Vp3-4) [2–4]. Chemotherapy is the traditional first choice

0168-8278/$32.00
doi:10.1016/j.jhep.2006.07.032

for the treatment of unresectable solid tumors; however, these drugs are not effective in promoting tumor regression and prolonging survival in HCC [5,6]. In addition, conventional therapeutic modalities such as transcatheter arterial embolization, radiofrequency ablation and microwave coagulation therapy are not recommended when portal vein tumor thrombosis (PVTT) is present because of low efficacy and potential complications [7,8]. Therefore, a new effective modality is needed to treat advanced HCC, especially in those cases with portal vein involvement.

Interferon (IFN) has a variety of biological properties including immunomodulation and anti-tumor activity. The anti-tumor effect of IFN against HCC was tested in several studies. From a randomized controlled trial, Llovet et al. [9] concluded that IFN used alone provides no clinical benefit for HCC patients with respect to tumor progression rate and survival. However, several other investigators reported a strong anti-tumor activity for IFN in HCC, when used in combination with some other chemotherapeutic agents. Urabe et al. [10] found that treatment with a combination of subcutaneous IFNα injection and intra-arterial infusion of 5-fluorouracil (FU), cisplatin and methotrexate for HCC with PVTT achieved a response rate of 46.7%. In addition, Patt et al. [11] reported that combination treatment with FU and IFN promoted anti-tumor has activity in HCC and could be tolerated even by cirrhotic patients. We also previously reported the beneficial results of subcutaneous IFNα injection and intra-arterial 5-FU infusion against HCC with PVTT [12–14]. This therapy showed an anti-tumor effect with a response rate approaching 50%, including several complete remissions of the tumor and prolonged survival without major adverse effects. From these results, we proposed that the combination chemotherapy of IFNα and 5-FU should become a standard therapy for advanced HCC.

We have already reported the synergistic effects of IFNα and 5-FU in influencing cell-cycle progression into the S phase via $p27^{Kip1}$, inducing apoptosis by downregulating Bcl-xl, and modulating the immune response via the TRAIL/TRAIL-receptor pathway [15–17]. The present study is an extension of this previous work, to investigate the role of the Fas/FasL pathway in the IFNα/5-FU treatment effect. Fas/FasL signaling participates in an apoptosis-inducing mechanism related to cytotoxic T Lymphocytes (CTL) and natural killer (NK) cells, which was implicated as a major pathway of T-cell-mediated cytotoxicity and a mediator of apoptosis via an IFN-stimulated gene [18]. In addition, we also investigated the mechanism underlying the apoptosis-enhancing effect of IFNα/5-FU that acts via the Fas/FasL pathway.

2. Materials and methods

2.1. Cells

Human HCC cell lines (HuH7, PLC/PRF/5, HLE, HLF and HepG2) were obtained from the Japan Cancer Research Resources Bank (JCRB) (Osaka, Japan) and the human HCC cell line, Hep3B, was obtained from the Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). These cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin at 37 °C in a humidified incubator with 5% CO_2 in air. A non-tumorigenic SV40-immortalized human liver epithelial cell line (THLE-2) was obtained from American Type Culture Collection (Manassas, VA). THLE-2 cells were maintained as an adherent monolayer in Bronchial epithelial medium (BEGM bullet kit, Combrex, NJ) from which remove the gentamicin/amphotericin and epinephrine and to which add extra 5 ng/ml EGF, 70 ng/ml phosphoethanolamine and 10% fetal bovine serum (FBS).

2.2. Reagents

Purified human IFNα was obtained from Otsuka Pharmaceutical Co. (Tokushima, Japan), and 5-FU was kindly provided by Kyowa Hakko Co. (Tokyo, Japan). Antibodies against Fas (UB-2, CH-11 and ZB4) were obtained from Medical and Biological Laboratories (Nagoya, Japan). Caspase-3-specific inhibitor (Z-DEVD-FMK), caspase-8-specific inhibitor (Z-IETD-FMK) and caspase-9-specific inhibitor (Z-LEHD-FMK) were purchased from Calbiochem (San Diego, CA).

2.3. Flow cytometric analysis of Fas expression

HCC cells were characterized for their surface expression of Fas receptors by flow cytometry. Cells (1×10^6) were incubated with 2.5 μg/ml of anti-Fas antibody (IgG, UB-2) for 30 min at 4 °C. After washing with PBS, the cells were analyzed on a FACScan (BD Transduction Laboratories, Lexington, KY), and data were processed using Cell Quest™ software (BD Transduction Laboratories).

2.4. Cell growth assay

Cell growth was assessed by the 3-(4-,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, 3×10^4 cells were seeded on a 96-well plate in 100 μl of medium and left overnight to adhere. Several concentrations of the test drugs in 100 μl volumes were added, and the cells were incubated for 48 h. After treatment, 10 μl of MTT solution was added to each well and incubated for another 4 h at 37 °C. Then 100 μl of acid–isopropanol was added, and after 24 h at 4 °C, reduced MTT was measured spectrophotometrically in a dual-beam microtiter plate reader at 570 nm with a 650 nm reference.

2.5. Flow cytometric analysis of annexin V-FITC binding

The binding of annexin V-FITC was used as a sensitive method for measuring apoptosis, according to a modification of a previously described method [19]. Briefly, after treatment with IFNα/5-FU and/or anti-Fas antibody CH-11, the cultured cells (1×10^6) were incubated with binding buffer (10 mM Hepes, 140 mM NaCl and 2.5 mM $CaCl_2$, pH 7.4) containing saturating concentrations of annexin V-FITC (BioVision Research Products, Mountain View, CA) and propidium iodide (PI) for 15 min at room temperature. After incubation, the cells were pelleted and analyzed on a FACScan (BD), and data were processed using Cell Quest™ software (BD).

2.6. Caspase activity

After treatment with the test drugs and/or anti-Fas antibody, cytosolic extracts were prepared using lysis buffer. The caspase activity in the cell cytosol was measured using a Caspase Colorimetric Protease Assay Kit (MBL) as per the instructions provided by the manufacturer. This assay is based on the spectrophotometric detection of the chromophore, *p*-nitroanilide after cleavage from the labeled substrate. Caspase-3, -8 and -9 assay kits were used in this study.

2.7. Real-time PCR

The LightCycler PCR and detection system (Roche Diagnostics, Mannheim, Germany) was used for amplification and quantification. For detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the amplification products of some apoptotic factors, the LightCycler DNA Master SYBR Green I (Boehringer Mannheim, Mannheim, Germany) was used. Real-time PCRs were performed in a sample mixture containing each primer (final concentration, 0.2 μM), 1× LC-DNA Master SYBR Green I, 4 mM of $MgCl_2$, and 2 μl of cDNA as a template using the following primers: human GAPDH (forward: 5′-CAACTACATGGTTTACATGTTC-3′, reverse: 5′-GCCAGTGGACTCCACGAC-3′); Bcl-xl (forward: 5′-GTAAACTGGGGTCGCATTGT-3′, reverse: 5′-TGGATCCAAGGCTCTAG GTG-3′), and Bax (forward: 5′-CCAGCTGCCTTGGACTGT-3′, reverse: 5′-ACCCCCTCAAGACCACTCTT-3′) yielding products of 182, 146 and 135 bp, respectively [20]. The GAPDH PCR cycle conditions were set up as follows: one cycle of 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 10 s and 72 °C for 20 s. Fluorescence was acquired at the end of every 72 °C extension phase. The annealing temperatures for Bcl-xl and Bax were 60 and 61 °C, respectively. Quantitative analysis of data was performed using the LightCycler™ analysis software (Roche Diagnostics).

2.8. Western blot analysis

The sub-confluent growing cells were washed with PBS (Sigma) and lysed in an ice-cold RIPA buffer [25 mM Tris (pH 7.5), 50 mM NaCl, 0.5% sodium deoxycholate, 2% Nonidet P-40, 0.2% SDS, 1 mM phenylmethylsulfonyl fluoride and 500 KIE/ml "Trasylol"® proteinase inhibitor (Bayer Leverkusen, Germany)]. Total protein concentration was determined using the Bradford protein assay (Bio-Rad, Hercules, CA) and Western blot analysis was performed as described in our previous study [17]. The antibodies were used in dilutions of 1:100 for FLIPS/L (sc-5276; Santa Cruz Biotechnology, Santa Cruz, CA), 1:1000 for actin (A-2066; Sigma), and 1:2000 for secondary donkey anti-rabbit (NA934V; Amersham Biosciences, Buckinghamshire, UK) antibodies. The expression of proteins was evaluated by measuring the optical densities of protein bands, using the National Institute of Health Image analysis software version 1.61 and the expression value was calculated relative to that of actin.

2.9. Cytotoxicity assay

Target cells (1×10^6) were labeled with 40 μCi $Na^{51}CrO_4$ for 45 min at 37 °C. ^{51}Cr-labeled target cells (1×10^4) and effector cells (peripheral blood mononuclear cells, PBMCs) were mixed in U-bottomed wells of a 96-well microplate at the indicated *E/T* ratios. After 8 h of incubation, the cell-free supernatants were collected and counted on a gamma counter. The percent-specific cytotoxicity was calculated using the formula: [100 × (experimental release − spontaneous release)/(total release − spontaneous release)]. Total or spontaneous release was determined in the presence of 1% NP-40 or medium alone. For blocking, anti-Fas mAb ZB-4 was added at a final concentration of 500 ng/ml before the cytotoxicity assay for 1 h in accordance with the manufacturer's instructions.

2.10. Magnetic sorting

PBMCs obtained from a healthy volunteer were prepared by Ficoll–Hypaque centrifugation. $CD4^+$ T cells, $CD8^+$ T cells, and $CD4^-CD8^-$ cells were isolated from PBMCs by using anti-CD4 and anti-CD8 immunomagnetic beads and a Magnetic Cell Sorter (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of each subset was estimated at >95% by flow cytometry.

2.11. TUNEL assay

To detect apoptosis, we used the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) method, using the Apop Tag in situ apoptosis detection Kit (Chemicon International, Inc., Temecula, CA) as described previously [17]. This method can detect fragmented DNA ends of apoptotic cells. Briefly, the paraffin-embedded sections were deparaffinized in xylene and rehydrated in a graded series of ethanol baths. The sections were treated with 20 μg/ml of proteinase K in distilled water for 10 min at room temperature. The adherent cultured THLE-2 cells were fixed in 1% paraformaldehyde for 10 min. To block endogenous peroxidase, the slides were incubated in methanol containing 0.3% hydrogen peroxide for 20 min. The remaining procedures were performed according to the instructions provided by the manufacturer. For quantification of apoptosis, five microscopic fields were randomly selected at high power magnification (200×) and the average counts of TUNEL-positive cells were calculated.

2.12. Statistical analysis

Statistical analysis was performed using the StatView J-5.0 program (Abacus Concepts, Inc., Berkeley, CA). Data are expressed as means ± SD. Differences between groups were examined for statistical significance using the Dunnett method and Student's *t*-test. $P < 0.05$ denotes a statistically significant difference.

3. Results

3.1. Fas expression in human hepatoma cell lines

Flow cytometry using an anti-Fas antibody (UB-2) revealed expression of Fas receptor on the cell surface in three of the six cell lines (HLE, HLF and HepG2), but not on HuH7, PLC/PRF/5 and Hep3B (Fig. 1).

3.2. Response to agonistic anti-Fas antibody with dose escalation

We confirmed the response described above using the agonistic anti-Fas monoclonal antibody, CH-11, which is used widely to replace FasL in vitro. The 48-h MTT assay showed that CH-11 treatment inhibited the growth of three Fas-positive hepatoma cell lines (HLE, HLF and HepG2) in a dose-dependent manner. In contrast, no Fas-negative cell lines (HuH7, PLC/PRF/5 and Hep3B) were growth-responsive to CH-11 (Fig. 2a). Dose dependency of IFNα and 5-FU was examined with various CH-11 concentrations. This effect was synergistic and observed in the combination of CH-11 and 5-FU in the doses of 0–0.5 μg/ml of 5-FU. There was seen little difference between 0.5 and 1 μg/ml of 5-FU (Fig. 2b).

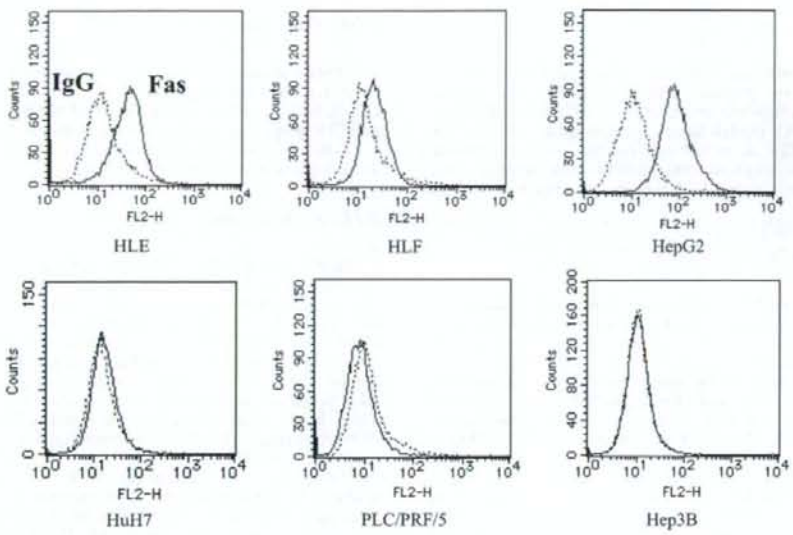

Fig. 1. Fas expression in six hepatoma cell lines assessed using flow cytometry with an anti-human Fas antibody (IgG, clone UB2). Histogram with dotted line represents cells stained with secondary antibody alone; histogram with solid line is those cells stained with anti-Fas antibody. Fas was expressed on the surface of three of the six cell lines (HLE, HLF and HepG2). All experiments were performed three times independently.

3.3. Influence of IFNα and/or 5-FU on apoptosis induced by agonistic anti-Fas antibody

We next evaluated the effects of IFNα, 5-FU and combination treatments on growth inhibition induced by CH-11 using the MTT assay. In Fas-expressing cell lines, the inhibitory effect of CH-11 was enhanced with IFNα or 5-FU alone, but the maximum effect was observed with a combination treatment of both agents (Fig. 3a). In the HepG2 cells, the anti-proliferating effect of CH-11 alone was $26.7 \pm 1.8\%$, and the effect of either IFNα or 5-FU used alone was $18.0 \pm 4.7\%$, which did represent a significant enhancement. However, the combination treatment (CH-11 + IFNα/5-FU) yielded a markedly increased effect of $83.8 \pm 6.3\%$ ($P = 0.01$). Without CH-11, none of the agents, whether used alone or in combination, had any anti-proliferative effects. Similar results were obtained in the other Fas-positive cell lines, HLE and HLF.

To confirm these results, we performed an annexin-V assay to detect Fas-mediated apoptosis. Results with the HepG2 cells were comparable with those from the MTT assay (Fig. 3b, Table 1), in that an increase in apoptotic cell numbers induced by CH-11 was found with stimulation by IFNα alone, 5-FU alone, and particularly strongly with the combination treatment. In contrast, the effects of CH-11 and the influence of IFNα/5-FU were not observed with the three Fas-negative cell lines (HuH7, PLC/PRF/5 and Hep3B) in both the MTT and annexin-V assay (Fig. 3a; MTT assay, data not shown; annexin-V assay).

3.4. Regulation of Fas expression by IFNα and/or 5-FU

To investigate the mechanism underlying the upregulation of Fas-mediated apoptosis, we analyzed the relationship between the change in Fas expression and the regulation of apoptosis. Out of the six cell lines, 5-FU increased Fas expression in the HepG2 cells only, while IFNα also increased Fas in the HuH7 and PLC/PRF/5 cells (Fig. 4). No additional effects were seen with the combination of IFNα and 5-FU compared with each drug used alone. In the other three hepatoma cell lines (HLE, HLF and Hep3B), neither IFNα nor 5-FU affected the level of cell surface Fas.

3.5. Caspase activation after stimulation with agonistic anti-Fas antibody and/or IFNα/5-FU

Results indicated that the change in Fas expression seen in this study would not be related to the CH-11 and IFNα/5-FU-mediated effects on apoptosis. Therefore, we next tested for variations in caspase activity in the HepG2 cells, as the representative Fas-positive cell line, using a caspase colorimetric protease assay kit (Fig. 5a). Caspase-3 activity (downstream of caspase cascades) was increased after 12 h of CH-11 stimulation. IFNα and 5-FU alone enhanced this upregulation, with the combination treatment having a further effect (Fig. 5a); 5-FU alone, IFNα alone and combination therapy did not affect caspase-3 activity without CH-11. Caspase-8 activity also

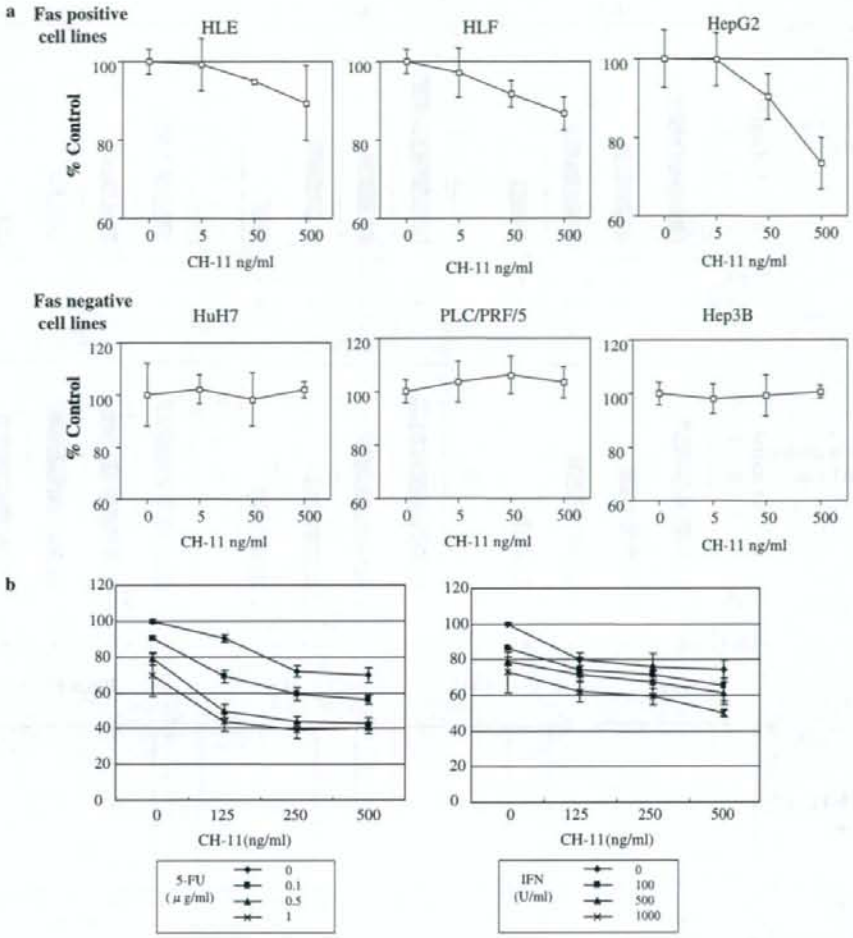

Fig. 2. Susceptibility of six hepatoma cell lines to agonistic anti-Fas monoclonal antibody CH-11-mediated apoptosis was measured by MTT assay. (a) Each cell line was incubated with CH-11 at various concentrations for 48 h. Three Fas-positive cell lines (HLE, HLF and HepG2) were naive to CH-11 in a dose–response manner. Fas-negative cell lines (HuH7, PLC/PRF/5 and Hep3B) were resistant to Fas-mediated apoptosis. (b) Dose dependency of combination treatment was examined. CH-11 and 5-FU or IFNα were added to HepG2 cells with indicated doses. Results are expressed as percent of cell growth of each untreated cell. Data represent means ± SD of at least triplicate samples. Similar results were observed in three independent experiments.

increased with CH-11 stimulation compared with the control and further increased with CH-11 + IFNα/5-FU; there was no significant difference between stimulation of CH-11 + IFNα and the combination. On the other hand, caspase-9 activity showed a different tendency from the data for caspase-3 and -8. Stimulation with CH-11+5-FU or CH-11 + IFNα/5-FU slightly increased the caspase-9 activity, but the effects were much less pronounced than for caspase-3 and caspase-8, and they were not significant (Fig. 5a). To confirm the significance of caspase activities in the apoptotic effect in Fas/FasL system, MTT assay using specific caspase inhibitors was performed. All specific caspase inhibitors blocked the apoptotic effect of CH-11 with IFNα/5-FU totally or partially in the dose-dependent manner (data not shown). Caspase-3-specific inhibitor (Z-DEVD-FMK) and caspase-8-specific inhibitor (Z-IETD-FMK) almost completely blocked CH-11 induced apoptosis enhanced by IFNα/5-FU (Fig. 5b). Caspase-9-specific inhibitor (Z-LEHD-FMK) showed only partial blocking effect. These results were compatible to the results of caspase assay (Fig. 5a). Colorimetric caspase assay using specific caspase inhibitors showed Z-DEVD-FMK blocked caspase-3 activation induced by CH-11 and IFNα/5-FU (Fig. 5c).

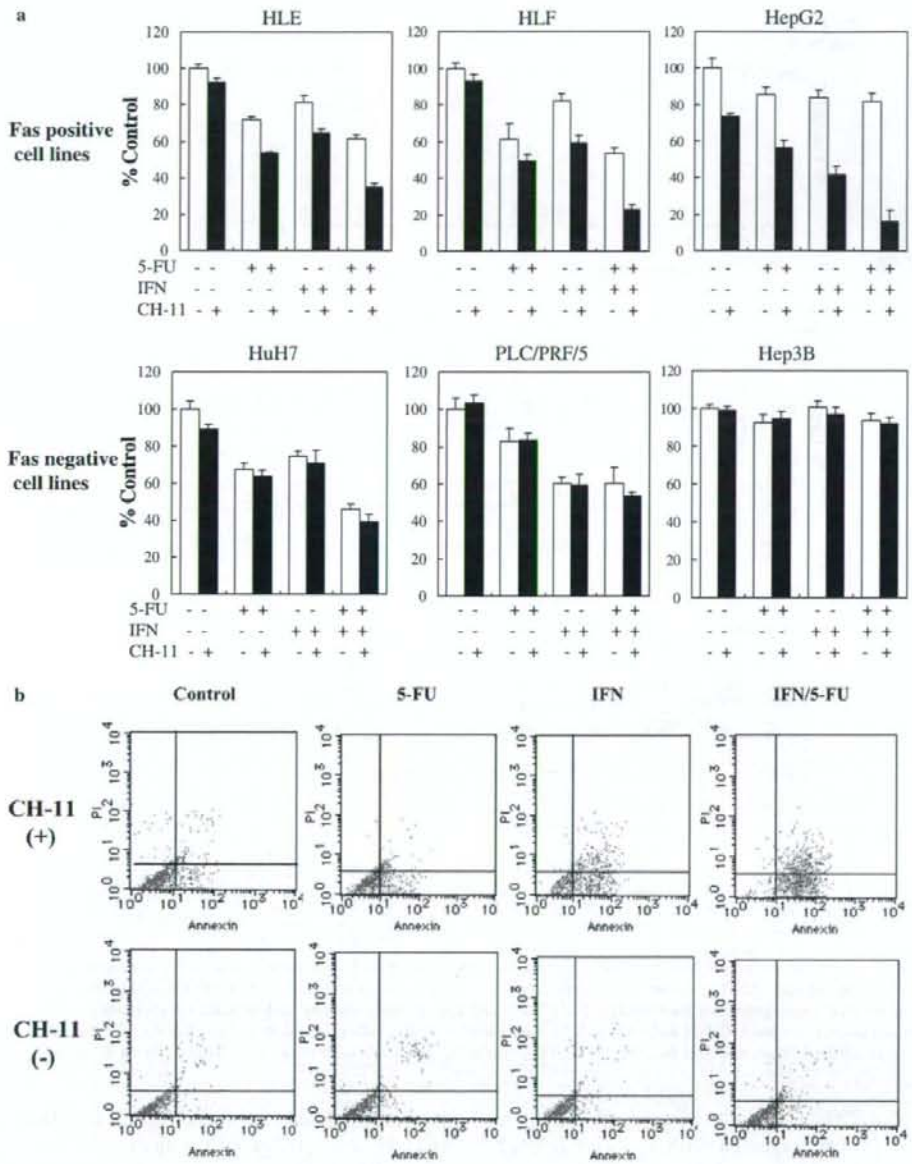

Fig. 3. (a) Effects of IFNα and/or 5-FU on Fas-mediated apoptosis in six hepatoma cell lines measured by MTT assay. All cells were incubated with IFNα (500 U/ml) and/or 5-FU (0.5 μg/ml) and with agonistic anti-Fas monoclonal antibody CH-11 (500 ng/ml) (□) or without CH-11 (■) for 48 h. The susceptibility of Fas-positive hepatoma cells to Fas-mediated apoptosis was significantly enhanced by IFNα or 5-FU alone, and further so in the combination treatment. Results were expressed as percent of cell viability of untreated cells. Data represent means ± SD values of at least triplicate samples. Similar results were observed in three independent experiments. (b) Apoptotic cells were determined using the annexin-V assay (HepG2). [This figure appears in colour on the web.]

3.6. Regulation of FLIP

Several factors involved in apoptosis were next examined at the mRNA expression level. The expression of FLIP (FLICE/caspase-8 inhibitory protein), which is an inhibitor of caspase-8 [21,22], was markedly decreased by treatment with IFNα or 5-FU, as shown by the FLIP/GAPDH ratio, compared with untreated

Table 1
Comparison of results of MTT and annexin-V assays

	Cell toxicity (%)	Annexin (+) cells (%)
Control	0.0	5.8
5-FU	14.4	9.5
IFN	16.2	10.0
IFN + 5FU	18.0	19.7
CH-11	26.7	27.8
CH-11 + 5FU	43.8	37.6
CH-11 + IFN	58.0	62.4
CH-11 + IFN + 5FU	83.8	93.5

The two independent assays showed similar tendency. Annexin-V assay indicated that the growth inhibition effect noted in MTT assay was caused by apoptosis.

cells with the combination treatment again providing the most significant decrease (Fig. 6a). Western blot analysis was performed to examine the change of FLIP at protein level. Expression of FLIP long was decreased by the combination treatment (Fig. 6b).

3.7. Regulation of apoptotic factors

Caspase-9 acts through mitochondria and is regulated by certain apoptotic factors [23]. Although the levels of caspase-9 were not increased, we checked the expression of the apoptotic factors Bax and Bcl-xl (Fig. 6c). The IFNα/5-FU combination significantly increased Bax expression, although the effect was not dramatic. Each of the above treatments in turn reduced Bcl-xl expression, although in this case the IFNα/5-FU combination produced no enhancement.

3.8. Involvement of the Fas/FasL pathway in IFNα/5-FU-induced PBMC cytotoxicity against HCC cells

We performed ^{51}Cr-release assay to evaluate the interaction between PBMC and hepatoma cell lines via the Fas/FasL pathway by which IFNα/5-FU appear to exert their influence (Fig. 7). In the Fas-positive HepG2 cells, we first established the optimal E/T ratio, which is directly proportional to the increase in released ^{51}Cr (Fig. 7a). The blocking effect of neutralizing anti-Fas antibody, ZB-4, is shown in Fig. 7b. Based on these data, an E/T ratio of 20 was chosen, to produce the most distinct difference in the presence and absence of ZB4 (Fig. 7c). Fig. 7c shows that IFNα increased the released ^{51}Cr and that this enhanced cytotoxicity was blocked by ZB4. Next, we tried to identify the main proponent of the cytotoxic effect using a magnetic sorting technique. $CD4^+$ cells, $CD8^+$ cells, and $CD4^-CD8^-$ cells were isolated from PBMCs and used as effecter cells in the ^{51}Cr-release assay after pretreatment with IFNα and/or 5-FU (Fig. 7d). The HepG2 target cells received no pretreatment. The results show that the $CD4^-CD8^-$ cells were the most cytotoxic and that IFNα enhanced this effect more than 5-fold. This IFNα-induced cytotoxicity was markedly inhibited by ZB4. Lastly, we pretreated both effecter and target cells with IFNα/5-FU

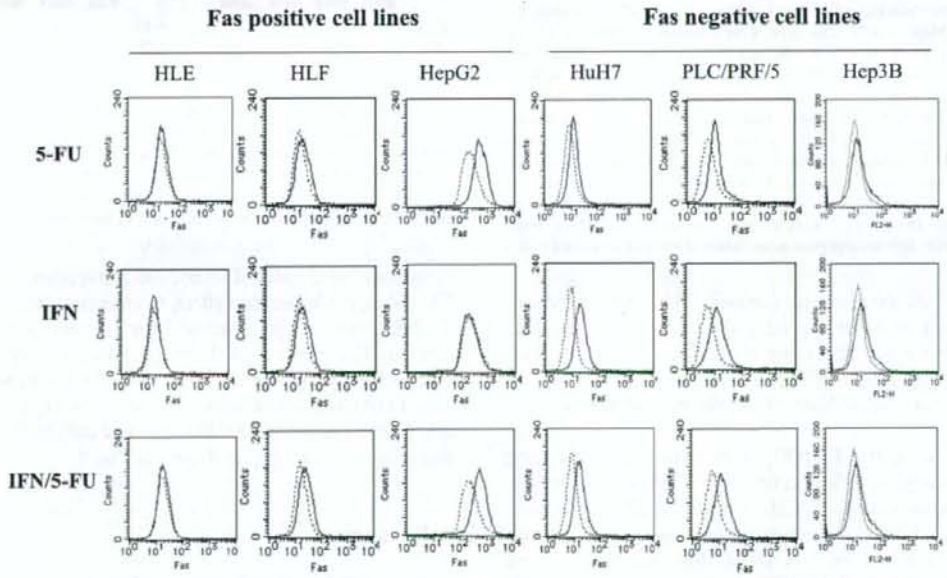

Fig. 4. Regulation of Fas expression induced by IFNα and/or 5-FU. Adherent cells were incubated with IFNα (500 U/ml) and/or 5-FU (0.5 μg/ml) for 24 h. Cell surface Fas was detected by flow cytometry using a mouse monoclonal anti-human Fas IgG (UB2). Histogram with dotted line shows untreated; histogram with solid line represents the drug-treated cells.