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EDITORIAL

Precise role of *H pylori* in duodenal ulceration

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Abstract

The facts that *H pylori* infection is commoner in duodenal ulcer (DU) patients than in the normal population, and that eradication results in most cases being cured, have led to the belief that it causes DU. However, early cases of DU are less likely than established ones to be infected. *H pylori*-negative cases are usually ascribed to specific associated factors such as non-steroidal anti-inflammatory drugs (NSAIDs), Crohn's disease, and hypergastrinaemia, but even after excluding these, several *H pylori*-negative cases remain and are particularly common in areas of low prevalence of *H pylori* infection. Moreover, this incidence of *H pylori* negative DU is not associated with a fall in overall DU prevalence when compared with countries with a higher *H pylori* prevalence. In countries with a high *H pylori* prevalence there are regional differences in DU prevalence, but no evidence of an overall higher prevalence of DU than in countries with a low *H pylori* prevalence. There is no evidence that virulence factors are predictive of clinical outcome. After healing following eradication of *H pylori* infection DU can still recur. Medical or surgical measures to reduce acid output can lead to long-term healing despite persistence of *H pylori* infection. Up to half of cases of acute DU perforation are *H pylori* negative. These findings lead to the conclusion that *H pylori* infection does not itself cause DU, but leads to resistance to healing, i.e., chronicity. This conclusion is shown not to be incompatible with the universally high prevalence of DU compared with controls.

Key words: Duodenal ulceration; *H pylori* infection; Not causal; Delays healing

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INTRODUCTION

The award of the Nobel Prize to Warren and Marshall for the discovery of *H pylori*[1] was rightly acclaimed by the medical profession, because the eradication of the organism turns a chronic, relapsing disease into one that in most (but not all) cases can be readily cured. The prevalence of infection with the organism is greater in patients with, than in subjects free of, duodenal ulcer; the organism is present in most (but not all) cases of the disease; and its removal results in most (though not all) cases being cured without relapse. The inference usually drawn from this combination of events is that *H pylori* actually causes duodenal ulcer.

In that case, why do most individuals infected with the organism not develop the disease? There has been considerable work done exploring the concept that the presence or absence of virulence factors explains this anomaly, but in a recent publication[2] we explain why we do not find this hypothesis convincing.

This unsatisfactory situation, the facts that most patients with infection do not have a duodenal ulcer, that, geographically, duodenal ulcer prevalence is not related to the prevalence of *H pylori*, and many other anomalies, has been highlighted in several review articles, such as *H pylori*: the African Enigma[3]. The enigma is two-fold: firstly, that DU prevalence is not higher in countries with a higher prevalence of *H pylori* infection, secondly, that within these countries, despite an uniformly high *H pylori* prevalence, regional differences in DU prevalence are found, that as described later, are related to diet but not to smoking, genetic or other factors.

Our continuing search for an explanation of the anomalies led us to review all the papers that provide evidence about the following: (1) Is *H pylori* infection present at the onset of DU? (2) Is there a dose-response relationship between *H pylori* and DU? (3) Does recurrence or non-recurrence of DU after successful treatment correlate with *H pylori*-status? (4) Can we deduce anything of importance from the prevalence and distribution of *H pylori*-negative DU?

The present paper analyses the evidence of the literature on these subjects and we show how the higher prevalence of *H pylori* in DU than in the normal population and some of the other anomalies can be explained by a self-consistent theory, but only by relinquishing the belief

that *H pylori* causes DU.

To the best of our knowledge, no paper has been published that contradicts statements in this review. We shall be obliged to readers for letting us know about any exceptions that we have missed.

Is H pylori infection present at the onset of DU?

One is entitled to expect that the cause always precedes its effect. The problem with duodenal ulceration is that the patient usually presents to the physician some time after the symptoms start: it is unlikely that evidence of infection has been sought before the symptoms began. It occurred to us to examine the *H pylori*-status of patients with duodenal ulcer in relation to the length of history before the initial biopsy that established the diagnosis of DU. Assuming the organism was the cause of the ulcer, we expected the infection to be manifest at least as often in the first six months as it was more than six months after symptoms started. We studied 37 patients: to our surprise, 5 whose history was of less than six months were *H pylori*-negative, the remaining 32, all with a length of history greater than six months, were *H pylori*-positive. The odds against this happening by chance were (Fisher's exact test) greater than 1000 to 1[4]. It looked as though the DU was causing the infection, rather than the other way about.

Searching the literature at that time (2001), we could find only one paper that gave figures for infection status, distinguished by different lengths of history[5]. Repeating the search in 2005, we were only able to find one further paper with the appropriate data[6]. Both these papers showed a higher infection rate later compared with earlier in the ulcer disease.

There are two reports[7,8] which suggest that pre-existing *H pylori* infection predisposes to DU based on seropositivity. The first paper refers to young inductees into the Israeli army and the second to an older population in Hawaii. In the first paper it is noteworthy that 7 of the 29 reported DU cases were *H pylori*-negative at the time of diagnosis. However, seropositivity does not mean that infection is present. After eradication, it can take 5-10 years for seropositive cases to become seronegative. Many children serorevert after childhood infection and in adults the conversion/reversion rates per annum vary between 0.5% and 1%. An approach along these lines therefore lacks scientific reliability.

Dose-response relationship?

The smallest dose is zero. Many patients develop a DU in the absence of infection with *H pylori*. This fact is usually explained by invoking 'special' causes of DU such as Zollinger-Ellison syndrome and non-steroidal anti-inflammatory agents (NSAIDs) (see later), but such causes are not always apparent.

Before the *H pylori* era, excess gastric hydrochloric acid was the favoured aetiology of DU. 'No acid, no ulcer' has been dogma since Schwartz[9], and there has been no contrary evidence. However, acid is 'statistically' greater than the normal range in only about 15% of (patients with) DU, the rest having acid in the normal range[10], albeit with a tendency to be greater than in the normal population.

In many countries there is no information about the actual prevalence of DU in the overall population, and available information is based on figures obtained from hospital statistics or small population surveys. From information that is available, there is no evidence that there is a higher overall prevalence of DU in those countries where there is a higher prevalence of *H pylori* infection than in other countries. There is evidence from India, China and Africa, however, of differences in DU prevalence between areas known to have a high prevalence of *H pylori* infection[3,11-18] these differences being related to the staple diets of the regions[15-24] rather than to smoking, genetic or other factors such as duodenal gastric metaplasia. There is evidence that different foods contain agents that are either ulcerogenic or ulceroprotective[24-27].

In developed countries, where *H pylori* prevalence is about 35% overall, DUs are about 70% *H pylori*-positive[6,28-38]; in developing countries, *H pylori* prevalence is about 70% overall, and DUs are *H pylori*-positive in about 90% of patients[16,28,29,39-50]. Therefore in all countries *H pylori*-positivity is greater in DU than in non-DU. With reference to further discussion, it is notable that in areas of low *H pylori* prevalence there is a high prevalence of *H pylori*-negative, non-NSAID, ulcers[4,6,16,28,29,34,38,39,51-63].

As mentioned above there is no evidence that the prevalence of DU is any greater in countries with a high prevalence of *H pylori* than in those with a low prevalence (this finding in itself argues against *H pylori* being the prime cause of DU). The prevalence of DU in London is about 11%[10]. Applying this figure to other developed countries, from the figures quoted previously in every 100 of the population, 35 are *H pylori*-positive and 65 *H pylori*-negative. There are 11 patients with DU, of whom perhaps only one is *H pylori*-negative, so only 10 of the 35 *H pylori*-positive subjects get DU. The remaining 25 (25/35, 71%) do not. A similar argument in the developing countries (again assuming an 11% prevalence) yields an estimate that only 10 of the 70 *H pylori*-positive subjects get DU. The remaining 60 of the 70 (86%) do not. The belief that *H pylori* is the prime cause of DU demands an explanation of why in 71%-86% of individuals it does *not* produce a DU.

Weight of infection can be measured by use of the breath test, but no-one has suggested that the greater the weight of infection, the greater is the risk of developing DU. However, there is excellent evidence that the risk of developing DU increases with the rate at which the subject secretes gastric juice when maximally stimulated with intravenous histamine[10]. The *H pylori*-lobby suggests that we cannot demonstrate a dose-relationship of DU with *H pylori* because only some strains of the organism possess virulence factors (of which many have been reported). It is beyond the scope of this paper to go into that topic, but there is considerable evidence that virulence factors have no relationship to clinical outcome[2].

Does recurrence of DU correspond with H pylori-status?

The answer is, apparently not very well. Ulcers heal with effective medical suppression of acid without eradication

of *H pylori*[64,65], and after surgical procedures ulcers remain healed despite persistent *H pylori* infection[66-75].

When *H pylori* was discovered and the first results came in about the effect of its extirpation, it was claimed that removing *H pylori* 'cured' the disease and that relapses never occurred. As time passed that picture had to be modified: there is no doubt that relapses are much less frequent and many patients have no further trouble, but there is increasing evidence of a significant recurrence rate after eradication of *H pylori* despite lack of recurrence of the infection. Excluding subjects taking NSAIDs, 9 papers, involving 2928 DU patients in whom *H pylori* had been eradicated as proven by multiple tests, reported recurrent ulceration in 182 (6.1%) over a period up to 5 years[76-84]. One meta-analysis by Laine of 7 trials subjected to strict criteria reports a recurrence rate of 20% within 6 mo[81]. Interestingly, a recurrence rate of 6.6% (571/8693) up to 2 years is given in 12 papers (including 6 meta-analyses)[76,84-96] involving 8693 cases of DU, not excluding NSAIDs, in whom *H pylori* had been eradicated. These recurrence rates, with and without NSAIDs, are virtually identical ($P = 0.4883$). In other words, recurrence after eradication of *H pylori* cannot be attributed to NSAIDs. The use of multiple tests for *H pylori* reduced the risk that we are dealing here with difficulty in demonstrating the presence of *H pylori* after eradication.

What does H pylori-negative DU tell us?

The phenomenon of *H pylori*-negative DU is an argument against a blanket role for *H pylori* as a "cause preceding effect". As stated above, this prevalence is greater in countries with a low, compared with countries with a high prevalence of the organism, even after excluding DU-associated factors such as Crohn's disease and the taking of NSAIDs. There are 20 such reports[4,6,28,29,34,38,39,51-63] from countries with a low prevalence of *H pylori* infection giving a mean of 14.4% (829/5745) of *H pylori*-negative DU and 5 reports[47,96-99] with a mean of 3.9% (52/1325), $P < 0.0001$) from countries with a high prevalence.

Three papers[100-102] suggest that despite the low *H pylori* prevalence in a population with an increased prevalence of *H pylori*-negative DU, there is no decrease in overall DU prevalence. If *H pylori* were the primary cause of DU then one would expect a lower prevalence of DU.

We can offer one supplementary consideration. Perforation of duodenal ulceration might reasonably be expected to signify an especially large secretion of gastric hydrochloric acid. In this context it is interesting that perforation does not seem to be associated with *H pylori*. In 4 reports about patients operated on for perforated duodenal ulcer *H pylori* prevalence was significantly less than in uncomplicated duodenal ulceration[103-106] (in 2[103,104] they were indistinguishable from normal controls). The only dissenting evidence was from a report by Matsukara[107].

INTERPRETATION

How are we to interpret the undoubted relationships between DU, *H pylori* and gastric acid? The present majority view is that *H pylori* causes DU, not that DU causes *H pylori*. The favourable evidence for the former inference is the greater proportion of *H pylori*-positive cases in DU compared with non-DU subjects, and the fact that clearing the organisms converts the clinical course of DU from chronic relapsing to (mostly) stable healing. However, the second of these points is not proof of initial causation, merely of an interference with healing leading to chronicity of the ulceration.

If H pylori is not the initial cause of DU?

If we reject *H pylori* as the cause of DU, how can we explain the greater proportion of *H pylori* in DU compared with non-DU subjects? *H pylori* can only live within a relatively narrow band of pH. Both highly acid and highly alkaline conditions kill the organism[108,109]. For example, in pernicious anaemia the patient is usually *H pylori*-positive in the early stages (acid production is reduced but still abundant) and then becomes *H pylori*-negative in the later stages (when all acid production has ceased and the stomach is exposed to alkaline reflux from the duodenum)[110,111].

These facts suggest that some patients who develop a DU may well have so much acid that they are *H pylori*-negative. When treated with acid suppression for their early symptoms, the gastric acidity may fall enough to encourage infection with the organism. At this stage they are investigated and found to be *H pylori*-positive. Strange as it may at first seem, we are postulating that one interpretation of the link between *H pylori* and DU in cases that are initially *H pylori*-negative is that DU (*via* its treatment) causes the infection. This would explain the greater prevalence of *H pylori* in the subjects with DU as an outcome of treatment with acid-suppressing drugs. If the likelihood of a first infection increases with the presence of virulence factors in the organism - as seems reasonable - this explains why the virulence factors are more prevalent in the DU than in the non-DU subjects[2].

In developing countries with a high prevalence of *H pylori* infection, and where people do not have access to acid-suppressing drugs and only come to hospital with long-standing chronic conditions, there is another possible explanation. With a high *H pylori* prevalence of 70% it follows that 30% of DU patients initially would be *H pylori* negative and 85% of them will not be hypersecretors of acid (See 'Dose Relationship' above). As a result of continued exposure to the high prevalence of *H pylori* a number of these may become infected, resulting in their ulceration becoming unremitting and chronic, and causing them to seek medical help. The result again would be a higher prevalence of *H pylori* infection in those diagnosed with duodenal ulceration.

In addition, the known lability of *H pylori* infection could result in some *H pylori* positive DUs healing as a result of spontaneous disappearance of the infection, leaving a preponderance of DU cases with persisting infection, thus resulting in a higher prevalence of infection in the DU population.

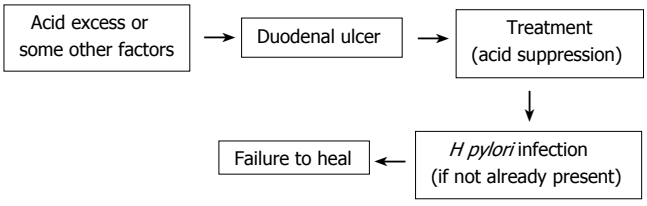
It is possible to calculate the relationship between the fraction of established duodenal ulcer patients who are

H pylori-positive, the fraction of the whole population who are *H pylori*-positive, and the fraction, X, of those initially *H pylori*-negative duodenal ulcer patients who become infected so as to produce the observed increase in established DU patients. We have performed these calculations on data from 18 reports from countries with a high prevalence[16,28,29,35,39-50,112] and 13 reports from countries with a low prevalence of *H pylori* infection[5,28-38,113]. The values of X were 0.6 in the developing countries, 0.58 in the developed. The congruence between these two estimates, while certainly not proof of our hypothesis, at least suggests that our hypothesis should not be rejected out of hand. Details of the derivation of the formula and the statistical interpretations are given in the Appendix.

There remain two important problems. (1) Most patients with DU have gastric secretion within the range of normal (though the chance of getting DU does increase with the rate of secretion). There must be some other factor to explain why some get an ulcer and others do not. Our work[114] demonstrated that this factor was unlikely to be *H pylori*. We favour the idea that the causative agent nevertheless involves interference with wound-healing. NSAIDs can interfere with the healing of *H pylori*-negative ulcer, so they may be responsible for part of the problem, but there is almost certainly more to discover; (2) We still do not know how *H pylori* (which can only live in gastric, not in true duodenal mucosa), makes the ulcer difficult to heal. The presence of colonised gastric mucosa within the duodenum might be a factor. *H pylori* infection inhibits healing of wounded duodenal epithelium in vitro due to vacA[115]. Another factor might be the effect that the organism has of increasing sub-maximally (gastrin-) stimulated gastric juice[116]. While the mechanism is in doubt, the relationship is clear and is the fundamental reason why the discovery of *H pylori* is of such enormous importance for the treatment of duodenal ulcer.

CONCLUSION

Our present view is that the relationship between duodenal ulcer, *H pylori* and gastric acid secretion is most likely to be:



APPENDIX

Fraction (= X) of *H pylori* -ve duodenal ulcers becoming *H pylori* + ve, possibly as a result of antacid treatment

$$X = \frac{\%\ \text{Hp+ve DU minus \% Hp+ve NUD or Controls}}{\%\ \text{Hp -ve NUD or Controls}}$$

Hypothesis

Whether or not a subject is *H pylori*-positive (+ve) or *H pylori*-negative (-ve) makes no difference to the likelihood that s/he will develop a duodenal ulcer (DU).

The diagnosis of most subjects with a DU is only made after the patient has already been treated with agents that reduce gastric acid secretion.

Reduction in gastric secretion is likely to increase the chance that a -ve patient becomes +ve.

When first diagnosed, most DU patients will include some who were +ve before they developed DU and others who had been -ve but became positive during their initial treatment and before diagnosis.

Calculation

Let

P = population

U = fraction having DU

F = fraction of population *H pylori* +ve

(so 1-F = fraction negative)

Then

Confirmed DU (say after 6/12)

$= U \times P$

However, the confirmed DU is made up of two moieties, one originally +ve, the other originally negative.

The originally positive DU number

$= F \times U \times P$.

The originally negative DU number

$= (1\text{-}F) \times U \times P$.

Let a fraction X of the originally negative DU be infected as a result of acid-suppression. Then after 6/12 these will number

$= (1 - F) \times U \times P \times X$.

Therefore the observed positive DUs can be expressed as

$(F \times U \times P) + X[(1\text{-}F) \times U \times P]$.

Therefore, the observed proportion of DUs who are positive is given by

$\{(F \times U \times P) + X(1\text{-}F) \times U \times P\}/(U \times P)$

or, dividing by $U \times P$,

$F + X(1\text{-}F)$.

Therefore, observed +ve DU/(total DU) = $\{F + X(1\text{-}F)\}$

$= F + X - FX$

Therefore, $X(1\text{-}F) = [\text{observed DU+ve/total DU}] - F$

And $X = \{[\text{observed DU+ve/total DU}] - F\}/(1 - F)\}$

Analysis of the figures quoted

High prevalence countries: Mean Hp+ DUs = 89.395; (SD 7.602, SE 1.700). Mean Hp+ NUD = 68.350 (SD 12.759, SE 2.853. Mean X = 60.565 (SD 26.466, SE 5.918).

Low prevalence countries: Mean Hp+DU = 70.221; (SD 14.319, SE 3.183). Mean Hp + NUD = 33.286; (SD 16.014, SE 4.280). Mean X = 58.279; (SD 17.773, SE 4.750)

Difference between high prevalence and low prevalence countries: for Hp + DU, $P = 0.0002$ (highly significant); for X, $P = 0.4109$ definitely non-significant.

This hypothesis suggests that in all countries there is much the same chance of originally *H pylori*-negative becoming infected as the ulcer progresses. This fact provides circumstantial evidence, though certainly not proof, of our hypothesis.

REFERENCES

1 **Marshall BJ**, Warren JR. Unidentified curved bacilli in the

stomach of patients with gastritis and peptic ulceration. *Lancet* 1984; **1**: 1311-1315
2 **Tovey FI**, Hobsley M, Holton J. *Helicobacter pylori* virulence factors in duodenal ulceration: A primary cause or a secondary infection causing chronicity. *World J Gastroenterol* 2006; **12**: 6-9
3 **Segal I**, Ally R, Sitas F, Walker AR. Co-screening for primary biliary cirrhosis and coeliac disease. *Helicobacter pylori*: the African enigma. *Gut* 1998; **43**: 300-301
4 **Boulos PB**, Botha A, Hobsley M, Holton J, Oshowo AO, Tovey FI. Possible absence of *Helicobacter pylori* in the early stages of duodenal ulceration. *QJM* 2002; **95**: 749-752
5 **Pest P**, Zarate J, Varsky C, Man F, Schraier M. *Helicobacter pylori* in recently-diagnosed versus chronic duodenal ulcer. *Acta Gastroenterol Latinoam* 1996; **26**: 273-276
6 **Bytzer P**, Teglbjaerg PS. *Helicobacter pylori*-negative duodenal ulcers: prevalence, clinical characteristics, and prognosis--results from a randomized trial with 2-year follow-up. *Am J Gastroenterol* 2001; **96**: 1409-1416
7 **Gdalevich M**, Cohen D, Ashkenazi I, Mimouni D, Shpilberg O, Kark JD. *Helicobacter pylori* infection and subsequent peptic duodenal disease among young adults. *Int J Epidemiol* 2000; **29**: 592-595
8 **Nomura A**, Stemmermann GN, Chyou PH, Perez-Perez GI, Blaser MJ. *Helicobacter pylori* infection and the risk for duodenal and gastric ulceration. Ann Intern Med 1994; 120: 977-81
9 **Schwarz K**. Ueber penetrierende magen- und jejunal geshwure. *Beitr Klin Chir* 1910; **67**: 96-128
10 **Hobsley M**, Whitfield PF. The likelihood of a disease in relation to the magnitude of a risk factor. The example of duodenal ulcer. *Theoretical Surgery* 1987; **2**: 6-9
11 **Holcombe C**. *Helicobacter pylori*: the African enigma. *Gut* 1992; **33**: 429-431
12 **Holcombe C**, Omatara BA, Eldridge J, Jones DM. *H. pylori*, the most common bacterial infection in Africa: a random serological study. *Am J Gastroenterol* 1992; **87**: 28-30
13 **Holcombe C**, Omatara BA, Padonu MK, Bassi AP. The prevalence of symptoms of dyspepsia in north eastern Nigeria. A random community based survey. *Trop Geogr Med* 1991; **43**: 209-214
14 **Segal I**, Ally R, Mitchell H. *Helicobacter pylori*--an African perspective. *QJM* 2001; **94**: 561-565
15 **Tovey FI**, Hobsley M, Segal I, Jayaraj AP. Duodenal ulcer in South Africa: home-pounded versus milled maize. *J Gastroenterol Hepatol* 2005; **20**: 1008-1011
16 **Tovey FI**, Hobsley M, Kaushik SP, Pandey R, Kurian G, Singh K, Sood A, Jehangir E. Duodenal gastric metaplasia and *Helicobacter pylori* infection in high and low duodenal ulcer-prevalent areas in India. *J Gastroenterol Hepatol* 2004; **19**: 497-505
17 **Wong BC**, Ching CK, Lam SK, Li ZL, Chen BW, Li YN, Liu HJ, Liu JB, Wang BE, Yuan SZ, Xu CP, Hou XH, Zhang AT, Zheng ZT. Differential north to south gastric cancer-duodenal ulcer gradient in China. China Ulcer Study Group. *J Gastroenterol Hepatol* 1998; **13**: 1050-1057
18 **Ching CK**, Lam SK. *Helicobacter pylori* epidemiology in relation to peptic ulcer and gastric cancer in south and north China. *J Gastroenterol Hepatol* 1994; **9** Suppl 1: S4-S7
19 **Jayaraj AP**, Tovey FI, Clark CG. Possible dietary protective factors in relation to the distribution of duodenal ulcer in India and Bangladesh. *Gut* 1980; **21**: 1068-1076
20 **Jayaraj AP**, Tovey FI, Lewin MR, Clark CG. Duodenal ulcer prevalence: experimental evidence for the possible role of dietary lipids. *J Gastroenterol Hepatol* 2000; **15**: 610-616
21 **Tovey F**. Peptic ulcer in India and Bangladesh. *Gut* 1979; **20**: 329-347
22 **Tovey FI**, Tunstall M. Duodenal ulcer in black populations in Africa south of the Sahara. *Gut* 1975; **16**: 564-576
23 **Tovey FI**. Duodenal ulcer in China. *J Gastroenterol Hepatol* 1992; **7**: 427-431
24 **Jayaraj AP**, Tovey FI, Clark CG, Hobsley M. Dietary factors in relation to the distribution of duodenal ulcer in India as assessed by studies in rats. *J Gastroenterol Hepatol* 2001; **16**: 501-505
25 **Jayaraj AP**, Rees KR, Tovey FI, White JS. A molecular basis of peptic ulceration due to diet. *Br J Exp Pathol* 1986; **67**: 149-155
26 **Jayaraj AP**, Tovey FI, Clark CG, Rees KR, White JS, Lewin MR. The ulcerogenic and protective action of rice and rice fractions in experimental peptic ulceration. *Clin Sci* (Lond) 1987; **72**: 463-466
27 **Paul Jayaraj A**, Tovey FI, Hobsley M. Duodenal ulcer prevalence: research into the nature of possible protective dietary lipids. *Phytother Res* 2003; **17**: 391-398
28 **Jyotheeswaran S**, Shah AN, Jin HO, Potter GD, Ona FV, Chey WY. Prevalence of *Helicobacter pylori* in peptic ulcer patients in greater Rochester, NY: is empirical triple therapy justified? *Am J Gastroenterol* 1998; **93**: 574-578
29 **Borody TJ**, George LL, Brandl S, Andrews P, Ostapowicz N, Hyland L, Devine M. *Helicobacter pylori*-negative duodenal ulcer. *Am J Gastroenterol* 1991; **86**: 1154-1157
30 **Dwyer B**, Sun NX, Kaldor J, Tee W, Lambert J, Luppino M, Flannery G. Antibody response to Campylobacter pylori in an ethnic group lacking peptic ulceration. *Scand J Infect Dis* 1988; **20**: 63-68
31 **Jones DM**, Eldridge J, Fox AJ, Sethi P, Whorwell PJ. Antibody to the gastric campylobacter-like organism ("Campylobacter pyloridis")--clinical correlations and distribution in the normal population. *J Med Microbiol* 1986; **22**: 57-62
32 **Kang JY**, Wee A, Math MV, Guan R, Tay HH, Yap I, Sutherland IH. *Helicobacter pylori* and gastritis in patients with peptic ulcer and non-ulcer dyspepsia: ethnic differences in Singapore. *Gut* 1990; **31**: 850-853
33 **Kochhar R**, Siddeshi ER, Ayyagiri A, Bhasin DK, Metha SH. Campylobacter pylori in dyspeptic patients: a report from North India. *Trans Roy Soc Trop Med Hygiene* 1989; **83**: 135
34 **Lahaie RG**, Lahaie M, Boivin M, Gagnon M, Lemoyne M, Nguyen B, Plourde V, Poitras S, Sahai A. Changing prevalence of *Helicobacter pylori* infection in endoscopically demonstrated duodenal ulcer. *Gut* 2000; **47** Suppl 1: A77-A78
35 **Li YY**, Hu PJ, Du GG, Hazell SL. The prevalence of *Helicobacter pylori* infection in the Peoples Republic of China. *Am J Gastroenterol* 1991; **86**: 446-449
36 **Azim Mirghani YA**, Ahmed S, Ahmed M, Ismail MO, Fedail SS, Kamel M, Saidia H. Detection of *Helicobacter pylori* in endoscopic biopsies in Sudan. *Trop Doct* 1994; **24**: 161-163
37 **Saita H**, Murakami M, Yoo JK, Teramura S, Dekigai H, Takahashi Y, Kita T. Link between *Helicobacter pylori*-associated gastritis and duodenal ulcer. *Dig Dis Sci* 1993; **38**: 117-122
38 **Uyub AM**, Raj SM, Visvanathan R, Nazim M, Aiyar S, Anuar AK, Mansur M. *Helicobacter pylori* infection in north-eastern peninsular Malaysia. Evidence for an unusually low prevalence. *Scand J Gastroenterol* 1994; **29**: 209-213
39 **Meucci G**, Di Battista R, Abbiati C, Benassi R, Bierti L, Bortoli A, Colombo E, Ferrara A, Prada A, Spinzi G, Venturelli R, de Franchis R. Prevalence and risk factors of *Helicobacter pylori*-negative peptic ulcer: a multicenter study. *J Clin Gastroenterol* 2000; **31**: 42-47
40 **Prasad S**, Mathan M, Chandy G, Rajan DP, Venkateswaran S, Ramakrishna BS, Mathan VI. Prevalence of *Helicobacter pylori* in southern Indian controls and patients with gastroduodenal disease. *J Gastroenterol Hepatol* 1994; **9:** 501-506
41 **Al-Saadi AM**, Al-Khayat JQ, Muhammad IM, Anwar SA. The role of *Helicobacter pylori* in esophagitis and peptic ulcer disease in Iraq. *Saudi Med J* 2004; **25**: 1216-1222
42 **Bakka AS**, El-Gariani AB, AbouGhrara FM, Salih BA. Frequency of *Helicobacter pylori* infection in dyspeptic patients in Libya. *Saudi Med J* 2002; **23**: 1261-1265
43 **Hu PJ**, Li YY, Zhou MH, Chen MH, Du GG, Huang BJ, Mitchell HM, Hazell SL. *Helicobacter pylori* associated with a high prevalence of duodenal ulcer disease and a low prevalence of gastric cancer in a developing nation. *Gut* 1995; **36**: 198-202
44 **Kate V**, Ananthakrishnan N, Badrinath S, Ratnakar C. Prevalence of *Helicobacter pylori* infection in disorders of the upper gastrointestinal tract in south India. *Natl Med J India* 1998; **11**: 5-8
45 **Kidd M**, Louw JA, Marks IN. *Helicobacter pylori* in Africa: observations on an 'enigma within an enigma'. *J Gastroenterol*

Hepatol 1999; **14**: 851-858
46 **Lachlan GW**, Gilmour HM, Jass JJ. Campylobacter pylori in central Africa. *Br Med J* (Clin Res Ed) 1988; **296**: 66
47 **Lee HR**, Han KS, Yoo BC, Park SM, Cha YJ. Prevalence of *Helicobacter pylori* infection in patients with peptic ulcer diseases and non-ulcer dyspepsia. *Korean J Intern Med* 1993; **8**: 73-77
48 **Nishikawa K**, Sugiyama T, Kato M, Ishizuka J, Komatsu Y, Kagaya H, Katagiri M, Nishikawa S, Hokari K, Takeda H, Asaka M. Non-*Helicobacter pylori* and non-NSAID peptic ulcer disease in the Japanese population. *Eur J Gastroenterol Hepatol* 2000; **12**: 635-640
49 **Ogutu EO**, Kang'ethe SK, Nyabola L, Nyong'o A. Endoscopic findings and prevalence of *Helicobacter pylori* in Kenyan patients with dyspepsia. *East Afr Med J* 1998; **75**: 85-89
50 **Rouvroy D**, Bogaerts J, Nsengiumwa O, Omar M, Versailles L, Haot J. Campylobacter pylori, gastritis, and peptic ulcer disease in central Africa. *Br Med J* (Clin Res Ed) 1987; **295**: 1174
51 **Ciociola AA**, McSorley DJ, Turner K, Sykes D, Palmer JB. *Helicobacter pylori* infection rates in duodenal ulcer patients in the United States may be lower than previously estimated. *Am J Gastroenterol* 1999; **94**: 1834-1840
52 **Henry A**, Batey RG. Low prevalence of *Helicobacter pylori* in an Australian duodenal ulcer population: NSAIDitis or the effect of ten years of H. pylori treatment? *Aust N Z J Med* 1998; **28**: 345
53 **Gisbert JR**, Blanco M, Mateos JM, Fernandez-Salazar L, Fernandez-Bernejo M, Cantero J, Pajares JM. H pylori-negative duodenal ulcer prevalence and causes in 74 patients. *Dig Dis Sci* 1999; **11**: 2295-2302
54 **Nensey YM**, Schubert TT, Bologna SD, Ma CK. *Helicobacter pylori*-negative duodenal ulcer. *Am J Med* 1991; **91**: 15-18
55 **Pilotto A**, Franceschi M, Costa MC, Di Mario F, Valerio G. *Helicobacter pylori* test-and-eradication strategy. *Lancet* 2000; **356**: 1683-1684
56 **Arents NL**, Thijs JC, van Zwet AA, Kleibeuker JH. Does the declining prevalence of *Helicobacter pylori* unmask patients with idiopathic peptic ulcer disease? Trends over an 8 year period. *Eur J Gastroenterol Hepatol* 2004; **16**: 779-783
57 **Kalaghchi B**, Mekasha G, Jack MA, Smoot DT. Ideology of *Helicobacter pylori* prevalence in peptic ulcer disease in an inner-city minority population. *J Clin Gastroenterol* 2004; **38**: 248-251
58 **Arroyo MT**, Forne M, de Argila CM, Feu F,Arenas J, de la Vega J, Garrigues V, Mora F, Castro M, Bujanda L, Cosme A, Castiella A, Gisbert JP, Hervas A, Lanas A. The prevalence of peptic ulcer not related to *Helicobacter pylori* or non-steroidal anti-inflammatory drug use is negligible in southern Europe. *Helicobacter* 2004; **9**: 249-254
59 **Sprung DJ**, Apter MN. What is the role of *Helicobacter pylori* in peptic ulcer and gastric cancer outside the big cities? *J Clin Gastroenterol* 1998; **26**: 60-63
60 **Sprung DJ**, Apter M, Allen B, Cook L, Allen B, Guarda L. The prevalence of *Helicobacter pylori* in duodenal ulcer disease. A community based study. *Am J Gastroenterol* 1996; **81**: A169
61 **Xia HH**, Phung N, Kalantar JS, Talley NJ. Demographic and endoscopic characteristics of patients with *Helicobacter pylori* positive and negative peptic ulcer disease. *Med J Aust* 2000; **173**: 515-519
62 **Gislason GT**, Emu B, Okolo 111 P, Pasncha PJ, Kalioo AN. Where have all the *Helicobacter pylori* gone? Etiologic factors in patients with duodenal ulcer presenting to a University Hospital. *Gastrointest Endosc* 1997; **45**: 263
63 R**aj SM**, Yap K, Haq JA, Singh S, Hamid A. Further evidence for an exceptionally low prevalence of *Helicobacter pylori* infection among peptic ulcer patients in north-eastern peninsular Malaysia. *Trans R Soc Trop Med Hyg* 2001; **95**: 24-27
64 **Bytzer P**, Aalykke C, Rune S, Weywadt L, Gjorup T, Eriksen J, Bonnevie O, Bekker C, Kromann-Andersen H, Kjaergaard J, Rask-Madsen J, Vilien M, Hansen J, Justesen T, Vyberg M, Teglbjaerg PS. Eradication of *Helicobacter pylori* compared with long-term acid suppression in duodenal ulcer disease. A randomized trial with 2-year follow-up. The Danish Ulcer Study Group. *Scand J Gastroenterol* 2000; **35**: 1023-1032
65 **Prach AT**, Malek M, Tavakoli M, Hopwood D, Senior BW, Murray FE. H2-antagonist maintenance therapy versus *Helicobacter pylori* eradication in patients with chronic duodenal ulcer disease: a prospective study. *Aliment Pharmacol Ther* 1998; **12**: 873-880
66 **Martin IG**, Diament RH, Dixon MF, Axon AT, Johnston D. *Helicobacter pylori* and recurrent ulceration after highly selective vagotomy. *Eur J Gastroenterol Hepatol* 1995; **7**: 207-209
67 **Svoboda P**, Krpensky A, Munzova H, Kunovska M. *Helicobacter pylori* after proximal selective vagotomy. *Vnitr Lek* 1991; **37**: 772-775
68 **Mitrokhina TV**, Fitilev SB, Grafskaia ND, Pavlova MV. Role of *Helicobacter pylori* in the etiology of duodenal ulcer recurrence after selective proximal vagotomy. *Khirurgiia* (Mosk) 1996: 39-42
69 **Kunzle JE**, Modena JL, Ziliotto Junior A, Mendes JA. *Helicobacter pylori* after surgery for duodenal ulcer. *Hepatogastroenterology* 1997; **44**: 599-603
70 **Huang WH**, Wang HH, Wu WW, Lai HC, Hsu CH, Cheng KS. *Helicobacter pylori* infection in patients with ulcer recurrence after partial gastrectomy. *Hepatogastroenterology* 2004; **51**: 1551-1553
71 **Archimandritis A**, Apostolopoulos P, Sougioultzis S, Delladetsima I, Davaris P, Tzivras M. The CLO test is unreliable in diagnosing *H. pylori* infection in post-surgical stomach; is there any role of *H. pylori* in peptic ulcer recurrence? *Eur J Gastroenterol Hepatol* 2000; **12**: 93-96
72 **Lee YT**, Sung JJ, Choi CL, Chan FK, Ng EK, Ching JY, Leung WK, Chung SC. Ulcer recurrence after gastric surgery: is *Helicobacter pylori* the culprit? *Am J Gastroenterol* 1998; **93**: 928-931
73 **Leivonen M**, Nordling S, Haglund C. The course of *Helicobacter pylori* infection after partial gastrectomy for peptic ulcer disease. Hepatogastroenterology 1998; **45**: 587-591
74 **Leivonen MK**, Haglund CH, Nordling SF. *Helicobacter pylori* infection after partial gastrectomy for peptic ulcer and its role in relapsing disease. *Eur J Gastroenterol Hepatol* 1997; **9**: 371-374
75 **Ludtke FE**, Maierhof S, Kohler H, Bauer FE, Tegeler R, Schauer A, Lepsien G. *Helicobacter pylori* colonization in surgical patients. *Chirurg* 1991; **62**: 732-738
76 **Miwa H**, Sakaki N, Sugano K, Sekine H, Higuchi K, Uemura N, Kato M, Murakami K, Kato C, Shiotani A, Ohkusa T, Takagi A, Aoyama N, Haruma K, Okazaki K, Kusugami K, Suzuki M, Joh T, Azuma T, Yanaka A, Suzuki H, Hashimoto H, Kawai T, Sugiyama T. Recurrent peptic ulcers in patients following successful *Helicobacter pylori* eradication: a multicenter study of 4940 patients. *Helicobacter* 2004; **9**: 9-16
77 **Louw JA**, Lucke W, Jaskiewicz K, Lastovica AJ, Winter TA, Marks IN. *Helicobacter pylori* eradication in the African setting, with special reference to reinfection and duodenal ulcer recurrence. *Gut* 1995; **36**: 544-547
78 **Martino G**, Paoletti M, Marcheggiano A, D`Ambra G, Delle Fave G, Annibale B. Duodenal ulcer relapse is not always associated with recurrence of *Helicobacter pylori* infection: a prospective 3 year follow-up study. *Helicobacter* 1999; **4**: 213-217
79 **Tepes B**, Kavcic B, Gubina M, Krizman I. A four-year follow-up of duodenal ulcer patients after *Helicobacter pylori* eradication. *Hepatogastroenterology* 1999; **46**: 1746-1750
80 **Marshall BJ**, Goodwin CS, Warren JR, Murray R, Blincow ED, Blackbourn SJ, Phillips M, Waters TE, Sanderson CR. Prospective double-blind trial of duodenal ulcer relapse after eradication of Campylobacter pylori. *Lancet* 1988; **2**: 1437-1442
81 **Laine L**, Hopkins RJ, Girardi LS. Has the impact of *Helicobacter pylori* therapy on ulcer recurrence in the United States been overstated? A meta-analysis of rigorously designed trials. *Am J Gastroenterol* 1998; **93**: 1409-1415
82 **Van der Hulst RW**, Rauws EA, Koycu B, Keller JJ, Bruno MJ, Tijssen JG, Tytgat GN. Prevention of ulcer recurrence after eradication of *Helicobacter pylori*: a prospective long-term follow-up study. *Gastroenterology* 1997; **113**: 1082-1086
83 **Bayerdorffer E**, Miehlke S, Mannes GA, Sommer A, Hochter W, Weingart J, Heldwein W, Klann H, Simon T, Schmitt W. Double-blind trial of omeprazole and amoxicillin to cure *Helicobacter pylori* infection in patients with duodenal ulcers.

Gastroenterology 1995; **108**: 1412-1417
84 **Fujioka T**, Uribe RU, Kubota T, Murakami K, Kawasaki H, Nasu M. Peptic ulcer recurrence after *Helicobacter pylori* eradication: a 5-year follow-up study. *Eur J Gastroenterol Hepatol* 1995; **7** Suppl 1: S35-S38
85 **Yang JC**, Chen WH, Wang JJ, Lin JJ, Wang TH. *Helicobacter pylori* infection and recurrence of duodenal ulceration. A prospective long-term follow- up study. *Gut* 1998; **43** Suppl 2: A97
86 **Huang JQ**, Chen Y, Wilkinson J, Hunt RH. Does initial choice of *Helicobacter pylori* treatment regime influence the recurrence rate of duodenal ulcer? A meta-analysis. *Gut* 1996; **43** Suppl 3: A142
87 **Hentschel E**, Brandstatter G, Dragosics B, Hirschl AM, Nemec H, Schutze K, Taufer M, Wurzer H. Effect of ranitidine and amoxicillin plus metronidazole on the eradication of *Helicobacter pylori* and the recurrence of duodenal ulcer. *N Engl J Med* 1993; **328**: 308-312
88 **Hildebrand P**, Bardhan P, Rossi L, Parvin S, Rahman A, Arefin MS, Hasan M, Ahmad MM, Glatz-Krieger K, Terracciano L, Bauerfeind P, Beglinger C, Gyr N, Klan AK. Recrudescence and reinfection with *Helicobacter pylori* after eradication therapy in Bangladeshi adults. *Gastroenterology* 2002; **123**: 653-654
89 **O'Morain C**, Dettmer A, Rambow A, von Fritsch E, Fraser AG. Double-blind, multicenter, placebo-controlled evaluation of clarithromycin and omeprazole for *Helicobacter pylori*-associated duodenal ulcer. *Helicobacter* 1996; **1**: 130-137
90 **Logan RP**, Bardhan KD, Celestin LR, Theodossi A, Palmer KR, Reed PI, Baron JH, Misiewicz JJ. Eradication of *Helicobacter pylori* and prevention of recurrence of duodenal ulcer: a randomised, double-blind, multi-centre trial of omeprazole with and without clarithromycin. *Aliment Pharmacol Ther* 1995; **9**: 417-423
91 **Rauws EA**, Tytgat GN. Cure of duodenal ulcer associated with eradication of *Helicobacter pylori*. *Lancet* 1990; **335**: 1233-1235
92 Hopkins RJ, Girardi LS, Turney EA. Relationship between *Helicobacter pylori* eradication and reduced duodenal and gastric ulcer recurrence: a review. *Gastroenterology* 1996; **110**: 1244-1252
93 **Coghlan JG**, Gilligan D, Humphries H, McKenna D, Dooley C, Sweeney E, Keane C, O'Morain C. Campylobacter pylori and recurrence of duodenal ulcers--a 12-month follow-up study. *Lancet* 1987; **2**: 1109-1111
94 **Penston JG**. Review article: *Helicobacter pylori* eradication--understandable caution but no excuse for inertia. *Aliment Pharmacol Ther* 1994; **8**: 369-389
95 **Ford A**, Delaney B, Moy Maayedi P. A systematic review of *Helicobacter pylori* eradication therapy in duodenal and gastric ulcer healing and maintenance. *Gut* 2003; **52** Suppl 1: A17-A18
96 **Xia HH**, Wong BC, Wong KW, Wong SY, Wong WM, Lai KC, Hu WH, Chan CK, Lam SK. Clinical and endoscopic characteristics of non-*Helicobacter pylori*, non-NSAID duodenal ulcers: a long-term prospective study. *Aliment Pharmacol Ther* 2001; **15**: 1875-1882
97 **Higuchi K**, Arakawa T, FujiwaraY, Uchida T, Tominaga K, Watanabe T, Kuroki T. Is *Helicobacter pylori*-negative duodenal ulcer masked by the high prevalence of *H. pylori infection* in the general population? *Am J Gastroenterol* 1999; **94**: 3083-3084
98 **Aoyama N**, Shinoda Y, Matsushima Y, Shirasaka D, Kimoshita Y, Kasuga M, Chiba T. *Helicobacter pylori*-negative peptic ulcer in Japan: which contributes most to peptic ulcer development, *Helicobacter pylori*, NSAIDs or stress? *J Gastroenterol* 2000; **35** Suppl 12: 63-67
99 **Vu C**, Ng YY. Prevalence of *Helicobacter pylori* in peptic ulcer disease in a Singapore hospital. *Singapore Med J* 2000; **41**: 478-481
100 **Kurata JH**, Nogawa AN. Meta-analysis of risk factors for peptic ulcer. Nonsteroidal antiinflammatory drugs, *Helicobacter pylori*, and smoking. *J Clin Gastroenterol* 1997; **24**: 2-17
101 **Kroupa R**, Dite P, Milnzova H, Husova L. Incidence of gastroduodenal ulcer *Helicobacter pylori* positive and negative in the years 1996-2000 in region of Czech Republic. *Gut* 2002; **51** Suppl 2: A63
102 **Gunay A**. Absence of *Helicobacter pylori* infection in patients with duodenal peptic ulcer. *Gut* 2002; **51** Suppl 2: A63
103 **Reinbach DH**, Cruickshank G, McColl KEL. Acute perforated duodenal ulcer is not associated with *Helicobacter pylori* infection. *Gut* 1993; **34**: 1344-1347
104 **Kate V**. Prevalence of *Helicobacter pylori* in normal controls and patients with upper alimentary disorders with special reference to complications of duodenal ulcer: a study in South India. PhD Thesis. 2000. Pondicherry University
105 **Sakaguchi M**, Oka H, Amemoto K, Honda M, Nakajima F, Kibi S, Lee K, Shimada M, Taniguchi K, Yamamoto N. Clinical investigation of perforated duodenal ulcer--with special reference to the presence of *Helicobacter pylori* infection and rate of recurrence. *Nihon Shokakibyo Gakkai Zasshi* 2002; **99**: 1197-1204
106 **Chowdhary SK**, Bhasin DK, Panigrahi D, Malik AK, Kataria RN, Behra A, Roy P, Singh K. *Helicobacter pylori* infection in patients with perforated duodenal ulcer. *Trop Gastroenterol* 1998; **19**: 19-21
107 **Matsukura N**, Onda M, Tokunaga A, Kato S, Yoshiyuki T, Hasegawa H, Yamashita K, Tomtitchong P, Hayashi A. Role of *Helicobacter pylori* infection in perforation of peptic ulcer: an age- and gender-matched case-control study. *J Clin Gastroenterol* 1997; **25** Suppl 1: S235-S239
108 **Sjostrom JE**, Larsson H. Factors affecting growth and antibiotic susceptibility of *Helicobacter pylori*: effect of pH and urea on the survival of a wild-type strain and a urease-deficient mutant. *J Med Microbiol* 1996; **44**: 425-433
109 **Dykhuizen RS**, Fraser A, McKenzie H, Golden M, Leifert C, Benjamin N. *Helicobacter pylori* is killed by nitrite under acidic conditions. *Gut* 1998; **42**: 334-337
110 **Presotto F**, Sabini B, Cecchetto A, Plebani M, De Lazzari F, Pedini B, Betterle C. *Helicobacter pylori* infection and gastric autoimmune diseases: is there a link? *Helicobacter* 2003; **8**: 578-584
111 **Djurkov VG**, Grudeva-Popova JG, Houbavenska IN. A study of *Helicobacter pylori* infection in patients with pernicious anemia. *Folia Med* (Plovdiv) 2000; **42**: 23-27
112 **Gutierrez O**, Sierra F, Gomez MC, Camargo F, Campylobacter pylori in chronic environmental gastritis and duodenal ulcer patients. *Gastroenterology* 1988; **94** Suppl 1: A163
113 **Jain A**, Buddhiraja S, Khurana B, Singhal R, Nair D, Arora P, Gangwal P, Mishra SK, Uppal B, Gondal R, Kar P. Risk factors for duodenal ulcer in north India. *Trop Gastroenterol* 1999; **20**: 36-39
114 **Chandrakumaran K**, Vaira D, Hobsley M. Duodenal ulcer, *Helicobacter pylori*, and gastric secretion. *Gut* 1994; **35**: 1033-1036
115 **Tabel G**, Hoa NT, Tarnawski A, Chen J, Domek M, Ma TY. *Helicobacter pylori* infection inhibits healing of the wounded duodenal epithelium in vitro. *J Lab Clin Med* 2003; **142**: 421-430
116 **el-Omar EM**, Penman ID, Ardill JE, Chittajallu RS, Howie C, McColl KE. *Helicobacter pylori* infection and abnormalities of acid secretion in patients with duodenal ulcer disease. *Gastroenterology* 1995; **109**: 681-691

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EDITORIAL

Mannose-binding lectin and maladies of the bowel and liver

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Abstract

Mannose-binding lectin (MBL) is a pattern-recognition molecule that binds to characteristic carbohydrate motifs present on the surface of many different pathogens. MBL binding stimulates the immune system *via* the lectin pathway of complement activation. In certain clinical situations, often characterized by pre-existing immune compromise, MBL deficiency increases the risk of infectious and other disease-specific complications. Many of the key pathogenic processes inherent to common gastroenterological diseases, such as infection, immunological damage, and carcinogenesis, have been linked to MBL. This editorial reviews the biology of MBL, outlines key disease associations to document the breadth of influence of MBL, and finally, highlights the relevance of MBL to both gastroenterological health and disease.

Key words: Mannose-binding lectin; Collectins; Innate immunity; Polymorphism; Infection

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INTRODUCTION

Mannose-binding lectin (MBL) is an important component of the innate immune system. MBL is primarily produced by the liver, circulates throughout the body, and is able to recognize a wide array of common pathogens through repeating carbohydrate sequences present on microbial surfaces. MBL binding of pathogens initiates complement activation via the lectin pathway. There have been a large number of studies addressing the influence of MBL deficiency on infection, autoimmunity, and carcinogenesis, all critical processes in the pathogenesis of gastrointestinal disease. Genetically determined MBL deficiency increases the risk and manifestations of a wide range of diseases, particularly when the immune system is already compromised. This editorial provides an introduction to the structure, function and regulation of MBL and explores its clinical relevance, placing it in the context of common medical and, in particular, gastrointestinal conditions.

THE BIOLOGY OF MANNOSE-BINDING LECTIN

The MBL2 Gene

The capacity of MBL to recognize and eradicate pathogens is extremely variable. Within any given population there are individuals that have varying functional levels of circulating MBL. The relative sufficiency of MBL function for any given individual is largely determined by polymorphisms within the *MBL2* gene, on chromosome 10. Three missense mutations within the first exon of *MBL2* significantly effect MBL function (codon 54 '*B*', codon 57 '*C*', and codon 52 '*D*') (Figure 1). These coding mutations are collectively designated '*O*', and the wild-type, sufficient allele is represented by '*A*'. An Australian study of healthy blood donors found that the prevalence of *MBL2* wild-type coding genotype *A/A* was 57.6%, coding mutation heterozygosity *(A/O)* was 34.8% (*A/D* 11%, *A/B* 19.9%, *A/C* 3.8%), and coding mutation homozygosity *(O/O)* was 7.6% (*B/B* 2.1%, *B/C* 2.1%, *B/D* 2.5%, *D/D* 0.9%)[1]. These frequencies are consistent with other Caucasoid populations[2-4]. In Asian communities, the most common mutation is also the *'B'* allele, but the '*D*' allele is virtually absent[5].

Further variability of MBL function is due, at least in part, to other polymorphisms within the promoter (position -550, G to C substitution, alleles '*H*' and '*L*' and position -221, G to C substitution, alleles '*X*' and '*Y*'); and 5'-untranslated (position +4, C to T, alleles '*P*' and '*Q*') regions of the gene (Figure 1). When inherited in the context of a normal coding allele (*A*), the promoter region haplotypes

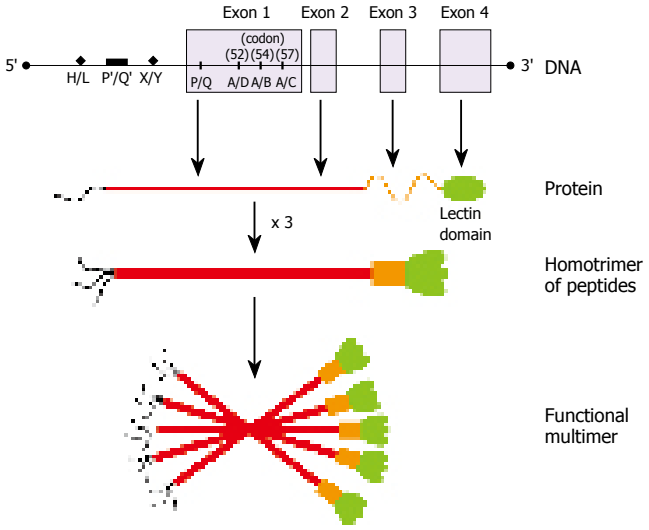

Figure 1 Schematic representation of the four exons of the *MBL2* gene, with the important polymorphisms identified. The peptides self associates into a homotrimer (structural subunit). Each peptide contains a lectin domain (green) to bind the specific, microbial carbohydrate motifs. Functional MBL circulates in higher-order multimers.

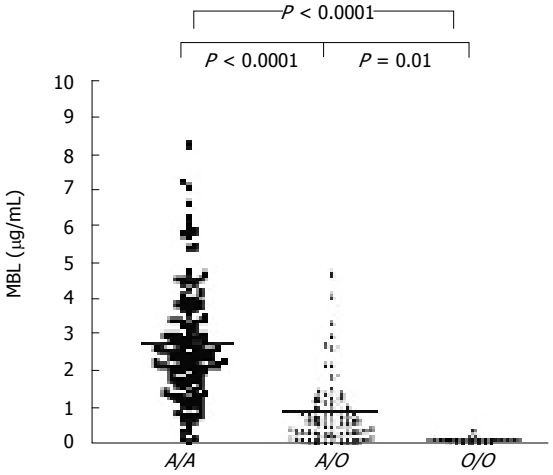

Figure 2 Distribution of plasma MBL levels within a normal population, stratified by *MBL2* genotype (Figure reproduced from Worthley *et al*[82] with permission). The *O/O* genotype is associated with the most extreme deficit in circulating MBL level (shown) and activity.

HY, *LY*, and *LX* are associated with high, intermediate, and low serum MBL concentrations, respectively. The genotypes *O/O, A/O* and *LXA/LXA*, are all associated with low antigenic and functional levels of MBL (compared to *A/A*). The *O/O* genotype is correlated with the most extreme MBL deficit (Figure 2). Low levels of MBL associated with the common polymorphic variants appear to result from impaired oligomerization of the MBL triple helix (see below) into functional higher order multimers[6], as well as increased susceptibility to degradation by metalloproteinases[7].

The MBL Protein

The basic structural subunit of MBL is a homotrimer of MBL peptides, entwined in a triple helix (Figure 1). Each peptide contains a lectin domain to bind the specific oligosaccharide motifs present on the surface of many different microorganisms[8]. Functional MBL circulates as a higher-order multimer (tetramers, pentamers and hexamers) of the basic MBL subunit. This oligomerization allows high-affinity interaction between MBL and the microorganism. Binding of MBL to pathogens causes a conformational change in the MBL multimer, and activation of associated molecules, the MBL-associated serine proteases (MASPs), that initiate the lectin-complement pathway.

The lectin-complement pathway

The enzymatic cascade of complement activation is a vital aspect of innate immunity. Complement-derived opsonization also provides an effective means of articulation with adaptive immunity through subsequent phagocytosis and antigen processing. The classical complement pathway is initiated by the binding of the C1 complex (C1q, r and s) to bound antibody on pathogen surfaces and the alternative pathway by binding of C3b to hydroxyl or amino groups on cell-surface molecules, as a result of spontaneous C3 turnover[9]. The lectin-complement pathway is the third arm of complement activation. Higher order MBL multimers circulate in a functional complex with three serine proteases MASP-1, MASP-2, MASP-3 and one non-protease molecule, MAp19[8]. This complex is analogous to the C1 complex that initiates the classical complement pathway, except that MBL binds to pathogens independently of antibody. Once activated, MASP-2, like its classical-pathway counterpart C1s, cleaves C4 to C4b, producing C4b2a, the C3 convertase. Subsequent production of C3b, also a key opsonin, generates the C5 convertase, which in turn produces the chemoattractant C5a, and, through C5b, the formation of the membrane-attack complex, C5b-C9 (Figure 3).

Recently, an additional mechanism of lectin-mediated complement activation, which bypasses the classical pathway proteins, has been described. Selander *et al*[10] demonstrated that an MBL-dependent alternative pathway mediated C3 deposition in C2 deficient serum. This bypass pathway may be of particular significance in the presence of complement deficiencies[11].

THE CLINICAL RELEVANCE OF MANNOSE-BINDING LECTIN

MBL binds a broad range of bacteria, viruses, fungi and protozoa (Table 1). Its affinity for Gram-negative and Gram-positive bacteria is mediated through cell surface components, such as lipopolysaccharide (endotoxin) and lipoteichoic acid, respectively. MBL deficiency increases *in vivo* susceptibility to many common bacterial infections, including *Neisseria meningitidis*[12], *Streptococcus pneumoniae*[13], and *Staphylococcus aureus*[14]. MBL deficiency may also increase the risk of several viral infections and some of the most compelling data in this area have been conducted in viral hepatitides, discussed below.

The balance of evidence suggests that MBL deficiency is most relevant when immunity is already compromised as a consequence of immunological immaturity, for example

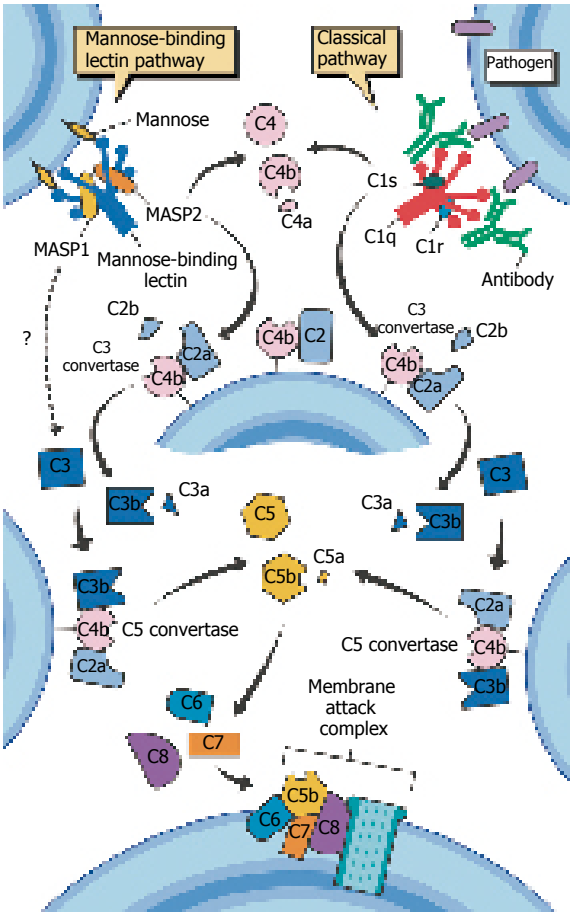

Figure 3 The lectin and classical complement pathways (Figure reproduced from Worthley *et al*[82] with permission).

Table 1 Some clinically relevant microorganisms recognized by MBL

Bacteria	Viruses	Fungi	Protozoa
Staphylococcus aureus	HIV-1 and 2	*Aspergillus fumigatus*	*Plasmodium falciparum*
Streptococcus pneumoniae	Herpes simplex 2	*Candida albicans*	*Cryptosporidium parvum*
Streptococcus pyogenes	Influenza A	*Cryptococcus neoformans*	*Trypanosoma cruzi*
Enterococcus spp.	Hepatitis B virus	*Saccharomyces cerevisiae*	
Listeria monocytogenes	Hepatits C virus		
Haemophilus influenzae			
Neisseria meningitidis			
Neisseria gonorrhoeae			
Escherichia coli			
Klebsiella spp.			
Pseudomonas aeruginosa			
Salmonella montevideo			
Salmonella typhimurium			
H pylori			
Chlamydia trachomatis			
Chlamydia pneumonia			
Proprionibacterium acnes			
Mycobacterium avium			
Mycobacterium tuberculosis			
Mycobacterium leprae			
Leishmania chagasi			

in young children[15], or is impaired by comorbidity or medical therapy, such as in cystic fibrosis[16], after chemotherapy[17,18], or following transplantation[19,20]. In the pediatric population, MBL exerts greatest influence during an immunological "window of vulnerability", between the decline in maternal passive immunity but before the development of a fully mature adaptive immune system. There is a strong association between MBL deficiency and childhood infection, which has been found for both milder respiratory tract infections managed within the community[21], as well as more severe infections requiring hospitalization[15]. In cystic fibrosis (CF), innate immunity is compromised in part by impaired mucociliary clearance and bronchiectasis. In one series of CF patients, those with mutant *MBL2* alleles had worse pulmonary function and shorter survival to end-stage CF[16]. The same investigators reported successful MBL replacement in the management of one patient with rapidly progressive CF[22]. Several studies have shown an association between MBL deficiency and risk or severity of infection following chemotherapy[17,18].

A number of autoimmune disorders are associated with MBL. This may in part relate to the role of MBL in removing pathogens and apoptotic bodies, thus minimizing the emergence of cross-reactivity or auto-immunogenic epitopes[23]. Inherited deficiencies within the classical complement pathway predispose to systemic lupus erythematosus (SLE), thus it was logical to evaluate the role of MBL in this condition. A recent meta-analysis concluded that deficient *MBL2* genotypes increase the risk of developing SLE[24]. Other studies have shown that MBL deficiency increases the risk of SLE-related complications, such as arterial thrombosis[25]. The effect of variant MBL and risk of vascular complications extend beyond patients with SLE. Several studies have now demonstrated an association between *MBL2* mutations and risk of coronary artery disease[26-28]. These results have been supported by a population-based study from Denmark, that genotyped 9 245 individuals for *MBL2* coding mutations[29]. Although MBL deficiency did not greatly increase the rate of morbidity or mortality within the population, those with biallelic mutations had a significantly greater risk of hospitalization for cardiovascular disease compared to those without deficient alleles [RR = 1.2 (1.0-1.4), $P = 0.02$][29].

To this point, all of the disease associations presented have identified the wild-type (*A*/*A*) *MBL2* gene as advantageous. The global preservation of *MBL2* -deficient haplotypes, however, hints at a selective advantage, at least under certain circumstances, of the deficient state. The concept of heterosis, whereby a heterozygous trait may demonstrate a selective advantage, has many well known examples, such as the ΔF508 mutation in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene and resistance to cholera toxin[30]. MBL-facilitated opsonization and phagocytosis could theoretically enhance the infectivity of some intracellular pathogens. The dichotomous nature of MBL deficiency is supported by several clinical

studies that show it to be protective against several obligate intracellular organisms, including *Mycobacterium leprae*[31], *M. tuberculosis*[32], and *Leishmania chagasi*[33].

Both plasma-derived as well as recombinant MBL are now available for therapeutic use, albeit that the indications for replacement are still evolving. The results from the first phase I trial conducted in healthy, MBL-deficient individuals, has been published[34]. This study confirmed that MBL replacement is a technically viable option. Phase II trials are eagerly awaited.

MALADIES OF THE BOWEL AND LIVER

Innate immunity has developed multiple strategies for protecting us against microbiological threats. Pattern-recognition molecules, such as toll-like receptors (TLR), *NOD2/CARD15*, and MBL, are particularly important in the alimentary tract, characterized by its large surface area and intimate relationship to the bowel contents, particularly the extreme microbial burden found within the colonic lumen. In addition to initiating inflammation, the capacity for immune tolerance is critical for normal bowel function. Although it is clear that the liver is the chief contributor to plasma MBL, mucosal MBL production may be relevant in localized immune defence, particularly within the alimentary tract[35]. The following examples present some of the better developed areas of gastroenterological MBL research, including inflammatory bowel disease, carcinogenesis, gastrointestinal and hepatotropic infection, and chronic liver disease.

MBL and inflammatory bowel disease

Inflammatory bowel disease (IBD) is a pathological spectrum encompassing ulcerative colitis (UC), Crohn's disease (CD), and indeterminate colitis. The resultant IBD phenotype is the consequence of multiple interactions between environmental factors, particularly enteric flora, and the host response to this environment, determined by immunogenetic, epithelial, and other non-immune genetic factors[36]. MBL, as an important component of innate immunity, has engendered considerable research interest. In an early study of 340 unrelated patients with IBD genotyped for *MBL2* exon 1 coding mutations, the frequency of deficient alleles was significantly lower in patients with UC than either the control group ($P = 0.02$), or those with CD ($P = 0.01$)[37]. This study suggests that MBL deficiency could be protective against UC; alternatively, it could be interpreted that MBL deficiency, in individuals otherwise predisposed to IBD, may skew the phenotype away from the UC spectrum of disease towards CD. This concept is supported by another study that genotyped *MBL2* in patients with CD, UC, or healthy controls[38]. In this study the allele frequency of coding mutations was approximately 30% in patients with CD, 8% in UC, and 16% in healthy controls. In addition, the frequency of homozygosity or compound heterozygosity for coding mutations (i.e. *O/O MBL2* genotype) within the IBD group was significantly higher than in the healthy control population and the association was strengthened if the small number of UC patients were excluded from the analysis (16% *vs* 0%; $P = 0.05$)[38]. The study also assessed anti-*Saccharomyces cerevisiae* antibody (ASCA) and MBL levels within the same subsets of patients, albeit slightly different numbers within each group[38]. CD patients with MBL deficiency were significantly more likely to be positive for ASCA and for their lymphocytes to proliferate in response to mannan. Thus, it appears that MBL deficiency could impair normal processing of mannan-expressing microbial antigens, such as those found on the cell surface of many common microorganisms. The accumulated antigens could then stimulate the immune system, and contribute to the production of ASCA and possibly the pathogenesis of Crohn's disease[38]. ASCA is a well established phenotypic marker of IBD, tending to aggregate with Crohn's rather than the ulcerative colitis phenotype, and within CD the presence of ASCA is particularly associated with the fibrostenosing phenotype and ileal inflammation[39]. Thus, MBL deficiency might act primarily to influence IBD-specific phenotype in these patients. It should be noted, however, that a follow-up study, testing a larger cohort of CD patients ($n = 241$), failed to confirm the significant association between variant MBL genotypes and ASCA positivity[40]. The observed trend, however, did show that the frequency of ASCA positivity was proportional to the relative deficiency of the coding genotype, with 54% ASCA positivity for *A/A,* 58% for *A/O,* and 67% for *O/O*[40]. Nevertheless, further studies into the role of MBL as a marker or regulator of IBD phenotype are warranted. Finally, the contribution of *M. avium* subspecies *paratuberculosis* to the pathogenesis of Crohn's disease is controversial[41]. Nevertheless, it would be interesting to investigate whether, in a fashion analogous to *M. tuberculosis,* MBL might predispose to Crohn's disease by facilitating the infectivity of this obligate intracellular pathogen.

MBL and coeliac disease

Coeliac disease is an important autoimmune disorder involving the alimentary tract[42]. The majority of patients with coeliac disease express the major histocompatability complex (MHC) molecule DQ2 and the remainder usually carry DQ8[42]. But HLA genes convey only about 40% of the genetic risk, and although 30%-40% of Caucasians carry DQ2 or DQ8, less than 3% of these will develop coeliac disease[42]. In one study, 117 patients with histologically and serologically confirmed coeliac disease were genotyped for *MBL2* exon 1 mutations, and compared to a healthy blood donor population. There was a significant difference in the frequency of the *O/O* genotype between those with coeliac disease (13%) and the control group (5%) ($P = 0.04$)[43]. A follow-up study included a detailed assessment of the coeliac disease patients' MHC[23]. HLA susceptibility alleles and *MBL2* exon 1 coding mutations were genotyped in 147 healthy controls and 149 patients with coeliac disease, enriched with 29 coeliac disease patients known to be negative for DQ2 and DQ8, which is extremely rare[23]. As in their first study, patients with coeliac disease had a greater frequency of the *O/O* genotype than healthy controls, but in addition, the association between coeliac disease and MBL deficiency was even stronger in the small number of patients negative for DQ2 and DQ8.

It is likely that in those rare cases of coeliac disease that are negative for DQ2 and DQ8, the non-HLA susceptibility genotypes would exert a greater effect. Their study also analyzed apoptosis within small intestinal biopsy specimens, and showed that MBL tended to aggregate to areas of apoptosis within the epithelium. MBL has been implicated in the normal clearance of apoptotic bodies[44,45]. The authors postulated that the association between MBL and coeliac disease, and indeed other autoimmune conditions, could relate to impaired apoptosis, whereby MBL deficiency impairs the normal removal and clearance of apoptotic cells, that may subsequently reveal previously hidden self-antigen, causing loss of self-tolerance, and spreading of autoimmunity[23]. The association between variant *MBL2* alleles and coeliac disease has also been confirmed within the Finnish population[46].

MBL and colorectal cancer

Experimentally there is the suggestion that MBL (both wild-type and the mutant *B* allele) may possess anti-colorectal cancer tumour activity[47]. *In vitro* MBL binds specifically to oligosaccharide moieties on colorectal cancer cell line SW1116[47]. The investigators transplanted SW1116 cells subcutaneously in nude mice, resulting in palpable tumor masses at three weeks. In order to evaluate the *in vivo* anti-tumoral activity of MBL, the mice were administered one of four different intra-tumoral injections. The first group received an injection with vaccinia virus carrying the wild-type (*'A'*) *MBL2* allele, the second group with the variant *'B'* allele and the two control groups received vaccinia virus alone, or saline alone. Intra-tumoral administration of the recombinant vaccinia virus carrying a *MBL2* gene (either the *'A'* or *'B'* allele) significantly reduced tumor size as compared with the two control groups ($P < 0.005$), and also prolonged survival[47]. These laboratory results have not, however, been reflected in clinical trials. In fact, patients with colorectal cancer have increased activation of the lectin-complement pathway and increased levels of serum MBL[48]. In patients undergoing surgery for colorectal cancer, however, low preoperative levels of serum MBL has been linked to an increased risk of developing post-colectomy pneumonia[49]. Most recently, increased preoperative serum levels of MASP2 predicted adverse outcome following colorectal cancer surgery, both in terms of disease recurrence ($P = 0.03$; HR = 1.4, 1.0-2.0) and survival ($P = 0.0005$; HR = 1.4, 1.2-1.7)[50]. There are several possible explanations for these results. A preoperative elevation in acute phase markers, such as CRP, is known to predict worse outcome[51], and the elevation in MASP2 may simply reflect a heightened inflammatory state. Alternatively, MASP2 may meaningfully influence tumor progression. Further studies are required to clarify the role of the lectin-complement pathway in cancer.

MBL and gastrointestinal infection

Despite the well-established role of MBL in innate immunity, there have been relatively few studies detailing the clinical effect of MBL deficiency in enteric infections. One notable exception analyzed the association between MBL deficiency and risk of *Cryptosporidium parvum* enteritis. This study included 72 African patients with acquired immunodeficiency syndrome (AIDS) and diarrhea. They were genotyped for exon 1 *MBL2* mutations and had their duodenal aspirates tested for MBL. Patients with biallelic coding mutations (*O/O)* had a significantly greater chance of cryptosporidiosis compared to those who were either wild-type or heterozygous for *MBL2* mutation (*A/A or A/O,* respectively) (OR = 8.2; 95% CI: 1.5-42; $P = 0.02$)[52]. This study places MBL's anti-microbial function back in the context of the 'window of vulnerability' hypothesis. Of further interest from this study was the detection of MBL within some of the duodenal aspirates. The presence of albumin in the intestinal lumen led the authors to postulate that MBL entered the bowel through mucosal leakage of serum; however, local intestinal production could not be excluded. The association between MBL deficiency and cryptosporidiosis was recently confirmed in a second case-control study, this time in young (< 3 year) Haitian children. Mean serum MBL levels were significantly lower in the cases (1110 *vs* 2395 ng/mL, $P = 0.002$), and 37% of the cases compared to only 10% of the healthy controls were found to be deficient in MBL (level $\leqslant$ 70 ng/mL) ($P = 0.005$)[53]. Unlike the earlier study, *MBL2* genotyping was not performed, and thus MBL deficiency secondary to enteric protein loss, as a consequence of cryptosporidiosis, could not be excluded. When considered together, however, these two studies present compelling evidence for the role of MBL in the host defense against *Cryptosporidium spp.* infection.

Another study analyzed serum MBL levels in a pediatric population presenting with *Escherichia coli* 0157: H7 colitis. MBL levels were measured in patients with uncomplicated *E. coli* 0157:H7 colitis, patients in whom the colitis was complicated by haemolytic uraemic syndrome (HUS), and in normal and disease (rotavirus enteritis) control groups[54]. MBL deficiency was not associated with an increased risk of either infection nor the complication of HUS, albeit that without analysis of *MBL2* genotype, overall MBL status may be more difficult to assess.

H pylori is one of the most common human bacterial infections, affecting approximately 50% of humans, although only 10%-20% of those affected will develop a clinical disorder[55]. Several immunogenetic polymorphisms are associated with clinical outcomes in *H pylori* infection[56], as well as with the risk of infection itself[57,58]. *H pylori* activates MBL *in vitro*[59], and a recent study demonstrated that *H pylori*-related chronic gastritis causes an increase in gastric mucosal MBL expression, but no association was found between *MBL2* genotype and risk of chronic gastritis[60]. A recent study was performed to investigate whether MBL deficiency increased the risk of *H pylori* infection[61]. Two normal populations (166 blood donors and 108 stem cell donors) were included in the analysis. All individuals were genotyped for *MBL2,* had their peripheral MBL activity characterized by level and functional assays, and were tested for serological evidence of *H pylori* infection. In this study, MBL deficiency did not increase the risk of *H pylori* infection, and in one population greater MBL activity actually increased the risk of infection.

It is worth noting that MBL has been implicated in

mediating gastrointestinal ischemia/reperfusion injury in mice[62]. MBL-null mice (deficient in the two murine genes encoding MBL) developed only minor gut injury after induced ischemia/reperfusion insult compared to the wild-type mice. MBL has been implicated as a mediator of ischemia/reperfusion injury in both the myocardium[63] and the kidney[64] and thus clinical correlation of MBL status and risk or outcome following mesenteric ischemia may yield interesting results.

MBL and viral hepatitides

MBL was first isolated from hepatocytes[65], and the liver produces most if not all of the circulating MBL[20]. There is obviously considerable functional reserve in hepatic MBL production, because in the setting of cirrhosis, unlike many other hepatic proteins, MBL production appears to be increased[66]. The viral hepatitides have stimulated considerable MBL-related research. In one study of chronic hepatitis B virus (HBV) infection, MBL codon 54 (*B*) mutations were significantly associated with risk of developing both symptomatic cirrhosis and spontaneous bacterial peritonitis (SBP)[67]. The increased risk of SBP in patients with MBL deficiency is biologically plausible, given that low levels of ascitic fluid opsonins are important in the pathogenesis of SBP, and MBL deficiency would be likely to compound this deficit[68]. A second study by the same investigators recently confirmed the association, extending the results from their previous study, to include the low expression haplotype *XA* as a risk factor, as well as the '*B*' allele. The odds ratio for developing cirrhosis and hepatocellular carcinoma was 1.97 for patients with *XA* and 1.90 for those with *YB* ($P = 0.002$)[69]. A study from the U.S. confirmed these findings, which is important given that the age and route of acquisition of HBV may vary between different countries[70]. It is likely that MBL plays an important role in the pathogenesis of HBV-related chronic disease, even though some small studies have failed to confirm the association[71,72]. It will be interesting to examine the influence of MBL status upon the rate or type of drug resistance that emerges in individuals during long-term antiviral therapy.

Many of the studies analyzing MBL in chronic hepatitis C virus (HCV) infection have investigated the role of MBL mutations on rate of sustained viral response following interferon alpha (IFN-α) monotherapy. Two studies, from the same Japanese group, reported that patients who failed to eradicate HCV following IFN monotherapy were more likely to have variant *MBL2* alleles, either the '*B*' coding mutation[73,74] or the '*LXPA*' haplotype[73]. A third Japanese study addressed whether MBL deficiency altered the course of HCV chronic liver disease[75]. In their cross-sectional study, 52 patients with chronic HCV and 50 controls were genotyped for the '*B*' coding mutation in exon 1 of *MBL2*. All patients with HCV had the stage and activity of their liver disease categorized as "chronic inactive hepatitis", "chronic active hepatitis" (CAH), or cirrhosis. No significant differences in the frequency of mutations was found between the patients and the controls, but within the HCV-infected group, all of the patients with heterozygous or homozygous codon 54 mutations had either CAH or cirrhosis, whilst none of those in the "chronic inactive hepatitis" group had mutations. This represented a significantly higher frequency of mutation in the advanced (CAH plus cirrhosis) liver disease group ($P = 0.0405$)[75]. A final study from Scotland sought to test the Japanese findings in a Caucasoid population[76]. This study failed to find an association between MBL deficiency and either progression of liver disease or response to IFN-α therapy. This study, however, did not perform *MBL2* genotyping, but stratified MBL concentrations into four groupings for comparison. On balance, there appears to be an association between MBL status and HCV in terms of both disease progression and response to monotherapy, at least in the Japanese population. The development of newer anti-viral treatment regimens, including pegylated-IFN in combination with ribavirin treatment, makes it necessary to re-evaluate immunogenetic influences, at least, any which are hoped to inform therapy.

MBL and liver transplantation

One of the more exciting recent reports regarding MBL and hepatobiliary disease, addressed the role of MBL deficiency following orthotopic liver transplantation (OLT)[20]. OLT provides the unique opportunity not only to evaluate the role of MBL in post-transplant infection, but also to assess the contribution of hepatic and extra-hepatic MBL production, because in many recipients the *MBL2* genotype will be different in the liver. The study reported the clinical results from 49 transplants, in which 49 of the donors were genotyped for *MBL2*, 25 of the recipients were genotyped, and serum samples were collected from 25 of the recipients to evaluate the change in serum MBL concentration post-transplant. There was an impressive correlation between the risk of post-transplant clinically significant infection and the relative deficiency of the donor *MBL2* genotype, with infection occurring in 12% receiving a wild-type *A/A* liver, 39% of those receiving an *A/O* liver, and in 67% of those receiving an *O/O* donor liver ($P = 0.01$)[20]. In addition, the post-transplantation serum MBL level was predicted by the hepatic, not the extra-hepatic, genotype. Nevertheless, as only 25 of the recipients, and thus only 25 extra-hepatic genotypes were analyzed, this study was too small to detect more subtle changes in the risk of infection, conferred through local, extra-hepatic MBL production[77].

MBL and hepatic synthesis

The site of MBL production has been a contentious area. Undoubtedly, the liver produces the majority of MBL and most if not all of the circulating MBL within peripheral blood. This view is supported by the liver transplantation study above, as well as a report documenting that successful allo-SCT failed to correct peripheral blood MBL deficiency[20,78]. Nevertheless, MBL mRNA is expressed in extra-hepatic tissue[35,79,80] possibly including haemopoietic lineages[35], and there have now been two allo-SCT studies that support a contribution from donor (i.e. hematopoietic) *MBL2* genotype, and risk of post-transplant infection[4,81]. The most important issue is not whether extra-hepatic MBL production significantly influences peripheral

blood MBL levels, but whether it contributes in a clinically meaningful way to local, tissue-specific immunity.

CONCLUSION

MBL has stimulated a great deal of basic and clinical gastroenterological research and has provided new insights to the pathogenesis of infectious and immune disorders within the bowel and liver. The possibility of a local, mucosal effect of MBL as suggested by gene expression[35,79,80], as well as clinical studies[23,60] is an exciting discovery. Further work is needed to clarify whether mucosal production occurs, and if so whether it contributes to local immune surveillance in health, or under certain situations, even exacerbates alimentary tract disease.

Despite the promise of replacement therapy and the value of MBL in predicting the risk of disease and disease-specific complications, for now investigation of MBL status remains primarily a research tool. Future studies more rigorously examining MBL status by both measurement of MBL levels and *MBL2* genotyping in large patient cohorts will help clarify the most important disease associations and identify those clinical settings in which MBL replacement therapy is most likely to be beneficial. Now that MBL replacement has been shown to be feasible, the first trials of MBL replacement therapy in several clinical settings, including recurrent infection, severe sepsis, and liver transplantation, are likely to be reported in the next few years. This prospect of MBL replacement therapy represents the culmination of several decades of basic and translational research and is an exciting advance in the field of innate immunity.

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REFERENCES

1 **Minchinton RM**, Dean MM, Clark TR, Heatley S, Mullighan CG. Analysis of the relationship between mannose-binding lectin (MBL) genotype, MBL levels and function in an Australian blood donor population. *Scand J Immunol* 2002; **56**: 630-641
2 **Madsen HO**, Garred P, Thiel S, Kurtzhals JA, Lamm LU, Ryder LP, Svejgaard A. Interplay between promoter and structural gene variants control basal serum level of mannan-binding protein. *J Immunol* 1995; **155**: 3013-3020
3 **Mullighan CG**, Heatley S, Bardy PG, Lester S, Rischmueller M, Gordon TP. Lack of association between mannose-binding lectin gene polymorphisms and primary Sjogren's syndrome. *Arthritis Rheum* 2000; **43**: 2851-2852
4 **Mullighan CG**, Heatley S, Doherty K, Szabo F, Grigg A, Hughes TP, Schwarer AP, Szer J, Tait BD, Bik To L, Bardy PG. Mannose-binding lectin gene polymorphisms are associated with major infection following allogeneic hemopoietic stem cell transplantation. *Blood* 2002; **99**: 3524-3529
5 **Ip WK**, To YF, Cheng SK, Lau YL. Serum mannose-binding lectin levels and mbl2 gene polymorphisms in different age and gender groups of southern Chinese adults. *Scand J Immunol* 2004; **59**: 310-314
6 **Larsen F**, Madsen HO, Sim RB, Koch C, Garred P. Disease-associated mutations in human mannose-binding lectin compromise oligomerization and activity of the final protein. *J Biol Chem* 2004; **279**: 21302-21311
7 **Butler GS**, Sim D, Tam E, Devine D, Overall CM. Mannose-binding lectin (MBL) mutants are susceptible to matrix metalloproteinase proteolysis: potential role in human MBL deficiency. *J Biol Chem* 2002; **277**: 17511-17519
8 **Holmskov U**, Thiel S, Jensenius JC. Collections and ficolins: humoral lectins of the innate immune defense. *Annu Rev Immunol* 2003; **21**: 547-578
9 **Medzhitov R**, Janeway C Jr. Innate immunity. *N Engl J Med* 2000; **343**: 338-344
10 **Selander B**, Martensson U, Weintraub A, Holmstrom E, Matsushita M, Thiel S, Jensenius JC, Truedsson L, Sjoholm AG. Mannan-binding lectin activates C3 and the alternative complement pathway without involvement of C2. *J Clin Invest* 2006; **116**: 1425-1434
11 **Atkinson JP**, Frank MM. Bypassing complement: evolutionary lessons and future implications. *J Clin Invest* 2006; **116**: 1215-1218
12 **Bax WA**, Cluysenaer OJ, Bartelink AK, Aerts PC, Ezekowitz RA, van Dijk H. Association of familial deficiency of mannose-binding lectin and meningococcal disease. *Lancet* 1999; **354**: 1094-1095
13 **Roy S**, Knox K, Segal S, Griffiths D, Moore CE, Welsh KI, Smarason A, Day NP, McPheat WL, Crook DW, Hill AV. MBL genotype and risk of invasive pneumococcal disease: a case-control study. *Lancet* 2002; **359**: 1569-1573
14 **Shi L**, Takahashi K, Dundee J, Shahroor-Karni S, Thiel S, Jensenius JC, Gad F, Hamblin MR, Sastry KN, Ezekowitz RA. Mannose-binding lectin-deficient mice are susceptible to infection with Staphylococcus aureus. *J Exp Med* 2004; **199**: 1379-1390
15 **Summerfield JA**, Sumiya M, Levin M, Turner MW. Association of mutations in mannose binding protein gene with childhood infection in consecutive hospital series. *BMJ* 1997; **314**: 1229-1232
16 **Garred P**, Pressler T, Madsen HO, Frederiksen B, Svejgaard A, Hoiby N, Schwartz M, Koch C. Association of mannose-binding lectin gene heterogeneity with severity of lung disease and survival in cystic fibrosis. *J Clin Invest* 1999; **104**: 431-437
17 **Neth O**, Hann I, Turner MW, Klein NJ. Deficiency of mannose-binding lectin and burden of infection in children with malignancy: a prospective study. *Lancet* 2001; **358**: 614-618
18 **Peterslund NA**, Koch C, Jensenius JC, Thiel S. Association between deficiency of mannose-binding lectin and severe infections after chemotherapy. *Lancet* 2001; **358**: 637-638
19 **Mullighan CG**, Bardy PG. Mannose-binding lectin and infection following allogeneic hemopoietic stem cell transplantation. *Leuk Lymphoma* 2004; **45**: 247-256
20 **Bouwman LH**, Roos A, Terpstra OT, de Knijff P, van Hoek B, Verspaget HW, Berger SP, Daha MR, Frolich M, van der Slik AR, Doxiadis II, Roep BO, Schaapherder AF. Mannose binding lectin gene polymorphisms confer a major risk for severe infections after liver transplantation. *Gastroenterology* 2005; **129**: 408-414
21 **Koch A**, Melbye M, Sorensen P, Homoe P, Madsen HO, Molbak K, Hansen CH, Andersen LH, Hahn GW, Garred P. Acute respiratory tract infections and mannose-binding lectin insufficiency during early childhood. *JAMA* 2001; **285**: 1316-1321
22 **Garred P**, Pressler T, Lanng S, Madsen HO, Moser C, Laursen I, Balstrup F, Koch C, Koch C. Mannose-binding lectin (MBL) therapy in an MBL-deficient patient with severe cystic fibrosis lung disease. *Pediatr Pulmonol* 2002; **33**: 201-207
23 **Boniotto M**, Braida L, Baldas V, Not T, Ventura A, Vatta S, Radillo O, Tedesco F, Percopo S, Montico M, Amoroso A, Crovella S. Evidence of a correlation between mannose binding lectin and celiac disease: a model for other autoimmune diseases. *J Mol Med* (Berl) 2005; **83**: 308-315
24 **Lee YH**, Witte T, Momot T, Schmidt RE, Kaufman KM, Harley JB, Sestak AL. The mannose-binding lectin gene polymorphisms and systemic lupus erythematosus: two case-control studies and a meta-analysis. *Arthritis Rheum* 2005; **52**: 3966-3974
25 **Ohlenschlaeger T**, Garred P, Madsen HO, Jacobsen S.

Mannose-binding lectin variant alleles and the risk of arterial thrombosis in systemic lupus erythematosus. *N Engl J Med* 2004; **351**: 260-267
26 **Madsen HO**, Videm V, Svejgaard A, Svennevig JL, Garred P. Association of mannose-binding-lectin deficiency with severe atherosclerosis. *Lancet* 1998; **352**: 959-960
27 **Rugonfalvi-Kiss S**, Endresz V, Madsen HO, Burian K, Duba J, Prohaszka Z, Karadi I, Romics L, Gonczol E, Fust G, Garred P. Association of Chlamydia pneumoniae with coronary artery disease and its progression is dependent on the modifying effect of mannose-binding lectin. *Circulation* 2002; **106**: 1071-1076
28 **Best LG**, Davidson M, North KE, MacCluer JW, Zhang Y, Lee ET, Howard BV, DeCroo S, Ferrell RE. Prospective analysis of mannose-binding lectin genotypes and coronary artery disease in American Indians: the Strong Heart Study. *Circulation* 2004; **109**: 471-475
29 **Dahl M**, Tybjaerg-Hansen A, Schnohr P, Nordestgaard BG. A population-based study of morbidity and mortality in mannose-binding lectin deficiency. *J Exp Med* 2004; **199**: 1391-1399
30 **Gabriel SE**, Brigman KN, Koller BH, Boucher RC, Stutts MJ. Cystic fibrosis heterozygote resistance to cholera toxin in the cystic fibrosis mouse model. *Science* 1994; **266**: 107-109
31 **Garred P**, Harboe M, Oettinger T, Koch C, Svejgaard A. Dual role of mannan-binding protein in infections: another case of heterosis? *Eur J Immunogenet* 1994; **21**: 125-131
32 **Soborg C**, Madsen HO, Andersen AB, Lillebaek T, Kok-Jensen A, Garred P. Mannose-binding lectin polymorphisms in clinical tuberculosis. *J Infect Dis* 2003; **188**: 777-782
33 **Santos IK**, Costa CH, Krieger H, Feitosa MF, Zurakowski D, Fardin B, Gomes RB, Weiner DL, Harn DA, Ezekowitz RA, Epstein JE. Mannan-binding lectin enhances susceptibility to visceral leishmaniasis. *Infect Immun* 2001; **69**: 5212-5215
34 **Valdimarsson H**, Vikingsdottir T, Bang P, Saevarsdottir S, Gudjonsson JE, Oskarsson O, Christiansen M, Blou L, Laursen I, Koch C. Human plasma-derived mannose-binding lectin: a phase I safety and pharmacokinetic study. *Scand J Immunol* 2004; **59**: 97-102
35 **Seyfarth J**, Garred P, Madsen HO. Extra-hepatic transcription of the human mannose-binding lectin gene (mbl2) and the MBL-associated serine protease 1-3 genes. *Mol Immunol* 2006; **43**: 962-971
36 **Lakatos PL**, Fischer S, Lakatos L, Gal I, Papp J. Current concept on the pathogenesis of inflammatory bowel disease-crosstalk between genetic and microbial factors: pathogenic bacteria and altered bacterial sensing or changes in mucosal integrity take "toll" ? *World J Gastroenterol* 2006; **12**: 1829-1841
37 **Rector A**, Lemey P, Laffut W, Keyaerts E, Struyf F, Wollants E, Vermeire S, Rutgeerts P, Van Ranst M. Mannan-binding lectin (MBL) gene polymorphisms in ulcerative colitis and Crohn's disease. *Genes Immun* 2001; **2**: 323-328
38 **Seibold F**, Konrad A, Flogerzi B, Seibold-Schmid B, Arni S, Juliger S, Kun JF. Genetic variants of the mannan-binding lectin are associated with immune reactivity to mannans in Crohn's disease. *Gastroenterology* 2004; **127**: 1076-1084
39 **Bossuyt X**. Serologic markers in inflammatory bowel disease. *Clin Chem* 2006; **52**: 171-181
40 **Joossens S**, Pierik M, Rector A, Vermeire S, Ranst MV, Rutgeerts P, Bossuyt X. Mannan binding lectin (MBL) gene polymorphisms are not associated with anti-Saccharomyces cerevisiae (ASCA) in patients with Crohn's disease. *Gut* 2006; **55**: 746
41 **Naser SA**, Ghobrial G, Romero C, Valentine JF. Culture of Mycobacterium avium subspecies paratuberculosis from the blood of patients with Crohn's disease. *Lancet* 2004; **364**: 1039-1044
42 **Green PH**, Jabri B. Celiac disease. *Annu Rev Med* 2006; **57**: 207-221
43 **Boniotto M**, Braida L, Spano A, Pirulli D, Baldas V, Trevisiol C, Not T, Tommasini A, Amoroso A, Crovella S. Variant mannose-binding lectin alleles are associated with celiac disease. *Immunogenetics* 2002; **54**: 596-598
44 **Nauta AJ**, Raaschou-Jensen N, Roos A, Daha MR, Madsen HO, Borrias-Essers MC, Ryder LP, Koch C, Garred P. Mannose-binding lectin engagement with late apoptotic and necrotic cells. *Eur J Immunol* 2003; **33**: 2853-2863
45 **Ogden CA**, deCathelineau A, Hoffmann PR, Bratton D, Ghebrehiwet B, Fadok VA, Henson PM. C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells. *J Exp Med* 2001; **194**: 781-795
46 **Iltanen S**, Maki M, Collin P, Mustalahti K, Kaukinen K, Partanen J, Hulkkonen J, Hurme M, Aittoniemi J. The association between mannan-binding lectin gene alleles and celiac disease. *Am J Gastroenterol* 2003; **98**: 2808-2809
47 **Ma Y**, Uemura K, Oka S, Kozutsumi Y, Kawasaki N, Kawasaki T. Antitumor activity of mannan-binding protein in vivo as revealed by a virus expression system: mannan-binding proteindependent cell-mediated cytotoxicity. *Proc Natl Acad Sci USA* 1999; **96**: 371-375
48 **Ytting H**, Jensenius JC, Christensen IJ, Thiel S, Nielsen HJ. Increased activity of the mannan-binding lectin complement activation pathway in patients with colorectal cancer. *Scand J Gastroenterol* 2004; **39**: 674-679
49 **Ytting H**, Christensen IJ, Jensenius JC, Thiel S, Nielsen HJ. Preoperative mannan-binding lectin pathway and prognosis in colorectal cancer. *Cancer Immunol Immunother* 2005; **54**: 265-272
50 **Ytting H**, Christensen IJ, Thiel S, Jensenius JC, Nielsen HJ. Serum mannan-binding lectin-associated serine protease 2 levels in colorectal cancer: relation to recurrence and mortality. *Clin Cancer Res* 2005; **11**: 1441-1446
51 **Nozoe T**, Matsumata T, Kitamura M, Sugimachi K. Significance of preoperative elevation of serum C-reactive protein as an indicator for prognosis in colorectal cancer. *Am J Surg* 1998; **176**: 335-338
52 **Kelly P**, Jack DL, Naeem A, Mandanda B, Pollok RC, Klein NJ, Turner MW, Farthing MJ. Mannose-binding lectin is a component of innate mucosal defense against Cryptosporidium parvum in AIDS. *Gastroenterology* 2000; **119**: 1236-1242
53 **Kirkpatrick BD**, Huston CD, Wagner D, Noel F, Rouzier P, Pape JW, Bois G, Larsson CJ, Alston WK, Tenney K, Powden C, O'Neill JP, Sears CL. Serum mannose-binding lectin deficiency is associated with cryptosporidiosis in young Haitian children. *Clin Infect Dis* 2006; **43**: 289-294
54 **Proulx F**, Wagner E, Toledano B, Decaluwe H, Seidman EG, Rivard GE. Mannan-binding lectin in children with Escherichia coli O157:H7 haemmorrhagic colitis and haemolytic uraemic syndrome. *Clin Exp Immunol* 2003; **133**: 360-363
55 **Suerbaum S**, Michetti P. Helicobacter pylori infection. *N Engl J Med* 2002; **347**: 1175-1186
56 **El-Omar EM**, Carrington M, Chow WH, McColl KE, Bream JH, Young HA, Herrera J, Lissowska J, Yuan CC, Rothman N, Lanyon G, Martin M, Fraumeni JF Jr, Rabkin CS. Interleukin-1 polymorphisms associated with increased risk of gastric cancer. *Nature* 2000; **404**: 398-402
57 **Thye T**, Burchard GD, Nilius M, Muller-Myhsok B, Horstmann RD. Genomewide linkage analysis identifies polymorphism in the human interferon-gamma receptor affecting Helicobacter pylori infection. *Am J Hum Genet* 2003; **72**: 448-453
58 **Zambon CF**, Basso D, Navaglia F, Germano G, Gallo N, Milazzo M, Greco E, Fogar P, Mazza S, Di Mario F, Basso G, Rugge M, Plebani M. Helicobacter pylori virulence genes and host IL-1RN and IL-1beta genes interplay in favouring the development of peptic ulcer and intestinal metaplasia. *Cytokine* 2002; **18**: 242-251
59 **Kuipers S**, Aerts PC, van Dijk H. Differential microorganism-induced mannose-binding lectin activation. *FEMS Immunol Med Microbiol* 2003; **36**: 33-39
60 **Bak-Romaniszyn L**, Cedzynski M, Szemraj J, St Swierzko A, Zeman K, Kaluzynski A, Planeta-Malecka I. Mannan-binding lectin in children with chronic gastritis. *Scand J Immunol* 2006; **63**: 131-135
61 **Worthley DL**, Mullighan CG, Dean MM, Gordon, DL, Phillips P, Heatley SL, Young GP, Bardy PG. Mannose-binding lectin deficiency does not increase the prevalence of *Helicobacter pylori* seropositivity. *Euro J Gastroenterol Hepatology* 2006; In press
62 **Hart ML**, Ceonzo KA, Shaffer LA, Takahashi K, Rother RP, Reenstra WR, Buras JA, Stahl GL. Gastrointestinal ischemia-

reperfusion injury is lectin complement pathway dependent without involving C1q. *J Immunol* 2005; **174**: 6373-6380

63 **Walsh MC**, Bourcier T, Takahashi K, Shi L, Busche MN, Rother RP, Solomon SD, Ezekowitz RA, Stahl GL. Mannose-binding lectin is a regulator of inflammation that accompanies myocardial ischemia and reperfusion injury. *J Immunol* 2005; **175**: 541-546

64 **Moller-Kristensen M**, Wang W, Ruseva M, Thiel S, Nielsen S, Takahashi K, Shi L, Ezekowitz A, Jensenius JC, Gadjeva M. Mannan-binding lectin recognizes structures on ischaemic reperfused mouse kidneys and is implicated in tissue injury. *Scand J Immunol* 2005; **61**: 426-434

65 **Summerfield JA**, Taylor ME. Mannose-binding proteins in human serum: identification of mannose-specific immunoglobulins and a calcium-dependent lectin, of broader carbohydrate specificity, secreted by hepatocytes. *Biochim Biophys Acta* 1986; **883**: 197-206

66 **Homann C**, Garred P, Hasselqvist P, Graudal N, Thiel S, Thomsen AC. Mannan-binding protein and complement dependent opsonization in alcoholic cirrhosis. *Liver* 1995; **15**: 39-44

67 **Yuen MF**, Lau CS, Lau YL, Wong WM, Cheng CC, Lai CL. Mannose binding lectin gene mutations are associated with progression of liver disease in chronic hepatitis B infection. *Hepatology* 1999; **29**: 1248-1251

68 **Bhuva M**, Ganger D, Jensen D. Spontaneous bacterial peritonitis: an update on evaluation, management, and prevention. *Am J Med* 1994; **97**: 169-175

69 **Chong WP**, To YF, Ip WK, Yuen MF, Poon TP, Wong WH, Lai CL, Lau YL. Mannose-binding lectin in chronic hepatitis B virus infection. *Hepatology* 2005; **42**: 1037-1045

70 **Thio CL**, Mosbruger T, Astemborski J, Greer S, Kirk GD, O' Brien SJ, Thomas DL. Mannose binding lectin genotypes influence recovery from hepatitis B virus infection. *J Virol* 2005; **79**: 9192-9196

71 **Hohler T**, Wunschel M, Gerken G, Schneider PM, Meyer zum Buschenfelde KH, Rittner C. No association between mannose-binding lectin alleles and susceptibility to chronic hepatitis B virus infection in German patients. *Exp Clin Immunogenet* 1998; **15**: 130-133

72 **Bellamy R**, Ruwende C, McAdam KP, Thursz M, Sumiya M, Summerfield J, Gilbert SC, Corrah T, Kwiatkowski D, Whittle HC, Hill AV. Mannose binding protein deficiency is not associated with malaria, hepatitis B carriage nor tuberculosis in Africans. *QJM* 1998; **91**: 13-18

73 **Matsushita M**, Hijikata M, Matsushita M, Ohta Y, Mishiro S. Association of mannose-binding lectin gene haplotype LXPA and LYPB with interferon-resistant hepatitis C virus infection in Japanese patients. *J Hepatol* 1998; **29**: 695-700

74 **Matsushita M**, Hijikata M, Ohta Y, Iwata K, Matsumoto M, Nakao K, Kanai K, Yoshida N, Baba K, Mishiro S. Hepatitis C virus infection and mutations of mannose-binding lectin gene MBL. *Arch Virol* 1998; **143**: 645-651

75 **Sasaki K**, Tsutsumi A, Wakamiya N, Ohtani K, Suzuki Y, Watanabe Y, Nakayama N, Koike T. Mannose-binding lectin polymorphisms in patients with hepatitis C virus infection. *Scand J Gastroenterol* 2000; **35**: 960-965

76 **Kilpatrick DC**, Delahooke TE, Koch C, Turner ML, Hayes PC. Mannan-binding lectin and hepatitis C infection. *Clin Exp Immunol* 2003; **132**: 92-95

77 **Worthley DL**, Bardy PG, Angus P, Harley H, Mullighan CG. Mannose-binding lectin and liver transplantation. *Gastroenterology* 2005; **129**: 1805-1806; author reply 1806-1807

78 **Kilpatrick DC**, Stewart K, Allan EK, McLintock LA, Holyoake TL, Turner ML. Successful haemopoietic stem cell transplantation does not correct mannan-binding lectin deficiency. *Bone Marrow Transplant* 2005; **35**: 179-181

79 **Wagner S**, Lynch NJ, Walter W, Schwaeble WJ, Loos M. Differential expression of the murine mannose-binding lectins A and C in lymphoid and nonlymphoid organs and tissues. *J Immunol* 2003; **170**: 1462-1465

80 **Uemura K**, Saka M, Nakagawa T, Kawasaki N, Thiel S, Jensenius JC, Kawasaki T. L-MBP is expressed in epithelial cells of mouse small intestine. *J Immunol* 2002; **169**: 6945-6950

81 **Granell M**, Urbano-Ispizua A, Lozano F, Rovira M, Fernandez-Aviles F, Ortega M, Uriburu C, Talarn C, Martínez C, Guardia A, Carreras E, Urbano-Ispizua A, Vives J, Montserrat E, Lozano F. Donor's Mannan-Binding Lectin (MBL) Gene Polymorphism Is Associated with Invasive Fungal Infection Following Allogeneic Stem Cell Transplantation. *Blood (ASH Annual Meeting Abstracts)* 2004; **104**: 2220

82 **Worthley DL**, Bardy PG, Mullighan CG. Mannose-binding lectin: biology and clinical implications. *Intern Med J* 2005; **35**: 548-555

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EDITORIAL

Emerging roles of the intestine in control of cholesterol metabolism

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Abstract

The liver is considered the major "control center" for maintenance of whole body cholesterol homeostasis. This organ is the main site for *de novo* cholesterol synthesis, clears cholesterol-containing chylomicron remnants and low density lipoprotein particles from plasma and is the major contributor to high density lipoprotein (HDL; good cholesterol) formation. The liver has a central position in the classical definition of the reverse cholesterol transport pathway by taking up periphery-derived cholesterol from lipoprotein particles followed by conversion into bile acids or its direct secretion into bile for eventual removal *via* the feces. During the past couple of years, however, an additional important role of the intestine in maintenance of cholesterol homeostasis and regulation of plasma cholesterol levels has become apparent. Firstly, molecular mechanisms of cholesterol absorption have been elucidated and novel pharmacological compounds have been identified that interfere with the process and positively impact plasma cholesterol levels. Secondly, it is now evident that the intestine itself contributes to fecal neutral sterol loss as a cholesterol-secreting organ. Finally, very recent work has unequivocally demonstrated that the intestine contributes significantly to plasma HDL cholesterol levels. Thus, the intestine is a potential target for novel anti-atherosclerotic treatment strategies that, in addition to interference with cholesterol absorption, modulate direct cholesterol excretion and plasma HDL cholesterol levels.

Key words: Cholesterol metabolism; Intestine; High density lipoprotein; Cholesterol absorption; Reverse cholesterol transport

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INTRODUCTION

Maintenance of cholesterol homeostasis in the body requires accurate metabolic cross-talk between processes that govern *de novo* cholesterol synthesis and turnover to adequately cope with (large) fluctuations in dietary cholesterol intake. Imbalance may lead to elevated plasma cholesterol levels and increased risk for cardiovascular diseases (CVD), the main cause of death in Western society. A multitude of epidemiological studies has shown the direct link between high plasma cholesterol, particularly of low density lipoprotein (LDL) cholesterol, and risk for CVD. Treatment of high plasma cholesterol has been focused for many years on interference with cholesterol synthesis by application of statins. Statins are competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-controlling enzyme in the cholesterol biosynthesis pathway[1]. Inhibition of cholesterol synthesis leads to reduced production of very low density lipoprotein (VLDL) particles by the liver and particularly, up-regulation of LDL receptor activity. Both processes contribute to lowering of plasma LDL-cholesterol levels[2]. Large clinical trials have established the beneficial effects of statin treatment[3]. However, a relative large number of hypercholesterolaemic patients do not adequately respond to statin therapy or remain at risk for CVD despite substantial reductions in LDL cholesterol[4,5]. Consequently, alternative strategies are currently actively pursued, particularly high density lipoprotein (HDL)-raising approaches. These approaches are considered particularly promising, as data from epidemiological studies indicate that every 1 mg/dL increase in HDL cholesterol reduces CVD risk by 2%-3%[6,7]. In addition, strategies aiming at interference with intestinal cholesterol metabolism are gaining interest. A major development has been the introduction of ezetimibe, a potent inhibitor of intestinal cholesterol absorption that reduces plasma LDL-cholesterol by approximately 20% in mildly

hypercholesteroleamic patients[8]. Likewise, phytosterol/stanol (esters)-enriched functional foods have successfully been introduced for lowering of plasma cholesterol levels through interference with cholesterol absorption[9].

Recently obtained insights in intestinal cholesterol trafficking may open even more promising avenues for further developments. It appears that the intestine actively excretes cholesterol and thereby, significantly contributes to fecal sterol excretion. In addition, it appears that the intestine is an important source of HDL-cholesterol, also known as "good" cholesterol. Thus, the intestine is an attractive target for new therapeutic strategies aimed to alter plasma cholesterol profiles and to reduce the risk for CVD. This review summarizes the important new findings regarding the mechanism(s) of intestinal cholesterol absorption, with specific focus on newly identified transporter proteins, the novel concept of direct intestinal cholesterol secretion and the role of the intestine in HDL biogenesis.

SOME BASIC FEATURES OF CHOLESTEROL

Cholesterol is essential for mammalian life as a structural component of cellular membranes, influencing membrane organization and thereby membrane properties[10]. Cholesterol is the precursor molecule of steroid hormones and therefore, essential for metabolic control. In the liver, cholesterol can be converted into bile salts, which represents the major pathway for cholesterol metabolism in quantitative sense. Bile salts are amphipathic molecules that facilitate the absorption of dietary cholesterol, fats and fat-soluble vitamins in the small intestine. Recently, it has become clear that bile salts are able to regulate gene expression through activation of the nuclear receptor, the farnesoid X receptor (FXR)[11-13]. Cholesterol or more correctly, oxidized cholesterol acts as a ligand for the nuclear liver X receptor (LXR or NRH2 or NRH3) and directly contributes to regulation of expression of genes involved in cholesterol, lipid, and glucose metabolism. Accumulation of free cholesterol rather than cholesterylesters, has been shown to induce apoptosis in macrophages by activating the Fas pathway[14]. Thus, cholesterol is a key component in cellular and whole-body physiology and cholesterol homeostasis is tightly regulated at a variety of levels.

Body cholesterol derives from two sources, i.e., *de novo* biosynthesis and diet. Cholesterol is synthesized from two-carbon acetyl-CoA moieties. The rate-controlling enzyme in the synthetic pathway is HMG-CoA reductase, a highly regulated enzyme that catalyses the conversion of HMG-CoA into mevalonate. Cholesterol itself regulates feed-back inhibition of HMG-CoA reductase activity, as accumulation of sterols in the endoplasmic reticulum (ER) membrane triggers HMG-CoA reductase to bind to Insig proteins, which leads to ubiquitination and degradation of HMG-CoA reductase[15,16]. In addition, cholesterol regulates the gene expression of HMG-CoA reductase indirectly by blocking the activation of the transcription factor, sterol regulatory element-binding protein 2 (SREBP2). Under low-cholesterol conditions, SREBP2 in the ER binds to the SREBP cleavage activating protein (SCAP), which escorts SREBP2 to the Golgi. In the Golgi, SREBP2 is cleaved to generate its transcriptionally active form, which activates transcription of the HMG-CoA reductase encoding gene. Upon accumulation of sterols in the ER-membrane, binding of cholesterol to the sterol-sensing domain of SCAP causes a conformation change, which induces binding of SCAP to the ER anchor protein Insig, preventing exit of SCAP-SREBP2 complexes to the Golgi thereby preventing activation of SREBP2[17].

The contribution of the two sources to the total pool of cholesterol differs between species and prevailing diet composition, but the total cholesterol pool is similar in rodents and humans when expressed on the basis of body weight[18]. Cholesterol synthesis in the liver is highly sensitive to the amount of (dietary) cholesterol that reaches the liver from the intestine via the chylomicron-remnant pathway[19]. The Western-type human diet provides approximately 400 mg of cholesterol per day. On top of this, the liver secretes approximately 1 gram of cholesterol into bile per day[20]. Intestinal cholesterol absorption efficiency in humans is highly variable, ranging from 15% to 85% in healthy subjects[21]. After uptake by enterocytes, cholesterol is packed with triglycerides into chylomicrons and secreted into the lymph. In the circulation, the triglycerides are rapidly hydrolyzed and free fatty acids are taken up by the peripheral tissues. Cholesterol-enriched chylomicron remnants are subsequently cleared by the liver. Since chylomicron remnants, which contain most of the cholesterol that is being absorbed from the intestine, are rapidly taken up by the liver, interference with the absorption process directly influences hepatic cholesterol synthesis.

The healthy liver is perfectly equipped for handling large amounts of cholesterol. When relatively large amounts of cholesterol reach the liver, *de novo* synthesis and LDL uptake are rapidly down-regulated. In addition, the liver can dispose excess cholesterol molecules in several ways. A rapid response involves esterification of cholesterol by Acyl CoA cholesterol acyltransferase (ACAT) 2 for storage as cholesterylesters in cytoplasmic lipid droplets. Cholesterylester can be hydrolyzed when necessary and this esterification/hydrolysis cycle provides cells with short-term buffering capacity for cholesterol. The liver, like the intestine, is able to produce and secrete VLDL particles, which consist of a neutral lipid core composed of cholesterylesters and triacylglycerols and a monolayer surface containing phospholipids, free cholesterol, and a variety of apolipoproteins. Finally, cholesterol can be converted into bile acids by the hepatocytes, followed by their secretion into the bile along with significant amounts of free cholesterol and phosphatidylcholine. In humans, cholesterol lost via the feces consists of approximately 50% acidic (= bile acids) and 50% neutral sterols, emphasizing the point that conversion into bile acids represents a major pathway for cholesterol elimination.

Peripheral cells, e.g., macrophages, muscle and fat cells, are not able to form lipoproteins or to metabolize cholesterol extensively. Therefore, these cell types depend massively on efflux pathways for removal of their excess cholesterol. It is generally assumed that HDL is the

primary acceptor for cholesterol efflux from cells. HDL cholesterol can subsequently be taken up by the liver for further processing. This pathway is generally referred to as the reverse cholesterol transport (RCT) pathway. The RCT pathway is particularly important for removal of excess cholesterol from macrophages, as accumulation of esterified cholesterol in these cells is considered a primary step in the development of atherosclerosis. Several epidemiological studies have shown that plasma HDL is an independent, negative risk factor for the development of CVD. The common hypothesis is that high HDL cholesterol levels decrease the risk for CVD by removing the excess of cholesterol from the macrophages and enhancing RCT. Recent work, however, indicates that this is an oversimplification and that current concepts of RCT require re-definition[22]. In addition, the anti-inflammatory and anti-oxidant features of molecules rather than cholesterol associated with the HDL particles, like paraoxonase, platelet activating factor-acetylhydrolase or lysophospholipids, are becoming increasingly apparent[23-25].

TOWARDS UNDERSTANDING OF INTESTINAL CHOLESTEROL ABSORPTION

In the past years, insight in regulation of cholesterol absorption has greatly increased by identification of transporter proteins involved. In addition, unraveling of molecular regulation of their expression is progressing. Yet, it should be realized that besides transporter proteins, the presence of bile acids in the intestinal lumen is an essential prerequisite for absorption to occur[26]. Micellar solubilization of (dietary/biliary) cholesterol is necessary for its absorption as exemplified by the fact that fractional cholesterol absorption is virtually zero in bile-diverted rats and *Cyp7a1*-deficient mice with a strongly diminished bile acid pool size[26].

Identification of novel proteins involved in cholesterol absorption

Cholesterol absorption has long been considered a merely passive process, despite the fact that the process is clearly selective since dietary cholesterol is absorbed with a relative high efficiency whereas structurally similar phytosterols are not. Several candidate intestinal cholesterol transporters have been proposed during the past couple of years, e.g., SR-B1[27] and aminopeptidase N[28], but their role (if any) has remained elusive so far. The recent identification of the Niemann-Pick C1 like 1 (NPC1L1) protein as a crucial molecule involved in cholesterol uptake by enterocytes[29] and of Abcg5 and Abcg8 proteins as (intestinal) cholesterol efflux transporters[30-32], has provided definite proof that cholesterol absorption is a protein-mediated, selective and active process.

The identification of NPC1L1 is strongly facilitated by the discovery of a powerful cholesterol absorption inhibitor named ezetimibe[33]. Ezetimibe and analogs comprise a new class of sterol absorption inhibitors that reduce diet-induced hypercholesterolemia in mice, hamsters, rats, rabbits, dogs, monkeys and humans[8,33-37]. Using a bioinformatics approach, Altmann *et al*[29] have identified the NPC1L1 protein as a putative cholesterol transporter in intestinal cells. NPC1L1 is expressed in the intestine at the brush border membrane and *Npc1l1*-deficient mice show a 69% reduction in fractional cholesterol absorption. Importantly, treatment with ezetimibe could not further reduce fractional cholesterol absorption efficiency in these mice, indicating that NPC1L1 at least is involved in a pathway targeted by ezetimibe[29]. In support of this, recent studies have shown that ezetimibe glucuronide, the active molecule, indeed binds to cells expressing NPC1L1[38]. Using intestinal brush border membrane (BBM) fractions, the authors showed that ezetimibe binds specifically to a single site in the brush border membrane and that this binding is lost in BBM fractions of *Npc1l1*- deficient mice[38]. The exact cellular localization of NPC1L1 is, however, still under debate. Iyer *et al*[39] showed that NPC1L1 is glycosylated and enriched in the BBM of rat enterocytes. Davies *et al*[40] who were the first to identify NPC1L1 as a homolog of the Niemann Pick type C (NPC) protein[40], showed in HepG2 cells that NPC1L1 is localized to a subcellular vesicular compartment but not in the plasma membrane. Using immortalized fibroblasts from wild-type and *Npc1l1* knock-out mice these authors also showed that lack of NPC1L1 activity causes dysregulation of caveolin transport and localization, suggesting that the observed sterol transport defect may be an indirect result of the inability of *Npc1l1*-deficient cells to properly target and/or regulate cholesterol transport in the cells.

Another possible mechanism of action of ezetimibe has been proposed by Smart and colleagues[41]. These authors described the presence of a stable complex of annexin (ANX) 2 and caveolin (CAV) 1 located in enterocytes of zebrafish and mouse. Disruption of this complex by morpholino antisense oligonucleotides in zebrafish prevented normal uptake of cholesterol. Ezetimibe treatment of zebrafish, C57Bl/6 mice fed a Western type diet and LDL receptor knock-out mice disrupted the ANX2-CAV1 complex, suggesting that ANX2 and CAV1 are components of an intestinal sterol transport complex and targets for ezetimibe. Interestingly, C57BL/6 mice fed a standard diet did not show disruption of the ANX2-CAV1 complex upon ezetimibe treatment, but did show decreased cholesterol absorption[41]. Moreover, recent research using CAV1-deficient mice revealed that inhibition of cholesterol absorption by ezetimibe did not require the presence of CAV1[42]. In addition, rabbits did not appear to form the ANX2-CAV1 complexes, yet, their cholesterol absorption efficiency was still inhibited by ezetimibe[43]. Collectively, these studies make a mode of action in which ezetimibe acts by deregulating the ANX2-CAV1 complex less likely.

Other proteins critical in control of sterol absorption are the ATP-binding cassette (ABC) transporter proteins, G5 and G8. ABCG5 and ABCG8 act as functional heterodimers[44] and are localized at the canalicular membrane of hepatocytes and at the brush border membrane of enterocytes. Mutations in the human genes encoding ABCG5 or ABCG8 have been shown to cause the inherited disease sitosterolemia[30-32], which is characterized by an accumulation of plant sterols

(e.g., sitosterol, campesterol) in blood and tissues due to their enhanced intestinal absorption and decreased biliary removal. Thus, ABCG5/ABCG8 limit plant sterol absorption by effective efflux back into the intestinal lumen. Since ABCG5/ABCG8 also accommodate cholesterol, as evidenced from the fact that *Abcg5/g8*-deficient mice show a strongly reduced biliary cholesterol secretion[45], this system also provides a means to control cholesterol absorption efficiency. Yet, *Abcg5* and/or *Abcg8* deficiency in mice clearly enhances phytosterol absorption[45-47], but reported effects on cholesterol absorption efficiency are minimal[45,46]. On the other hand, overexpression of *ABCG5* and *ABCG8* in mice as well as pharmacological induction of their expression did lead to a strongly decreased fractional cholesterol absorption[46,48,49], indicating that ABCG5 and ABCG8 play a role in control of cholesterol absorption under certain conditions.

Other transporter proteins, like the scavenger receptor BI (SR-BI) and ABCA1 have been suggested to play a role in control of cholesterol absorption. In the small intestine, SR-BI is localized both at the apical membrane and at the basolateral membrane of enterocytes, with different expression levels along the length of the small intestine[50]. It was reported that mice deficient in SR-B1, however, show only a small increase in fractional cholesterol absorption efficiency and a small decrease in fecal neutral sterol output[51]. On the other hand, intestine-specific overexpression of SR-BI in mice did lead to increased cholesterol and triglyceride absorption in short-term absorption experiments[52], indicating that SR-BI might have a role in cholesterol absorption.

Although earlier reports[53] have suggested an apical localization, it is evident that ABCA1 is localized at the basolateral membranes of chicken enterocytes[54] and human CaCo-2 cells[55]. The conflicting results yielded in studies assessing intestinal cholesterol absorption in mice lacking Abca1[56,57], suggest that the overall effect of Abca1 on absorption is very minor. However, as will be described later, this protein does have an important function in intestinal cholesterol metabolism.

After uptake, cholesterol is esterified by the enzyme ACAT 2 in the endoplasmic reticulum (ER) of enterocytes. It was reported that *Acat2*-deficiency in mice on a low-cholesterol chow diet did not affect cholesterol absorption efficiency, however, *Acat2*-deficient mice did show a clear reduction in cholesterol absorption upon feeding a high-fat/high-cholesterol diet and as a consequence, are resistant to diet-induced hypercholesterolemia[58]. Other proteins crucial for cholesterol absorption are those involved in chylomicron formation, like apolipoprotein B (apoB) and microsomal triglyceride transfer protein (MTP), and proteins involved in intracellular chylomicron trafficking such as SARA2. Mutations in the MTP gene result in abetalipoproteinemia, an inherited human disease characterized by extremely low plasma cholesterol and triglyceride levels and absence of apoB-containing particles. Patients suffer from fat and cholesterol malabsorption and neurological diseases due to malabsorption of lipid-soluble vitamins. Mutations in SARA2 cause chylomicron retention disease or Anderson disease[59], both of which are characterized by the inability to secrete chylomicrons

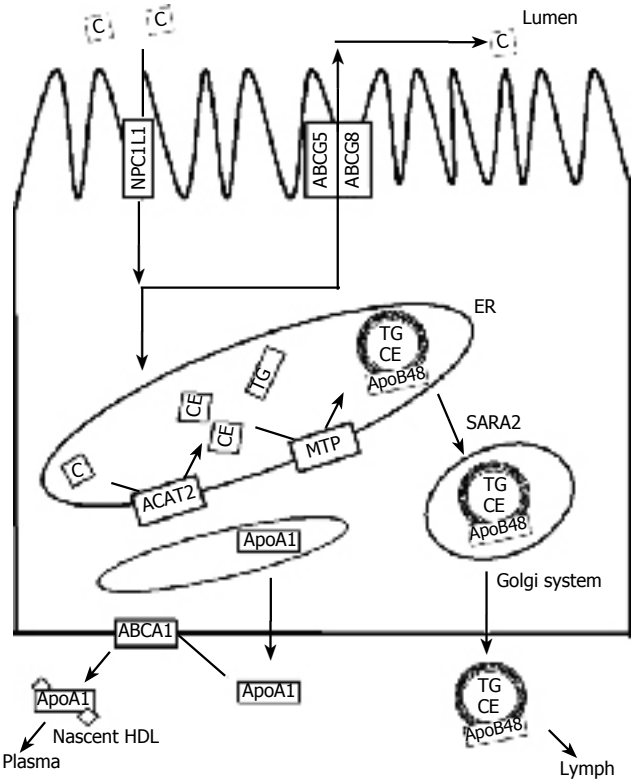

Figure 1 Schematic overview of the major routes of cholesterol in enterocytes. Dietary and biliary cholesterol are taken up via the action of NPC1L1. In the ER, cholesterol is esterified and incorporated into chylomicrons, which are subsequently secreted into lymph. Non-esterified sterols can be re-secreted into the intestinal lumen via the action of ABCG5/G8 or secreted towards ApoA1 *via* the action of ABCA1. ABCA1, ABCG5, ABCG8: ATP-binding cassette transporter A1, G5, G8; ACAT2: Acyl-coenzyme A: Cholesterol acyl transferase 2; ApoAI, ApoB48, apolipoprotein AI, B48; C: Cholesterol; CE: Cholesterylester; ER: Endoplasmatisch reticulum; MTP: Microsomal triglyceride transfer protein; NPC1L1: Niemann Pick C 1 like 1 protein; SARA2: Sar1-ADP-ribosylation GTPase 2; TG: Triglycerides.

causing severe fat malabsorption and accumulation of chylomicron-like particles in enterocytes. SARA2 belongs to the Sar1-ADP-ribosylation factor family of small GTPases and is involved in intracellular trafficking of chylomicrons through the secretory pathway[59].

The major routes of cholesterol in enterocytes and the proteins involved are depicted schematically in Figure 1.

Regulation of cholesterol absorption

As indicated above, cholesterol can be taken up from the intestinal lumen by NPC1L1 and effluxed back into the lumen *via* ABCG5 and ABCG8. When both processes are active and present in the same cells, a classical futile cycle arises, enabling very sensitive regulation. Interference with this system has a great potential for reducing plasma cholesterol.

An established application hereof is provided by ezetimibe that interferes with NPC1L1 activity[29,38]. Lowering of NPC1L1 expression provides another potential means to reduce cholesterol absorption. Mechanisms involved in transcriptional control of NPC1L1 are beginning to be unraveled. The nuclear receptor peroxisome proliferator-activated receptor (PPAR) δ/β (NR1C2) has been shown to decrease cholesterol absorption, presumably by decreasing

NPC1L1 expression[60]. Activation of PPARδ/β by the synthetic agonist GW610742 resulted in a 43% reduction of cholesterol absorption in mice, which coincides with unchanged intestinal expression of *Abcg5* and *Abcg8* but a decreased intestinal expression of *Npc1l1*. Treatment of human colon-derived CaCo-2 cells with ligands for PPARδ/β, but not for PPARγ or PPARα, decreased *NPC1L1* expression as well[60]. Whether PPARδ/β regulates NPC1L1 directly or indirectly via transcriptional repression, is still under investigation.

The major regulatory pathways in cholesterol metabolism are controlled by the nuclear receptor liver X receptor (LXR). Two LXR isotypes have been identified in mammals, i.e., LXRα (NR1H3) which is mainly expressed in the liver, kidney, intestine, spleen and adrenals, and LXRβ (NR1H2) which is expressed ubiquitously. Natural ligands for both LXRs are oxysterols. After activation, LXR heterodimerizes with retinoid X receptor (RXR)[61,62]. Activated RXR/LXR heterodimers bind to specific LXR response elements (LXREs) in the promoter regions of their target genes and activate gene transcription. LXR target genes include many genes involved in cellular cholesterol efflux like *ABCA1*, *ABCG1*, *ABCG5*, and *ABCG8*[53,63,64], genes involved in bile acid synthesis [*cholesterol-7α-hydroxylase* (*Cyp7a1*)] in rodent models and genes involved in lipogenesis like sterol regulatory element-binding protein (SREBP) 1C, fatty acid synthase (FAS) and *acetyl-CoA* carboxylase (ACC). Global LXR activation by synthetic agonists therefore has a plethora of effects including elevated HDL levels, hypertriglyceridemia, hepatic steatosis, increased biliary cholesterol excretion, reduced intestinal cholesterol absorption efficiency and increased neutral sterol loss *via* the feces[65,66]. The decreased intestinal cholesterol absorption is primarily due to increased cholesterol efflux of cholesterol towards the intestinal lumen due to increased *Abcg5* and *Abcg8* expression, as fractional cholesterol absorption was reduced upon LXR activation in wild-type mice but remained unaltered in *Abcg5/g8*-deficient mice[49] and *Abcg5*-deficient mice[46] under these conditions. Other mechanisms, such as reduced intestinal *Npc1l1* expression after LXR activation contribute to reduced cholesterol absorption, as recently shown in *ApoE2*-knock-out mice[67].

Dietary phytosterols and phytostanols and their esters have been introduced in functional foods to suppress intestinal cholesterol absorption and hence to reduce the risk for CVD[9]. Phytosterols and stanols are thought to decrease cholesterol absorption by competing with cholesterol for incorporation into mixed micelles in the intestinal lumen[68]. However, several recent studies suggested additional mechanisms involving alterations of intestinal gene expression. Igel and colleagues[69] showed for the first time that phytosterols and stanols are actually taken up by the enterocytes and subsequently re-secreted into the gut lumen, most probably through the action of Abcg5/Abcg8 transporters. This finding indicated that phytosterols and stanols, in addition to modes of action within the intestinal lumen, exert metabolic actions from inside the enterocytes. Moreover, dietary phytostanol consumption (2.5 g) once a day reduces LDL cholesterol as effective as consumption of 2.5 g phytostanols ingested in three daily portions[70], suggesting that luminal concentrations may not be the key to the control of metabolic actions. The identification of a phytosterol-derived agonist for the nuclear receptor LXR[71] has led to the proposal that phytosterols and stanols decrease cholesterol absorption via activation of intestinal LXR. *In vitro* studies in CaCo-2 cells indicated that phytostanols indeed are able to induce the expression of *ABCA1*, an established LXR target gene[72]. Recent *in vivo* studies, however, showed that dietary phytosterols and phytostanols decrease cholesterol absorption without activating LXR in rodent models. Field *et al*[73] showed that addition of 2% phytostanols to a chow diet do not affect intestinal expression of *ABC sterol transporters* and *Npc1l1* in male golden Syrian hamsters. Likewise, Calpe-Berdien *et al*[74] showed very recently that decreased cholesterol absorption upon addition of 2% phytosterol to a Western type diet is not associated with transcriptional changes in *Abca1*, *Abcg5*, *Abcg8* or *Npc1l1* in C57BL/6J, ApoE$^{-/-}$ and LDLr$^{-/-}$ mice. Plösch and colleagues[66] have shown similar results using 0.5% phytosterol or phytostanol in a semi-synthetic diet containing 0.2% cholesterol in C57BL mice. Additionally, these authors showed that the plant sterol/stanol-induced reduction of cholesterol absorption in mice is not influenced by *Abcg5*-deficiency (J. Nutr., in press), indicating that intra-luminal events are most relevant for the inhibitory effect of these dietary compounds.

The modes of action of the different cholesterol absorption decreasing compounds are schematically depicted in Figure 2.

NOVEL ROLE OF THE INTESTINE IN REVERSE CHOLESTEROL TRANSPORT

It is clear that the intestine plays a major role in cholesterol homeostasis as a cholesterol absorbing organ. However, recent studies revealed that the intestine also acts as an excretory organ in the reverse cholesterol transport (RCT) pathway[66,75]. This pathway is classically defined as the HDL-mediated flux of cholesterol from peripheral cells to the liver, followed by its secretion into bile and disposal *via* the feces. RCT is extremely important in prevention of CVD as it removes excess cholesterol from macrophages present in the arterial vessel wall. The amount of cholesterol secreted into bile is substantial. As only part of it is absorbed by the intestine, it contributes significantly to cholesterol loss *via* the feces. However, a novel pathway that contributes to fecal cholesterol loss has recently been established.

Already in the nineteen-sixties, it was suggested that non-dietary cholesterol present in the intestinal lumen consists of a fraction secreted by the liver into the bile and a second fraction directly secreted by the intestine. Measuring dietary cholesterol, cholesterol absorption and cholesterol loss *via* the feces in patients with complete obstruction of common bile duct due to carcinoma of the head of the pancreas unequivocally established the presence of intestinally secreted cholesterol in the feces[76]. By intestinal perfusion studies in humans, Simmonds *et al*[77] have tried to quantify this route. In a triple lumen tube system, perfusion studies were carried out using micellar solutions

with radio-labeled cholesterol. Decrease in specific activity was interpreted as secretion of endogenous cholesterol from the intestine and the contribution of endogenously secreted cholesterol from the intestine was estimated to be about 44% of total fecal output, but direct proof for the existence of this pathway could not be provided[77].

Since these early experiments, the focus of research has shifted more towards the liver. Biliary cholesterol and bile acid secretions are believed to represent the major pathways for removal of excess cholesterol. However, recent calculations of cholesterol fluxes in different mouse models again emphasize the relevance of intestinal cholesterol secretion. A striking example is provided by the *Cyp7a1*-deficient mouse[78]. Cyp7a1 is important for converting cholesterol into bile acids and catalyzing the formation of 7α-hydroxycholesterol[79]. As Cyp7a1 is rate-controlling in this pathway, it is regulated in a complex manner involving multiple nuclear receptors. *Cyp7a1*-deficiency in mice leads to significantly decreased fecal bile acid loss and bile acid pool size. Surprisingly, fecal neutral sterol output is increased two times in *Cyp7a1*$^{-/-}$ mice, although biliary cholesterol concentration remains unaffected[78]. As dietary intake and cholesterol absorption are known, it can be calculated from these data that direct intestinal cholesterol secretion contributes at least 30% to the increased fecal neutral sterol output.

Plösch and colleagues[66] showed that the pathway of intestinal cholesterol secretion can be induced in mice by treatment with the synthetic LXR agonist T0901317. In C57BL/6 mice, efflux of cholesterol from the intestinal epithelium into the lumen, calculated from the difference between dietary and biliary input minus fecal output, contributed up to 36% of the total fecal cholesterol loss. Pharmacological LXR activation in these mice tripled the intestinal cholesterol secretion, showing that this represents a valid, inducible pathway for removal of cholesterol in mice.

To further characterize this route, Kruit *et al*[75] studied the effects of LXR activation by the synthetic agonist GW3965 in wild-type and *Mdr2*-deficient mice. Mdr2-Pgp (or Abcb4 according to the new nomenclature) mediates the ATP-dependent translocation of phospholipids at the canalicular membrane of hepatocytes. Consequently, *Mdr2*-deficiency leads to the inability to secrete phospholipids into the bile. Due to the tight coupling of phospholipid and cholesterol secretion, these mice also show a severely impaired biliary cholesterol secretion[80,81]. Despite the impaired biliary cholesterol secretion, chow-fed *Mdr2*$^{-/-}$ mice showed a similar fecal neutral sterols loss as wild-type mice, suggesting that the intestine indeed contributes to the fecal neutral sterol loss. LXR activation increased fecal neutral sterol output to a similar extent in *Mdr2*$^{-/-}$ and wild-type mice, although biliary cholesterol secretion remained impaired in *Mdr2*$^{-/-}$ mice but increased in wild-type mice. These data show that the increased fecal cholesterol loss upon LXR activation is independent of biliary cholesterol secretion. Although fractional cholesterol absorption decreased to a greater extent in *Mdr2*$^{-/-}$ mice compared to wild-type mice upon LXR activation, it could be calculated that at least 57% of fecal cholesterol originates from intestinal secretion in *Mdr2*$^{-/-}$ mice[75].

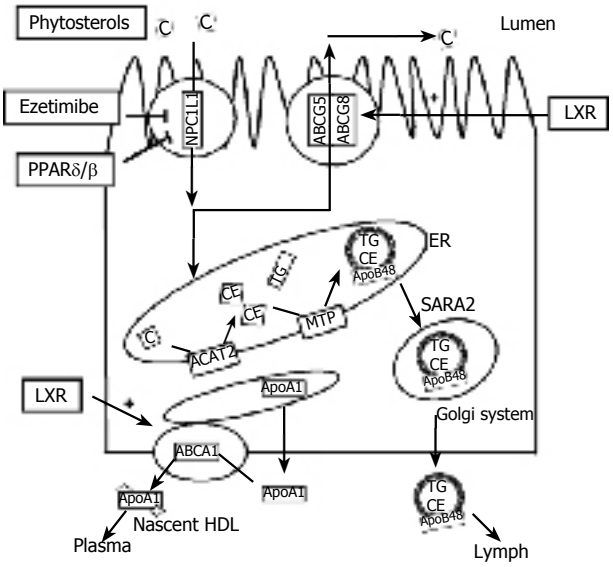

Figure 2 Schematic overview of the regulation of cholesterol transport in enterocytes. Plant sterols, ezetimibe PPARδ/β agonists and LXR agonists all reduce cholesterol absorption through different mechanisms. Plant sterols interfere with micellisation of cholesterol. Ezetimibe binds to NPC1L1 and thereby interferes with the cholesterol uptake. Agonists for PPARδ/β reduce expression of NPC1L1 and thereby the amount of NPC1L1 protein. Agonists for LXR increase the expression of ABCG5 and ABCG8 and thereby enhance the efflux of cholesterol towards the intestinal lumen. LXR: Liver X Receptor; PPARδ/β: Peroxisome proliferators-activated receptor δ/β.

The most intriguing question, namely the origin of intestine-derived cholesterol has remained unanswered so far. Part of the cholesterol could, in theory, originate from enhanced sloughing of intestinal cells or reflect a consequence of increased intestinal *de novo* cholesterol synthesis. Indeed, increased intestinal cholesterol synthesis has been found in *Cyp7a1*$^{-/-}$ mice[78]. Upon LXR activation, however, intestinal *HMGCoA reductase* gene expression remained unchanged[66,75], indicative for unchanged cholesterol synthesis, while fecal sterol loss increased 3 times. Staining for the proliferation marker Ki-67 has revealed no signs of increased intestinal cell proliferation upon LXR activation, making the possibility of enhanced cell shedding less likely[75]. Using intravenously injected radiolabeled cholesterol as a marker, Kruit and colleagues[75] additionally showed that fecal loss of plasma-derived cholesterol is 1.7-fold higher upon LXR activation in *Mdr2*$^{-/-}$ mice, suggesting that the intestine plays an important role independently of biliary cholesterol in cholesterol transport from plasma to the feces.

Further research should be done to identify the putative proteins involved in this pathway. The sterol efflux proteins, ABCG5/ABCG8, seem to be good candidates, as increased fecal neutral sterol output upon LXR activation requires the presence of Abcg5 and Abcg8[49] and transgenic mice overexpressing human *ABCG5* and *ABCG8* (*hG5G8Tg*) showed significantly-increased fecal neutral sterol loss[48]. However, deficiency of *Abcg5* and/or *Abcg8* leads to only mild[45,49] or no[46] decrease in fecal neutral sterol loss and the increased fecal neutral sterol excretion loss in the *hG5G8Tg* mice was inhibited in *hG5G8Tg* mice lacking *Mdr2* (*Mdr2*$^{-/-}$*hG5G8Tg* mice),

suggesting that biliary cholesterol secretion is responsible for the increased fecal sterol loss in *hG5G8Tg* mice[82]. However, *hG5G8Tg* mice showed a high expression of human *ABCG5* and *ABCG8* in the liver but their expression in the intestine was far less pronounced[48]. Thus, the question whether intestinal ABCG5 and ABCG8 are important for intestinal cholesterol efflux under normal conditions still remains unanswered.

Virtually nothing is known about transporter systems involved in uptake of plasma cholesterol by enterocytes prior to its excretion into the intestinal lumen. LXR activation can upregulate a number of cholesterol transporters, of which only SR-BI is known to be involved in cholesterol uptake, at least in the liver. Chow-fed *SR-B1*$^{-/-}$ mice show only a small decrease in fecal neutral sterol loss, suggesting a relatively small contribution of intestinal SR-B1 to the control of fecal cholesterol excretion. However, basolaterally localized SR-B1 in enterocytes could theoretically play a role in cholesterol. When free cholesterol in enterocytes decreases due to activation of ABCG5 and ABCG8, uptake of the sterol from the plasma compartment may become energetically favorable.

INTESTINAL CONTRIBUTION TO HDL BIOGENESIS

The intestine along with the liver, has been known for many years to synthesize and secrete apolipoprotein A-I (ApoA-I), the principal apolipoprotein of HDL. Already in 1977, Glickman and Green[83] have described the synthesis of ApoA-I by the intestine of rats. One year later, Wu and Windmueller[84] estimated that intestinally synthesized ApoA-I contributes up to 56% of total plasma ApoA-I in rats. A potential role for the intestine in HDL particle assembly was initially suggested from experiments in hepatectomized dogs and studies describing the presence of HDL in mesenteric lymph[86-89]. More recently, *in vitro* studies using the human colon carcinoma cell line CaCo-2 showed that basolateral efflux of cholesterol occurs in high density ApoB-free, ApoA-I containing lipoproteins[90,91].

In addition to ApoA-I, ATP-binding cassette (ABC) transporter 1 (ABCA1) is of crucial importance for HDL formation. Three different groups have independently reported mutations of the ABCA1 gene as the cause of Tangier disease[92-94]. Tangier disease is characterized by almost complete absence of plasma HDL, abnormal accumulation of cholesteryl esters in reticuloendothelial cells of many tissues and early incidence of atherosclerosis. No abnormalities in the ApoA-I protein[95] or in protein synthesis have been found. These findings and the subsequent generation of *Abca1*$^{-/-}$ mice which also lack plasma HDL[57], underscore that ABCA1 is crucial for HDL formation.

ABCA1 performs the rate-controlling step in HDL formation by mediating the efflux of cholesterol and phospholipids to nascent ApoA-I. ABCA1 is widely expressed throughout the body[96], however not all tissues are important for the regulation of plasma HDL. Bone marrow transplantation studies in which bone marrow of wild-type and *Abca1*$^{-/-}$ mice was transplanted into *Abca1*$^{-/-}$ or wild-type mice, respectively, revealed that macrophage expression of *Abca1* contributes only minimally to plasma HDL[97]. Macrophage ABCA1 is, however, important for the development of atherosclerosis because deficiency of *Abca1* in bone marrow-derived cells increased the susceptibility to atherosclerosis in sensitive strains of mice[98,99]. Conversely, overexpression of *ABCA1* in bone marrow-derived cells inhibited the progression of atherosclerotic lesions in such mice[100].

As both the liver and intestine synthesize ApoA-I and express significant levels of ABCA1, they are prone to contribute to plasma HDL levels. Indeed, mice overexpressing human *ABCA1* in the liver and macrophages showed increased plasma HDL levels. Since macrophage ABCA1 can only minimally increase plasma HDL[97], this indicates that plasma HDL is controlled by hepatic ABCA1. A similar conclusion can be drawn from studies employing adenoviral *Abca1* transfer to mouse liver *in vivo*[101,102]. Basso *et al* showed that treatment of C57BL/6 mice with adenovirus containing *rABCA1-GFP* resulted in a 2-fold increase in plasma HDL levels. Wellington *et al*[102] treated mice with increasing doses of *ABCA1*-containing adenoviruses, resulting in a dose-dependent increase in hepatic ABCA1 protein expression. HDL cholesterol was increased in mice injected with low doses of *adABCA1*, but surprisingly higher doses did not further raise plasma HDL levels[102]. Liver-specific *Abca1* knockdown by 50% in mice using siRNA resulted in a 40% decrease of plasma HDL cholesterol levels, indicating that hepatic *Abca1* expression correlates with plasma HDL levels in mice[103].

The creation of liver-specific *Abca1* knock-out (*Abca1*$^{-L/-L}$) mice definitively showed that the liver is the major contributor to plasma HDL as liver-specific deficiency of *Abca1* results in a decrease of plasma HDL cholesterol levels by 80%. Further analysis revealed that *in vivo* catabolism of HDL ApoA-I isolated from wild-type mice was 2-fold higher in Abca1$^{-L/-L}$ mice due to a 2-fold higher rate of catabolism of ApoA-I in the kidneys[104]. These data unequivocally demonstrate that hepatic *Abca1* is responsible for the maintenance of the circulating plasma HDL by direct lipidation of lipid-poor ApoA-1 containing particles. These data also show that, although the liver is the major organ responsible for HDL levels, additional extra-hepatic sites also contribute to HDL biogenesis.

To address the contribution of intestinal *Abca1* to plasma HDL, intestine-specific *Abca1* knockout (*Abca1*$^{-i/-i}$) mice were created using the Cre/Lox system with the Cre transgene under the control of the villin promoter[105]. Brunham *et al* showed that intestinal *Abca1* deficiency resulted in a 30% decrease in plasma HDL cholesterol levels, indicating that intestinal *Abca1* is critically involved in HDL biogenesis. Combined deletion of both hepatic and intestinal *Abca1* resulted in a 90% decrease of plasma HDL, which was similar to the level found in the whole-body *Abca1*$^{-/-}$ mice, proving that the liver and intestine are really the two major sites for HDL biogenesis. Absence of intestinal *Abca1* resulted in decreased transport of dietary cholesterol into plasma HDL, but total intestinal cholesterol absorption was not affected. Surprisingly, lymphatic HDL content was hardly affected in *Abca1*$^{-i/-i}$ mice. In contrast, HDL was virtually

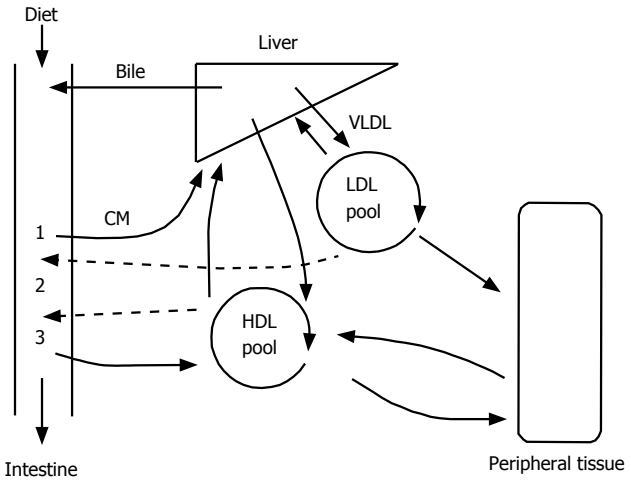

Figure 3 Schematic overview of the involvement of the intestine in cholesterol homeostasis. The intestine is critically involved in the control of plasma cholesterol due to its role in intestinal cholesterol absorption (1), direct cholesterol excretion into the intestinal lumen (2), and HDL biogenesis (3). CM: Chylomicron; HDL: High density lipoprotein; LDL: Low density lipoprotein; VLDL: Very low density lipoprotein.

absent in lymph of *Abca1*$^{-L/-L}$ mice, indicating that lymph HDL originates from the plasma compartment rather than directly from the intestine[105]. This finding has solved a long-lasting debate on the origin of lymphatic HDL[83,86-88,106,107]. It would be interesting to see whether lack of intestinal *Abca1* influences the development of atherosclerosis.

Modulation of plasma HDL by intestine- specific LXR activation

As discussed above, LXR is a major regulator of cholesterol metabolism and LXR agonists are considered promising candidates for novel treatment strategies against atherosclerosis. Indeed, treatment of *ApoE*$^{-/-}$ and *LDLr*$^{-/-}$ mice, both are sensitive to atherosclerosis development, with synthetic LXR agonists inhibited the development of atherosclerosis[108,109]. However, general LXR activation also leads to increased lipogenesis, hypertriglyceridemia and hepatic steatosis in rodents[65] and is therefore not recommended for its use in humans. Specific LXR activation in the intestine may be beneficial in this respect, as it can theoretically lead to decreased cholesterol absorption, increased intestinal cholesterol excretion and plasma HDL levels. The preliminary data from our laboratory, using an intestine-specific LXR agonist in Wistar rats, showed that intestine-specific LXR activation indeed has the desired effect in this model without adverse effects on triglyceride metabolism.

CONCLUSION

During the past 5 years, a number of developments have greatly contributed to appreciation of the important role of the intestine in maintenance of cholesterol homeostasis (Figure 3). The most important developments include the identification of transporter proteins involved in uptake and secretion of cholesterol by enterocytes, the establishment of the direct cholesterol excretion pathway of the intestine, and the definition of the role of the intestine in HDL biogenesis.

A wealth of data indicate that the intestine should be considered a promising target for development of anti-atherosclerotic drugs that, in addition to interference with cholesterol absorption, may directly modulate cholesterol excretion and plasma HDL cholesterol levels.

REFERENCES

1 **Endo A**. The discovery and development of HMG-CoA reductase inhibitors. *J Lipid Res* 1992; **33**: 1569-1582
2 **Brown MS**, Goldstein JL. A receptor-mediated pathway for cholesterol homeostasis. *Science* 1986; **232**: 34-47
3 **Ong HT**. The statin studies: from targeting hypercholesterolaemia to targeting the high-risk patient. *QJM* 2005; **98**: 599-614
4 **Illingworth DR**, Crouse JR 3rd, Hunninghake DB, Davidson MH, Escobar ID, Stalenhoef AF, Paragh G, Ma PT, Liu M, Melino MR, O'Grady L, Mercuri M, Mitchel YB. A comparison of simvastatin and atorvastatin up to maximal recommended doses in a large multicenter randomized clinical trial. *Curr Med Res Opin* 2001; **17**: 43-50
5 **Jones PH**, Davidson MH, Stein EA, Bays HE, McKenney JM, Miller E, Cain VA, Blasetto JW. Comparison of the efficacy and safety of rosuvastatin versus atorvastatin, simvastatin, and pravastatin across doses (STELLAR* Trial). *Am J Cardiol* 2003; **92**: 152-160
6 **Castelli WP**, Garrison RJ, Wilson PW, Abbott RD, Kalousdian S, Kannel WB. Incidence of coronary heart disease and lipoprotein cholesterol levels. The Framingham Study. *JAMA* 1986; **256**: 2835-2838
7 **Gordon T**, Kannel WB, Castelli WP, Dawber TR. Lipoproteins, cardiovascular disease, and death. The Framingham study. *Arch Intern Med* 1981; **141**: 1128-1131
8 **Sudhop T**, Lutjohann D, Kodal A, Igel M, Tribble DL, Shah S, Perevozskaya I, von Bergmann K. Inhibition of intestinal cholesterol absorption by ezetimibe in humans. *Circulation* 2002; **106**: 1943-1948
9 **Miettinen TA**, Puska P, Gylling H, Vanhanen H, Vartiainen E. Reduction of serum cholesterol with sitostanol-ester margarine in a mildly hypercholesterolemic population. *N Engl J Med* 1995; **333**: 1308-1312
10 **Maxfield FR**, Tabas I. Role of cholesterol and lipid organization in disease. *Nature* 2005; **438**: 612-621
11 **Makishima M**, Okamoto AY, Repa JJ, Tu H, Learned RM, Luk A, Hull MV, Lustig KD, Mangelsdorf DJ, Shan B. Identification of a nuclear receptor for bile acids. *Science* 1999; **284**: 1362-1365
12 **Parks DJ**, Blanchard SG, Bledsoe RK, Chandra G, Consler TG, Kliewer SA, Stimmel JB, Willson TM, Zavacki AM, Moore DD, Lehmann JM. Bile acids: natural ligands for an orphan nuclear receptor. *Science* 1999; **284**: 1365-1368
13 **Wang H**, Chen J, Hollister K, Sowers LC, Forman BM. Endogenous bile acids are ligands for the nuclear receptor FXR/BAR. *Mol Cell* 1999; **3**: 543-553
14 **Yao PM**, Tabas I. Free cholesterol loading of macrophages induces apoptosis involving the fas pathway. *J Biol Chem* 2000; **275**: 23807-23813
15 **Sever N**, Song BL, Yabe D, Goldstein JL, Brown MS, DeBose-Boyd RA. Insig-dependent ubiquitination and degradation of mammalian 3-hydroxy-3-methylglutaryl-CoA reductase stimulated by sterols and geranylgeraniol. *J Biol Chem* 2003; **278**: 52479-52490
16 **Sever N**, Yang T, Brown MS, Goldstein JL, DeBose-Boyd RA. Accelerated degradation of HMG CoA reductase mediated by binding of insig-1 to its sterol-sensing domain. *Mol Cell* 2003; **11**: 25-33
17 **Goldstein JL**, DeBose-Boyd RA, Brown MS. Protein sensors for membrane sterols. *Cell* 2006; **124**: 35-46
18 **Dietschy JM**, Turley SD. Control of cholesterol turnover in the mouse. *J Biol Chem* 2002; **277**: 3801-3804

19 **Dietschy JM**, Turley SD, Spady DK. Role of liver in the maintenance of cholesterol and low density lipoprotein homeostasis in different animal species, including humans. *J Lipid Res* 1993; **34**: 1637-1659

20 **Grundy SM**, Metzger AL. A physiological method for estimation of hepatic secretion of biliary lipids in man. *Gastroenterology* 1972; **62**: 1200-1217

21 **Grundy SM**. Absorption and metabolism of dietary cholesterol. *Annu Rev Nutr* 1983; **3**: 71-96

22 **Groen AK**, Oude Elferink RP, Verkade HJ, Kuipers F. The ins and outs of reverse cholesterol transport. *Ann Med* 2004; **36**: 135-145

23 **Watson AD**, Berliner JA, Hama SY, La Du BN, Faull KF, Fogelman AM, Navab M. Protective effect of high density lipoprotein associated paraoxonase. Inhibition of the biological activity of minimally oxidized low density lipoprotein. *J Clin Invest* 1995; **96**: 2882-2891

24 **Watson AD**, Navab M, Hama SY, Sevanian A, Prescott SM, Stafforini DM, McIntyre TM, Du BN, Fogelman AM, Berliner JA. Effect of platelet activating factor-acetylhydrolase on the formation and action of minimally oxidized low density lipoprotein. *J Clin Invest* 1995; **95**: 774-782

25 **Nofer JR**, van der Giet M, Tolle M, Wolinska I, von Wnuck Lipinski K, Baba HA, Tietge UJ, Godecke A, Ishii I, Kleuser B, Schafers M, Fobker M, Zidek W, Assmann G, Chun J, Levkau B. HDL induces NO-dependent vasorelaxation via the lysophospholipid receptor S1P3. *J Clin Invest* 2004; **113**: 569-581

26 **Voshol PJ**, Schwarz M, Rigotti A, Krieger M, Groen AK, Kuipers F. Down-regulation of intestinal scavenger receptor class B, type I (SR-BI) expression in rodents under conditions of deficient bile delivery to the intestine. *Biochem J* 2001; **356**: 317-325

27 **Hauser H**, Dyer JH, Nandy A, Vega MA, Werder M, Bieliauskaite E, Weber FE, Compassi S, Gemperli A, Boffelli D, Wehrli E, Schulthess G, Phillips MC. Identification of a receptor mediating absorption of dietary cholesterol in the intestine. *Biochemistry* 1998; **37**: 17843-17850

28 **Kramer W**, Girbig F, Corsiero D, Pfenninger A, Frick W, Jahne G, Rhein M, Wendler W, Lottspeich F, Hochleitner EO, Orso E, Schmitz G. Aminopeptidase N (CD13) is a molecular target of the cholesterol absorption inhibitor ezetimibe in the enterocyte brush border membrane. *J Biol Chem* 2005; **280**: 1306-1320

29 **Altmann SW**, Davis HR Jr, Zhu LJ, Yao X, Hoos LM, Tetzloff G, Iyer SP, Maguire M, Golovko A, Zeng M, Wang L, Murgolo N, Graziano MP. Niemann-Pick C1 Like 1 protein is critical for intestinal cholesterol absorption. *Science* 2004; **303**: 1201-1204

30 **Lu K**, Lee MH, Hazard S, Brooks-Wilson A, Hidaka H, Kojima H, Ose L, Stalenhoef AF, Mietinnen T, Bjorkhem I, Bruckert E, Pandya A, Brewer HB Jr, Salen G, Dean M, Srivastava A, Patel SB. Two genes that map to the STSL locus cause sitosterolemia: genomic structure and spectrum of mutations involving sterolin-1 and sterolin- 2, encoded by ABCG5 and ABCG8, respectively. *Am J Hum Genet* 2001; **69**: 278-290

31 **Lee MH**, Lu K, Hazard S, Yu H, Shulenin S, Hidaka H, Kojima H, Allikmets R, Sakuma N, Pegoraro R, Srivastava AK, Salen G, Dean M, Patel SB. Identification of a gene, ABCG5, important in the regulation of dietary cholesterol absorption. *Nat Genet* 2001; **27**: 79-83

32 **Berge KE**, Tian H, Graf GA, Yu L, Grishin NV, Schultz J, Kwiterovich P, Shan B, Barnes R, Hobbs HH. Accumulation of dietary cholesterol in sitosterolemia caused by mutations in adjacent ABC transporters. *Science* 2000; **290**: 1771-1775

33 **Salisbury BG**, Davis HR, Burrier RE, Burnett DA, Bowkow G, Caplen MA, Clemmons AL, Compton DS, Hoos LM, McGregor DG. Hypocholesterolemic activity of a novel inhibitor of cholesterol absorption, SCH 48461. *Atherosclerosis* 1995; **115**: 45-63

34 **Davis HR Jr**, Pula KK, Alton KB, Burrier RE, Watkins RW. The synergistic hypocholesterolemic activity of the potent cholesterol absorption inhibitor, ezetimibe, in combination with 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibitors in dogs. *Metabolism* 2001; **50**: 1234-1241

35 **Van Heek M**, France CF, Compton DS, McLeod RL, Yumibe NP, Alton KB, Sybertz EJ, Davis HR Jr. In vivo metabolism-based discovery of a potent cholesterol absorption inhibitor, SCH58235, in the rat and rhesus monkey through the identification of the active metabolites of SCH48461. *J Pharmacol Exp Ther* 1997; **283**: 157-163

36 **van Heek M**, Farley C, Compton DS, Hoos L, Alton KB, Sybertz EJ, Davis HR Jr. Comparison of the activity and disposition of the novel cholesterol absorption inhibitor, SCH58235, and its glucuronide, SCH60663. *Br J Pharmacol* 2000; **129**: 1748-1754

37 **van Heek M**, Compton DS, Davis HR. The cholesterol absorption inhibitor, ezetimibe, decreases diet-induced hypercholesterolemia in monkeys. *Eur J Pharmacol* 2001; **415**: 79-84

38 **Garcia-Calvo M**, Lisnock J, Bull HG, Hawes BE, Burnett DA, Braun MP, Crona JH, Davis HR Jr, Dean DC, Detmers PA, Graziano MP, Hughes M, Macintyre DE, Ogawa A, O'neill KA, Iyer SP, Shevell DE, Smith MM, Tang YS, Makarewicz AM, Ujjainwalla F, Altmann SW, Chapman KT, Thornberry NA. The target of ezetimibe is Niemann-Pick C1-Like 1 (NPC1L1). *Proc Natl Acad Sci USA* 2005; **102**: 8132-8137

39 **Iyer SP**, Yao X, Crona JH, Hoos LM, Tetzloff G, Davis HR Jr, Graziano MP, Altmann SW. Characterization of the putative native and recombinant rat sterol transporter Niemann-Pick C1 Like 1 (NPC1L1) protein. *Biochim Biophys Acta* 2005; **1722**: 282-292

40 **Davies JP**, Levy B, Ioannou YA. Evidence for a Niemann-pick C (NPC) gene family: identification and characterization of NPC1L1. *Genomics* 2000; **65**: 137-145

41 **Smart EJ**, De Rose RA, Farber SA. Annexin 2-caveolin 1 complex is a target of ezetimibe and regulates intestinal cholesterol transport. *Proc Natl Acad Sci USA* 2004; **101**: 3450-3455

42 **Valasek MA**, Weng J, Shaul PW, Anderson RG, Repa JJ. Caveolin-1 is not required for murine intestinal cholesterol transport. *J Biol Chem* 2005; **280**: 28103-28109

43 **Kramer W**, Corsiero D, Girbig F, Jahne G. Rabbit small intestine does not contain an annexin II/caveolin 1 complex as a target for 2-azetidinone cholesterol absorption inhibitors. *Biochim Biophys Acta* 2006; **1758**: 45-54

44 **Graf GA**, Li WP, Gerard RD, Gelissen I, White A, Cohen JC, Hobbs HH. Coexpression of ATP-binding cassette proteins ABCG5 and ABCG8 permits their transport to the apical surface. *J Clin Invest* 2002; **110**: 659-669

45 **Yu L**, Hammer RE, Li-Hawkins J, von Bergmann K, Lütjohann D, Cohen JC, Hobbs HH. Disruption of Abcg5 and Abcg8 in mice reveals their crucial role in biliary cholesterol secretion. *Proc Natl Acad Sci USA* 2002; **99**: 16237-16242

46 **Plösch T**, Bloks VW, Terasawa Y, Berdy S, Siegler K, Van Der Sluijs F, Kema IP, Groen AK, Shan B, Kuipers F, Schwarz M. Sitosterolemia in ABC-transporter G5-deficient mice is aggravated on activation of the liver-X receptor. *Gastroenterology* 2004; **126**: 290-300

47 **Klett EL**, Lu K, Kosters A, Vink E, Lee MH, Altenburg M, Shefer S, Batta AK, Yu H, Chen J, Klein R, Looije N, Oude-Elferink R, Groen AK, Maeda N, Salen G, Patel SB. A mouse model of sitosterolemia: absence of Abcg8/sterolin-2 results in failure to secrete biliary cholesterol. *BMC Med* 2004; **2**: 5

48 **Yu L**, Li-Hawkins J, Hammer RE, Berge KE, Horton JD, Cohen JC, Hobbs HH. Overexpression of ABCG5 and ABCG8 promotes biliary cholesterol secretion and reduces fractional absorption of dietary cholesterol. *J Clin Invest* 2002; **110**: 671-680

49 **Yu L**, York J, von Bergmann K, Lütjohann D, Cohen JC, Hobbs HH. Stimulation of cholesterol excretion by the liver X receptor agonist requires ATP-binding cassette transporters G5 and G8. *J Biol Chem* 2003; **278**: 15565-15570

50 **Cai L**, Eckhardt ER, Shi W, Zhao Z, Nasser M, de Villiers WJ, van der Westhuyzen DR. Scavenger receptor class B type I reduces cholesterol absorption in cultured enterocyte CaCo-2 cells. *J Lipid Res* 2004; **45**: 253-262

51 **Mardones P**, Quinones V, Amigo L, Moreno M, Miquel JF, Schwarz M, Miettinen HE, Trigatti B, Krieger M, VanPatten S, Cohen DE, Rigotti A. Hepatic cholesterol and bile acid metabolism and intestinal cholesterol absorption in scavenger receptor class B type I-deficient mice. *J Lipid Res* 2001; **42**: 170-180

52 **Bietrix F**, Yan D, Nauze M, Rolland C, Bertrand-Michel J, Comera C, Schaak S, Barbaras R, Groen AK, Perret B, Terce F, Collet X. Accelerated lipid absorption in mice overexpressing intestinal SR-BI. *J Biol Chem* 2006; **281**: 7214-7219

53 **Repa JJ**, Turley SD, Lobaccaro JA, Medina J, Li L, Lustig K, Shan B, Heyman RA, Dietschy JM, Mangelsdorf DJ. Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers. *Science* 2000; **289**: 1524-1529

54 **Mulligan JD**, Flowers MT, Tebon A, Bitgood JJ, Wellington C, Hayden MR, Attie AD. ABCA1 is essential for efficient basolateral cholesterol efflux during the absorption of dietary cholesterol in chickens. *J Biol Chem* 2003; **278**: 13356-13366

55 **Ohama T**, Hirano K, Zhang Z, Aoki R, Tsujii K, Nakagawa-Toyama Y, Tsukamoto K, Ikegami C, Matsuyama A, Ishigami M, Sakai N, Hiraoka H, Ueda K, Yamashita S, Matsuzawa Y. Dominant expression of ATP-binding cassette transporter-1 on basolateral surface of Caco-2 cells stimulated by LXR/RXR ligands. *Biochem Biophys Res Commun* 2002; **296**: 625-630

56 **Drobnik W**, Lindenthal B, Lieser B, Ritter M, Christiansen Weber T, Liebisch G, Giesa U, Igel M, Borsukova H, Buchler C, Fung-Leung WP, Von Bergmann K, Schmitz G. ATP-binding cassette transporter A1 (ABCA1) affects total body sterol metabolism. *Gastroenterology* 2001; **120**: 1203-1211

57 **McNeish J**, Aiello RJ, Guyot D, Turi T, Gabel C, Aldinger C, Hoppe KL, Roach ML, Royer LJ, de Wet J, Broccardo C, Chimini G, Francone OL. High density lipoprotein deficiency and foam cell accumulation in mice with targeted disruption of ATP-binding cassette transporter-1. *Proc Natl Acad Sci USA* 2000; **97**: 4245-4250

58 **Buhman KK**, Accad M, Novak S, Choi RS, Wong JS, Hamilton RL, Turley S, Farese RV Jr. Resistance to diet-induced hypercholesterolemia and gallstone formation in ACAT2-deficient mice. *Nat Med* 2000; **6**: 1341-1347

59 **Jones B**, Jones EL, Bonney SA, Patel HN, Mensenkamp AR, Eichenbaum-Voline S, Rudling M, Myrdal U, Annesi G, Naik S, Meadows N, Quattrone A, Islam SA, Naoumova RP, Angelin B, Infante R, Levy E, Roy CC, Freemont PS, Scott J, Shoulders CC. Mutations in a Sar1 GTPase of COPII vesicles are associated with lipid absorption disorders. *Nat Genet* 2003; **34**: 29-31

60 **van der Veen JN**, Kruit JK, Havinga R, Baller JF, Chimini G, Lestavel S, Staels B, Groot PH, Groen AK, Kuipers F. Reduced cholesterol absorption upon PPARdelta activation coincides with decreased intestinal expression of NPC1L1. *J Lipid Res* 2005; **46**: 526-534

61 **Janowski BA**, Willy PJ, Devi TR, Falck JR, Mangelsdorf DJ. An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. *Nature* 1996; **383**: 728-731

62 **Janowski BA**, Grogan MJ, Jones SA, Wisely GB, Kliewer SA, Corey EJ, Mangelsdorf DJ. Structural requirements of ligands for the oxysterol liver X receptors LXRalpha and LXRbeta. *Proc Natl Acad Sci USA* 1999; **96**: 266-271

63 **Repa JJ**, Berge KE, Pomajzl C, Richardson JA, Hobbs H, Mangelsdorf DJ. Regulation of ATP-binding cassette sterol transporters ABCG5 and ABCG8 by the liver X receptors alpha and beta. *J Biol Chem* 2002; **277**: 18793-18800

64 **Venkateswaran A**, Repa JJ, Lobaccaro JM, Bronson A, Mangelsdorf DJ, Edwards PA. Human white/murine ABC8 mRNA levels are highly induced in lipid-loaded macrophages. A transcriptional role for specific oxysterols. *J Biol Chem* 2000; **275**: 14700-14707

65 **Grefhorst A**, Elzinga BM, Voshol PJ, Plösch T, Kok T, Bloks VW, van der Sluijs FH, Havekes LM, Romijn JA, Verkade HJ, Kuipers F. Stimulation of lipogenesis by pharmacological activation of the liver X receptor leads to production of large, triglyceride-rich very low density lipoprotein particles. *J Biol Chem* 2002; **277**: 34182-34190

66 **Plösch T**, Kok T, Bloks VW, Smit MJ, Havinga R, Chimini G, Groen AK, Kuipers F. Increased hepatobiliary and fecal cholesterol excretion upon activation of the liver X receptor is independent of ABCA1. *J Biol Chem* 2002; **277**: 33870-33877

67 **Duval C**, Touche V, Tailleux A, Fruchart JC, Fievet C, Clavey V, Staels B, Lestavel S. Niemann-Pick C1 like 1 gene expression is down-regulated by LXR activators in the intestine. *Biochem Biophys Res Commun* 2006; **340**: 1259-1263

68 **Mel'nikov SM**, Seijen ten Hoorn JW, Eijkelenboom AP. Effect of phytosterols and phytostanols on the solubilization of cholesterol by dietary mixed micelles: an in vitro study. *Chem Phys Lipids* 2004; **127**: 121-141

69 **Igel M**, Giesa U, Lütjohann D, von Bergmann K. Comparison of the intestinal uptake of cholesterol, plant sterols, and stanols in mice. *J Lipid Res* 2003; **44**: 533-538

70 **Plat J**, van Onselen EN, van Heugten MM, Mensink RP. Effects on serum lipids, lipoproteins and fat soluble antioxidant concentrations of consumption frequency of margarines and shortenings enriched with plant stanol esters. *Eur J Clin Nutr* 2000; **54**: 671-677

71 **Kaneko E**, Matsuda M, Yamada Y, Tachibana Y, Shimomura I, Makishima M. Induction of intestinal ATP-binding cassette transporters by a phytosterol-derived liver X receptor agonist. *J Biol Chem* 2003; **278**: 36091-39608

72 **Plat J**, Mensink RP. Increased intestinal ABCA1 expression contributes to the decrease in cholesterol absorption after plant stanol consumption. *FASEB J* 2002; **16**: 1248-1253

73 **Field FJ**, Born E, Mathur SN. Stanol esters decrease plasma cholesterol independently of intestinal ABC sterol transporters and Niemann-Pick C1-like 1 protein gene expression. *J Lipid Res* 2004; **45**: 2252-2259

74 **Calpe-Berdiel L**, Escola-Gil JC, Ribas V, Navarro-Sastre A, Garces-Garces J, Blanco-Vaca F. Changes in intestinal and liver global gene expression in response to a phytosterol-enriched diet. *Atherosclerosis* 2005; **181**: 75-85

75 **Kruit JK**, Plosch T, Havinga R, Boverhof R, Groot PH, Groen AK, Kuipers F. Increased fecal neutral sterol loss upon liver X receptor activation is independent of biliary sterol secretion in mice. *Gastroenterology* 2005; **128**: 147-156

76 **Cheng SH**, Stanley MM. Secretion of cholesterol by intestinal mucosa in patients with complete common bile duct obstruction. *Proc Soc Exp Biol Med* 1959; **101**: 223-225

77 **Simmonds WJ**, Hofmann AF, Theodor E. Absorption of cholesterol from a micellar solution: intestinal perfusion studies in man. *J Clin Invest* 1967; **46**: 874-890

78 **Schwarz M**, Russell DW, Dietschy JM, Turley SD. Marked reduction in bile acid synthesis in cholesterol 7alpha-hydroxylase-deficient mice does not lead to diminished tissue cholesterol turnover or to hypercholesterolemia. *J Lipid Res* 1998; **39**: 1833-1843

79 **Russell DW**, Setchell KD. Bile acid biosynthesis. *Biochemistry* 1992; **31**: 4737-4749

80 **Oude Elferink RP**, Ottenhoff R, van Wijland M, Frijters CM, van Nieuwkerk C, Groen AK. Uncoupling of biliary phospholipid and cholesterol secretion in mice with reduced expression of mdr2 P-glycoprotein. *J Lipid Res* 1996; **37**: 1065-1075

81 **Smit JJ**, Schinkel AH, Oude Elferink RP, Groen AK, Wagenaar E, van Deemter L, Mol CA, Ottenhoff R, van der Lugt NM, van Roon MA. Homozygous disruption of the murine mdr2 P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. *Cell* 1993; **75**: 451-462

82 **Langheim S**, Yu L, von Bergmann K, Lutjohann D, Xu F, Hobbs HH, Cohen JC. ABCG5 and ABCG8 require MDR2 for secretion of cholesterol into bile. *J Lipid Res* 2005; **46**: 1732-1738

83 **Glickman RM**, Green PH. The intestine as a source of apolipoprotein A1. *Proc Natl Acad Sci USA* 1977; **74**: 2569-2573

84 **Wu AL**, Windmueller HG. Relative contributions by liver and intestine to individual plasma apolipoproteins in the rat. *J Biol Chem* 1979; **254**: 7316-7322

85 **Roheim PS**, Gidez LI, Eder HA. Extrahepatic synthesis of lipoproteins of plasma and chyle: role of the intestine. *J Clin Invest* 1966; **45**: 297-300

86 **Bearnot HR**, Glickman RM, Weinberg L, Green PH, Tall AR. Effect of biliary diversion on rat mesenteric lymph apolipoprotein-I and high density lipoprotein. *J Clin Invest* 1982; **69**: 210-217

87 **Forester GP**, Tall AR, Bisgaier CL, Glickman RM. Rat intestine secretes spherical high density lipoproteins. *J Biol Chem* 1983; **258**: 5938-5943

88 **Green PH**, Tall AR, Glickman RM. Rat intestine secretes

discoid high density lipoprotein. *J Clin Invest* 1978; **61**: 528-534
89 **Holt PR**, Wu AL, Clark SB. Apoprotein composition and turnover in rat intestinal lymph during steady-state triglyceride absorption. *J Lipid Res* 1979; **20**: 494-502
90 **Iqbal J**, Anwar K, Hussain MM. Multiple, independently regulated pathways of cholesterol transport across the intestinal epithelial cells. *J Biol Chem* 2003; **278**: 31610-31620
91 **Iqbal J**, Hussain MM. Evidence for multiple complementary pathways for efficient cholesterol absorption in mice. *J Lipid Res* 2005; **46**: 1491-1501
92 **Bodzioch M**, Orso E, Klucken J, Langmann T, Bottcher A, Diederich W, Drobnik W, Barlage S, Buchler C, Porsch-Ozcurumez M, Kaminski WE, Hahmann HW, Oette K, Rothe G, Aslanidis C, Lackner KJ, Schmitz G. The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease. *Nat Genet* 1999; **22**: 347-351
93 **Brooks-Wilson A**, Marcil M, Clee SM, Zhang LH, Roomp K, van Dam M, Yu L, Brewer C, Collins JA, Molhuizen HO, Loubser O, Ouelette BF, Fichter K, Ashbourne-Excoffon KJ, Sensen CW, Scherer S, Mott S, Denis M, Martindale D, Frohlich J, Morgan K, Koop B, Pimstone S, Kastelein JJ, Genest J Jr, Hayden MR. Mutations in ABC1 in Tangier disease and familial high-density lipoprotein deficiency. *Nat Genet* 1999; **22**: 336-345
94 **Rust S**, Rosier M, Funke H, Real J, Amoura Z, Piette JC, Deleuze JF, Brewer HB, Duverger N, Denefle P, Assmann G. Tangier disease is caused by mutations in the gene encoding ATP-binding cassette transporter 1. *Nat Genet* 1999; **22**: 352-355
95 **Assmann G**, Smootz E, Adler K, Capurso A, Oette K. The lipoprotein abnormality in Tangier disease: quantitation of A apoproteins. *J Clin Invest* 1977; **59**: 565-575
96 **Wellington CL**, Walker EK, Suarez A, Kwok A, Bissada N, Singaraja R, Yang YZ, Zhang LH, James E, Wilson JE, Francone O, McManus BM, Hayden MR. ABCA1 mRNA and protein distribution patterns predict multiple different roles and levels of regulation. *Lab Invest* 2002; **82**: 273-283
97 **Haghpassand M**, Bourassa PA, Francone OL, Aiello RJ. Monocyte/macrophage expression of ABCA1 has minimal contribution to plasma HDL levels. *J Clin Invest* 2001; **108**: 1315-1320
98 **Aiello RJ**, Brees D, Bourassa PA, Royer L, Lindsey S, Coskran T, Haghpassand M, Francone OL. Increased atherosclerosis in hyperlipidemic mice with inactivation of ABCA1 in macrophages. *Arterioscler Thromb Vasc Biol* 2002; **22**: 630-637
99 **van Eck M**, Bos IS, Kaminski WE, Orso E, Rothe G, Twisk J, Bottcher A, Van Amersfoort ES, Christiansen-Weber TA, Fung-Leung WP, Van Berkel TJ, Schmitz G. Leukocyte ABCA1 controls susceptibility to atherosclerosis and macrophage recruitment into tissues. *Proc Natl Acad Sci USA* 2002; **99**: 6298-6303
100 **Van Eck M**, Singaraja RR, Ye D, Hildebrand RB, James ER, Hayden MR, Van Berkel TJ. Macrophage ATP-binding cassette transporter A1 overexpression inhibits atherosclerotic lesion progression in low-density lipoprotein receptor knockout mice. *Arterioscler Thromb Vasc Biol* 2006; **26**: 929-934
101 **Basso F**, Freeman L, Knapper CL, Remaley A, Stonik J, Neufeld EB, Tansey T, Amar MJ, Fruchart-Najib J, Duverger N, Santamarina-Fojo S, Brewer HB Jr. Role of the hepatic ABCA1 transporter in modulating intrahepatic cholesterol and plasma HDL cholesterol concentrations. *J Lipid Res* 2003; **44**: 296-302
102 **Wellington CL**, Brunham LR, Zhou S, Singaraja RR, Visscher H, Gelfer A, Ross C, James E, Liu G, Huber MT, Yang YZ, Parks RJ, Groen A, Fruchart-Najib J, Hayden MR. Alterations of plasma lipids in mice via adenoviral-mediated hepatic overexpression of human ABCA1. *J Lipid Res* 2003; **44**: 1470-1480
103 **Ragozin S**, Niemeier A, Laatsch A, Loeffler B, Merkel M, Beisiegel U, Heeren J. Knockdown of hepatic ABCA1 by RNA interference decreases plasma HDL cholesterol levels and influences postprandial lipemia in mice. *Arterioscler Thromb Vasc Biol* 2005; **25**: 1433-1438
104 **Timmins JM**, Lee JY, Boudyguina E, Kluckman KD, Brunham LR, Mulya A, Gebre AK, Coutinho JM, Colvin PL, Smith TL, Hayden MR, Maeda N, Parks JS. Targeted inactivation of hepatic Abca1 causes profound hypoalphalipoproteinemia and kidney hypercatabolism of apoA-I. *J Clin Invest* 2005; **115**: 1333-1342
105 **Brunham LR**, Kruit JK, Iqbal J, Fievet C, Timmins JM, Pape TD, Coburn BA, Bissada N, Staels B, Groen AK, Hussain MM, Parks JS, Kuipers F, Hayden MR. Intestinal ABCA1 directly contributes to HDL biogenesis in vivo. *J Clin Invest* 2006; **116**: 1052-1062
106 **Oliveira HC**, Nilausen K, Meinertz H, Quintao EC. Cholesteryl esters in lymph chylomicrons: contribution from high density lipoprotein transferred from plasma into intestinal lymph. *J Lipid Res* 1993; **34**: 1729-1736
107 **Quintao EC**, Drewiacki A, Stechhaln K, de Faria EC, Sipahi AM. Origin of cholesterol transported in intestinal lymph: studies in patients with filarial chyluria. *J Lipid Res* 1979; **20**: 941-945
108 **Joseph SB**, McKilligin E, Pei L, Watson MA, Collins AR, Laffitte BA, Chen M, Noh G, Goodman J, Hagger GN, Tran J, Tippin TK, Wang X, Lusis AJ, Hsueh WA, Law RE, Collins JL, Willson TM, Tontonoz P. Synthetic LXR ligand inhibits the development of atherosclerosis in mice. *Proc Natl Acad Sci USA* 2002; **99**: 7604-7609
109 **Terasaka N**, Hiroshima A, Koieyama T, Ubukata N, Morikawa Y, Nakai D, Inaba T. T-0901317, a synthetic liver X receptor ligand, inhibits development of atherosclerosis in LDL receptor-deficient mice. *FEBS Lett* 2003; **536**: 6-11

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REVIEW

Trichinosis: Epidemiology in Thailand

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Abstract

Trichinosis is one of the most common food-borne parasitic zoonoses in Thailand and many outbreaks are reported each year. This paper reviews the history, species, and epidemiology of the disease and food habits of the people with an emphasis on the north, northeast, central and south regions of Thailand. The earliest record of trichinosis in Thailand was in 1962 in the Mae Sariang District, Mae Hong Son Province. Since then, about 130 outbreaks have been reported involving 7392 patients and 97 deaths (1962-2005). The highest number of cases, 557, was recorded in 1983. The annual epidemiological surveillance reports of the Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health, Thailand, show that trichinosis cases increased from 61 in 1997 to 351 in 1998. In contrast to these figures, the number of reported cases decreased to 16 in 1999 and 128 cases in 2000. There was no record of trichinosis in 2001, but then the figures for 2002, 2003 and 2004 were 289, 126 and 212 respectively. The infected patients were mostly in the 35-44 years age group and the disease occurred more frequently in men than women at a ratio of 1.7-2.0:1. There were 84 reported cases of trichinosis in Chiang Rai, Nan, Chiang Mai, Si Sa ket, Nakhon Phanom, Kalasin, Nakhon Ratchasima, Nakhon Nayok, Nakhon Pathom and Surat Thani, provinces located in different parts of Thailand in 2005. The outbreaks were more common in the northern areas, especially in rural areas where people ate raw or under-cooked pork and/or wild animals. This indicates the need for health education programs to prevent and control trichinosis as soon as possible in the high-risk areas.

Key words: Trichinosis; Epidemiology; Thailand

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INTRODUCTION

Trichinosis is one of the most widespread helminthic zoonoses. Unlike other parasitic infections, it has been a major public health problem and reported in many Asian countries, including China, Japan, Korea and Thailand[1-7]. Since 1835, controversy has surrounded the discovery and description of *Trichinella*. In that year the organism, *T. spiralis*, was initially observed by a first-year medical student, James Paget, later famous for other medical achievements, but it was named and described by his professor, Richard Owen. Until recently, that species was the only one known[8]. However, seven distinct species of *Trichinella* are now recognized, *T. spiralis, T. pseudospiralis, T. native, T. nelsoni*, *T. britovi*, *T. papuae* and *T.murrelli*[9-16]. In Thailand, human trichinosis was first reported in June 1962. The causative agent of most outbreaks of this disease has been identified as *T. spiralis*[1], but an outbreak in the Ta Sae District, Chumporn Province in 1994 was due to *T. pseudispiralis*[17]. Trichinosis infections in humans are characterized by an initial phase dominated by gastro-intestinal symptoms (vomiting and diarrhea), followed by a stage lasting about 2 mo, of fever, sub-cutaneous edema, muscle pain, cachexy, myocardiosis and weakness. Death occurs in up to 40% of cases either due to anaphylactic shock or the consequences of the myocardiosis[7,18-23]. In eight cases of childhood trichinosis reported in Thailand, major symptoms and signs were fever, myalgia, puffy face and eyelids. Laboratory examinations showed leukocytosis, eosinophilia and elevation of muscle enzymes[24]. The outbreaks of trichinosis in Thailand seem to depend in part on the density of *Trichinella* contamination in domestic and wild animals, but they are also influenced by social and other factors, including people's eating habits[25-30]. This study includes a review of the history, species, epidemiology of trichinosis and food habits of Thai people based on previous studies and the annual epidemiological surveillance reports from 2002-2005 from the Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health, Thailand.

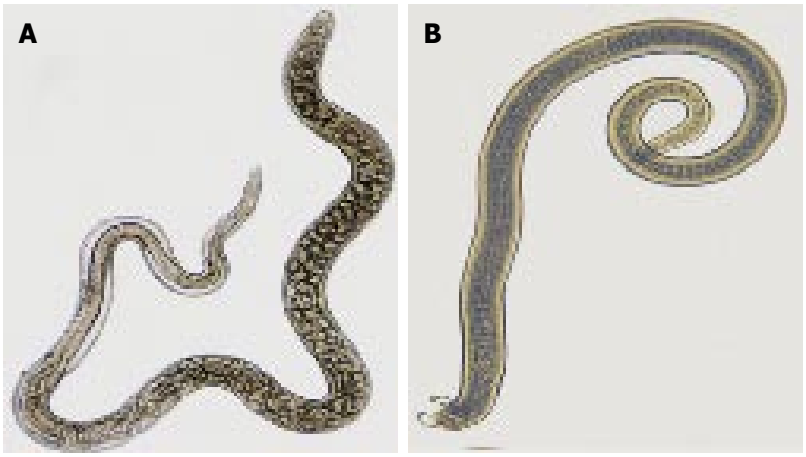

Figure 1 *T. spiralis* in Thailand[50]. **A**: Adult female *T. spiralis* with fully formed larvae in uterus; **B**: Adult male *T. spiralis* claspers on tail (lower end).

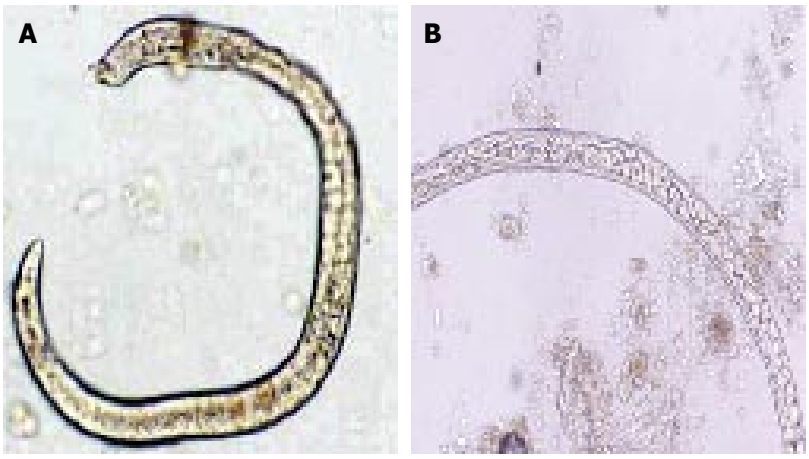

Figure 2 *T. pseudospiralis* in Thailand[51]. **A**: Adult male *T. pseudospiralis*; **B**: Adult female *T. pseudospiralis* containing eggs and larvae.

HISTORICAL REVIEW OF TRICHINOSIS IN THAILAND

The first outbreak of trichinosis in Thailand was in 1962 and involved 56 patients resulting in 11 deaths in the Mae Sariang District, Mae Hong Son Province. Meat from pigs was the source of outbreak[1]. The number of outbreaks has tended to increase in recent years. The annual epidemiological surveillance report dated 15 November 2005 recorded 7392 patients and 97 deaths. The highest annual number of hospital recorded trichinosis cases was 557 in 1983. This figure is considered an underestimation of the actual number of cases involved in the outbreaks[31-36]. In April 1973, an outbreak of trichinosis occurred in the Mae Sruay District, Chiang Rai Province. Thirty-one persons were involved, ranging from 9 to 72 years, and one adult female died[37]. In 1980, trichinosis was reported in the Pluak Dang District of Rayong Province, the infection being caused by the consumption of wild squirrel[38,39]. An epidemic of trichinosis involving 177 patients and 13 deaths occurred in April 1981 in Kok-Ta-Back Village, Nong-Pai District, Petchabun Province, and reported the fourteenth outbreak of human trichinosis in Thailand[40-42]. Khambooruang[43] reported 118 discrete outbreaks of the disease involving 5400 patients and 95 deaths. In the south of Thailand, an outbreak of trichinosis affecting 59 individuals resulting in one death occurred in Chumporn Province during 1994-1995. This was the first report of an epidemic of human infection caused by *T. pseudospiralis*[7]. Takahashi *et al*[35] reported 120 outbreaks from 1962 to 2000 involving nearly 6700 patients and 97 deaths. The highest number of cases was in Chiang Mai, Chiang Rai and Nan provinces, 1776, 1739 and 894 respectively. Chotmongkol *et al*[7] presented the case of a 49-year-old man with progressive generalized muscle hypertrophy and weakness for 3 mo. Histologic findings from muscle biopsy demonstrated a nurse cell-larva complex. Treatment with albendazole resulted in a very favorable outcome. Trichinosis remains a major public health problem in Thailand, often associated with rural people celebrating local and traditional festivals, such as the northern Thai New Year and wedding ceremonies, at which raw and/or under-cooked wild animals are eaten.

SPECIES OF TRICHINOSIS IN THAILAND

Seven species belonging to the *Trichinella* genus, five with encapsulated larvae and two with non-encapsulated larvae in host muscles and three additional genotypes, have been described to date: *T. spiralis*, *T. nativa*, *T. britovi, T. murrelli, T. nelsoni, T. pseudospiralis*, and *T. papuae*. In Southeast Asia, *T. spiralis* and *T. pseudospiralis* have been documented in domestic animals and/or humans in Cambodia, Indonesia (Bali and Sumatra), Lao PDR, Malaysia, Myanmar and Thailand[10,12,14,15,44]. In Thailand, the causative agent of most outbreaks of trichinosis has been identified as *T. spiralis*[1] (Figure 1). Meanwhile, Jongwutiwes *et al*[17] reported human infection by *T. pseudospiralis* (Figure 2). An outbreak

of trichinosis affecting 59 individuals, of whom one died, occurred in south Thailand during 1994-1995. After that, there were no reports of other species of *Trichinella* in this country in either humans or animals[35]. Until recently, *T. spiralis* and *T. pseudospiralis* were the only human- infecting species in Thailand.

EPIDEMIOLOGY OF TRICHINOSIS IN THAILAND

Trichinosis is more common in temperate regions than in tropical regions. The epidemiology of trichinosis was first reported in 1962 in patients who consumed pig meat[1]. The second outbreak was in 1963 at Prao District, Chiang Mai Province. Since then, outbreaks have occurred each year, mostly in the northern part of Thailand where people have eaten raw or under-cooked pork and/or wild animals[45-47]. The annual epidemiological surveillance reports indicated that trichinosis cases increased from 61 in 1997 to 351 in 1998. In 1999 and 2000, the number of reported cases decreased to 16 and 128 respectively. No cases were recorded in 2001, hospital based or by the Bureau of Epidemiology, that clearly showed a human trichinosis case this year, but then 289, 126 and 212 occurred in 2002, 2003 and 2004 respectively. In 2005, 75 cases were reported by the Bureau of Epidemiology, Department of Disease Control, and Ministry of Public Health (Figure 3). Since then, about 130 outbreaks have been reported totaling 7392 patients and 97 deaths.

Since 2002, the distribution of human trichinosis cases by age groups has been considered by the annual epidemiological surveillance reports which data were the hospital based. The youngest patient was about 1 year old. Charkrit[44] reported a patient of the same age. It is not uncommon to see patients in the 10-14 and 65+ age groups, but most patients are in the age 35-44 groups, morbidity rate was 0.04 per 100 000 of people (Figure 4). Infection occurs in men more frequently than women at the ratio of 1.7-2:1 (calculated from trichinosis cases of 2002-2005). This result was similar to the 1.2-2.6:1 found by Charkrit[44].

The epidemiological surveillance reports of trichinosis have been conducted almost every year and data investigation reveals that the outbreaks have occurred predominantly in rural areas. The north part of Thailand is responsible for 96.4% of all cases reported from 1962 to 2000[35]. The annual epidemiological surveillance reports from 2002 to 2005 found consistently high numbers of cases (289, 126, 30 and 60 respectively) in the north region. The figures for 2004 reported 124 in the northeast, the first time that a region other than the north has had the highest number of cases. Only small numbers of trichinosis cases were recorded in the central and south regions in 2005 (Figure 5). In 2005, 75 trichinosis cases were reported, the highest number occurring in October, August, March and September, 36, 16, 7 and 5 respectively. The cases were reported in Chiang Rai, Nan, Chiang Mai, Si Sa ket, Nakon Phanom, Kalasin, Nakhon Ratchasima, Nakhon Nayok and Surat Thani, all provinces located in different parts of Thailand. The main age group was 35-44 years and the youngest patient was 1.

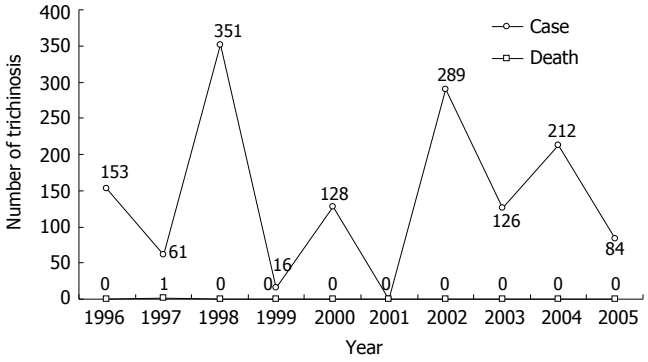

Figure 3 Trichinosis cases in Thailand from 1995-2005.

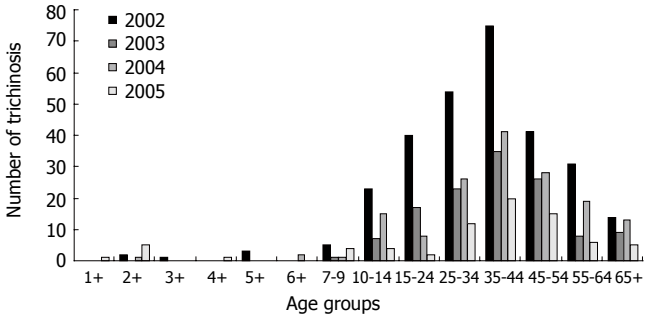

Figure 4 Human trichinosis cases in Thailand from 2002-2005.

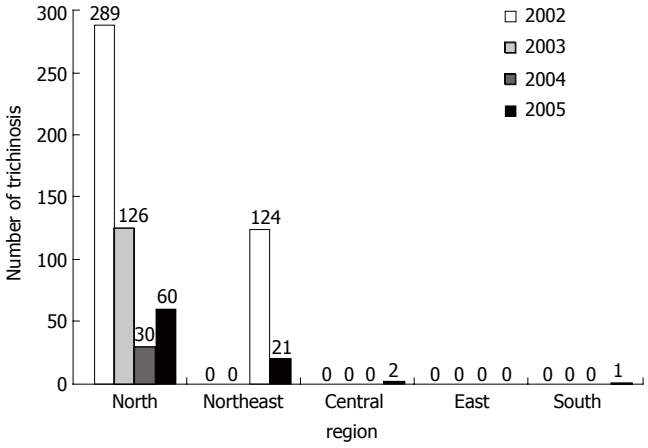

Figure 5 Human trichinosis by region in Thailand from 2002-2005.

Most outbreaks occurred in the north region, including 60.84% of all cases reported from 1962 to 2005. These results were different from those of Takahashi *et al* (2000) that reported the north region was responsible for 96.4% of all cases. The most severely affected areas in the north region were the highland provinces of Chiang Rai, Nan, Chiang Mai, Mae Hong Son and Payao. (Figure 6). The numbers of cases in other parts of Thailand were very few. In the central region, Uthai Thani, Karnchanaburi Nakhon Pathom and Nakhon Nayok provinces reported 0.28% of the total number of cases. Chumporn, Songkla and Surat Thani were the only three provinces of the south region in

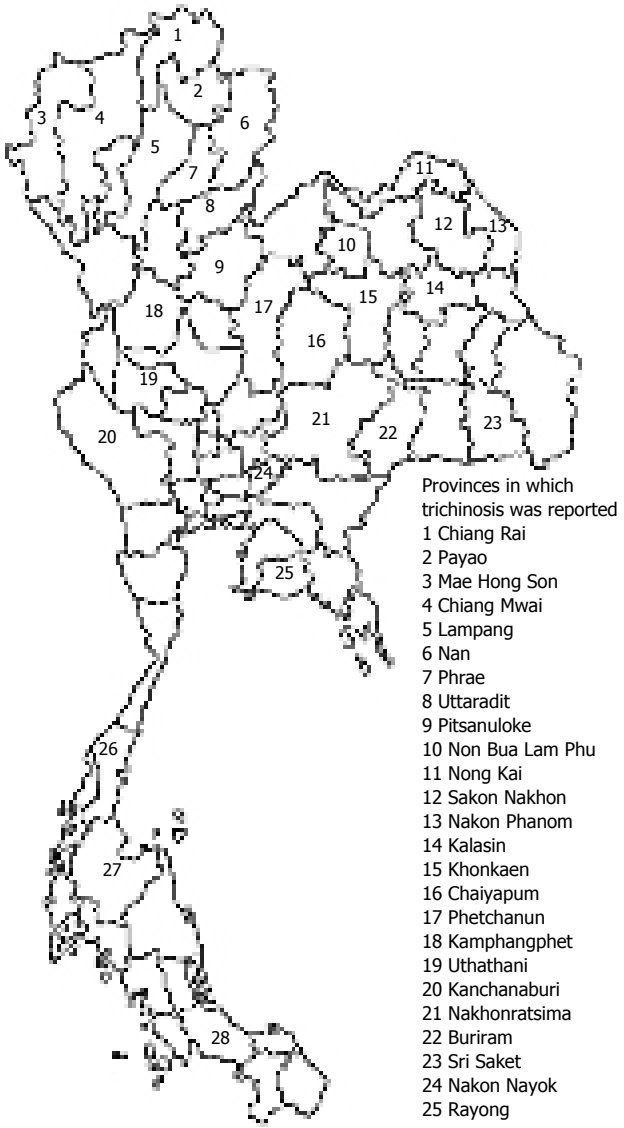

Figure 6 Provinces trichinosis reported in Thailand from 1962-2005.

Table 1 Distribution of trichinosis in humans by province in Thailand from 2002-2005

Region	Province	Total cases (%)
North	Chiang Mai, Chiang Rai, Nan, Phrae, Uttaradit, Payao, Mae Hong Son, Petchabun, Kamphaengpetch, Pitsanuloke, Lampang	60.87
Northeast	Nong Bua Lam Phu, Buri Rum, Kalasin, Sakol Nakorn, Sri Saket, Nong Kai, Khon Kaen, Nakhon Phanom, Nakhon Ratchasima	38.59
East	Rayong	0.18
Central	Uthai Thani, Karnchanaburi, Nakhon Nayok, Nakhon Pathom	0.18
South	Chumporn, Songkla, Surat Thani	0.18

which cases of trichinosis were observed, these accounting for only 0.28% of cases (Table 1). The northeast of Thailand was responsible for the highest number of cases in 2004 and the second highest (38.5%) in 2005. Provinces involved were Nong Bua Lam Phu, Buri Ram, Kalasin, Sakol Nakorn, Sri Saket, Nong Kai, Khon Kaen, Nakhon Phanom and Nakhon Ratchasima. These results showed trichinosis as a serious problem, particularly in the north and northeast regions of Thailand.

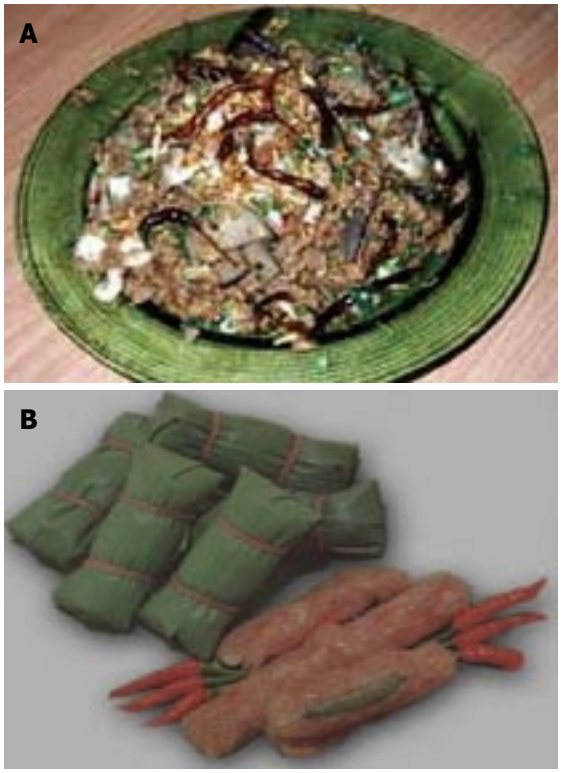

Figure 7 The dishes of raw or under-cooked wild boar are the favorite local foods that are the major sources of infection in Thailand. **A**: Lahb is made from wild pig or reptile; **B**: Nham is also made from wild pig and fermented for a few days.

SOURCE OF THAI HUMAN INFECTION

Trichinosis is a parasitic disease of mammals caused by the nematode parasite Trichinella spp. It has an important zoonosis with humans becoming infected by eating raw or inadequately cooked infected meat. Infection is more common in omnivores (horses, humans, pigs and rats) and carnivores (cats, dogs, and seals). Pigs and rodents seem to play the most important role in the epidemiology of the disease. The main source of infection in Thailand has been pigs, but wild boar, jackal and black bear were also reported as sources of trichinosis[45,46]. All trichinosis cases gave a history of having consumed raw pork in the form of "lahb" and "nahm," favorite dishes of north Thailand[37] (Figure 7). Lahb is made from chopped raw pork mixed with lemon juice, roasted rice powder, finely cut red onion and parsley (Figure 7A). Nahm is also made from chopped raw pork mixed with salt, garlic and chili, tightly wrapped in banana leaves for a few days for fermentation (Figure 7B)[35]. Some Thai dishes are proven as viable *T. spiralis* larvae sources due to cooking procedures[47]. Srikitjakarn *et al*[48] reported *T. spiralis* was found in 1.67% of 421 dogs in Tarae District, Sakonnakon Province**.** Raw dog meat was a source of infection in Kaeng Khlo District, Chaiyaphum Province

in December 1984[49]. The incidence of *T. spiralis* larvae in dog meat in the areas favoring dog meat consumption is a major public health problem in the future. The major source of infection is wild boar, free roaming pigs located in the north, and wild animals from Laos and Myanmar sold in Thailand[2].

In conclusion, various studies of trichinosis in Thailand since the first outbreak up until recent years have shown that most of the outbreaks occurred in the north of the country, an area in which some of the favorite traditional dishes involve meat from pigs and wild boars, often eaten raw or under-cooked. No vaccines have yet been developed. Treatment exists for humans if diagnosis is done promptly. Better prevention and control of trichinosis require health education to stop the consumption of infected and under-cooked meats.

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REFERENCES

1 **Boonthanom P**, Nawarat A. The outbreaks of trichinosis at Amphur Mae Sarialng. *Bull Pub Health* 1963; **33**: 301-308
2 **Yamaguchi T**. Present status of trichinellosis in Japan. *Southeast Asian J Trop Med Public Health* 1991; **22** Suppl: 295-301
3 **Miyazaki I**. Helminthic zoonoses. International Medical Foundation of Japan, Tokyo, Japan, 1991: 452-459
4 **Sohn WM**, Kim HM, Chung DI, Yee ST. The first human case of Trichinella spiralis infection in Korea. *Korean J Parasitol* 2000; **38**: 111-115
5 **Wang ZQ**, Cui J. The epidemiology of human trichinellosis in China during 1964-1999. *Parasite* 2001; **8**: S63-S66
6 **Wang ZQ**, Cui J, Xu BL. The epidemiology of human trichinellosis in China during 2000-2003. *Acta Trop* 2006; **97**: 247-251
7 **Chotmongkol V**, Intapan PM, Koonmee S, Kularbkaew C, Aungaree T. Case report: acquired progressive muscular hypertrophy and trichinosis. *Am J Trop Med Hyg* 2005; **72**: 649-650
8 **Markell EK**, John DT, Krotoshi WA. The blood and tissue-dwelling nematode: In Markell and Voge's Medical Parasitology. 8th ed. USA, 1999: 340-345
9 **Garkavi BL**. The species of Trichinella isolated from wild carnivores. *Veterinariia* 1972; **10**: 90-91
10 **Geller ER**, Malykhina AN, Silakova LN, Timonov EV. Morpho-physiologic criteria for the taxonomic independence of the species Trichinella pseudospiralis Garkavi, 1972. *Parazitologiia* 1977; **11**: 113-116
11 **Pozio E**, Khamboonruang C. Trichinellosis in Thailand: epidemiology and biochemical identification of the aethiological agent. *Trop Med Parasitol* 1989; **40**: 73-74
12 **Pozio E**, La Rosa G, Murrell KD, Lichtenfels JR. *Taxonomic revision of the genus Trichinella. J Parasitol* 1992; **78**: 654-659
13 **Pozio E**, Varese P, Morales MA, Croppo GP, Pelliccia D, Bruschi F. Comparison of human trichinellosis caused by Trichinella spiralis and by Trichinella britovi. *Am J Trop Med Hyg* 1993; **48**: 568-575
14 **Pozio E**, La Rosa G, Yamaguchi T, Saito S. Trichinella britovi from Japan. *J Parasitol* 1996; **82**: 847-849
15 **Pozio E**, La Rosa G. Trichinella murrelli n. sp: etiological agent of sylvatic trichinellosis in temperate areas of North America. *J Parasitol* 2000; **86**: 134-139
16 **Pozio E**. Taxonomy of Trichinella and the epidemiology of infection in the Southeast Asia and Australian regions. *Southeast Asian J Trop Med Public Health* 2001; **32** Suppl 2: 129-132
17 **Jongwutiwes S**, Chantachum N, Kraivichian P, Siriyasatien P, Putaporntip C, Tamburrini A, La Rosa G, Sreesunpasirikul C, Yingyourd P, Pozio E. First outbreak of human trichinellosis caused by Trichinella pseudospiralis. *Clin Infect Dis* 1998; **26**: 111-115
18 **Schalm S**, Eberhard HD. Fever, myalgia, and eyelid edema. *Med Klin* (Munich) 2000; **95**: 103
19 **Remig J**, Froscher W. Acute trichinosis. 193 cases in an epidemic. *Dtsch Med Wochenschr* 1987; **112**: 1855-1859
20 **Clausen MR**, Meyer CN, Krantz T, Moser C, Gomme G, Kayser L, Albrectsen J, Kapel CM, Bygbjerg IC. Trichinella infection and clinical disease. *QJM* 1996; **89**: 631-636
21 **Holstein A**, Wieczorek G, Nahrwold D, Egberts EH. Father and son with muscle pain and loss of muscle strength. Acute trichinosis. *Internist* (Berl) 1999; **40**: 673-677
22 **Levy Y**, Rimbrot S, Raz R. Myalgia, fever, abnormal muscle enzymes and blue urine in a farmworker from Thailand. *Isr Med Assoc J* 2001; **3**: 704
23 **Valdes PV**, Prieto A, Diaz A, Calleja M, Gomez JL. Microcalcifications of pectoral muscle in trichinosis. *Breast J* 2005; **11**: 150
24 **Limsuwan S**, Thisyakorn U. Trichinosis in children, report of 8 cases. *Thai J Pediatrics* 1993; **32**: 265-270
25 **Kapel CM**. Sylvatic and domestic Trichinella spp. in wild boars; infectivity, muscle larvae distribution, and antibody response. *J Parasitol* 2001; **87**: 309-314
26 **Petri WA Jr**, Holsinger JR, Pearson RD. Common-source outbreak of trichinosis associated with eating raw home-butchered pork. *South Med J* 1988; **81**: 1056-1058
27 **Kociecka W**, Majchrowicz H, Szulc M. Trichinellosis focus resulting from consumption of wild boar meat. *Przegl Epidemiol* 1992; **46**: 195-205
28 **Oivanen L**, Mikkonen T, Sukura A. An outbreak of trichinellosis in farmed wild boar in Finland. *APMIS* 2000; **108**: 814-818
29 **Pozio E**. Factors affecting the flow among domestic, synanthropic and sylvatic cycles of Trichinella. *Vet Parasitol* 2000; **93**: 241-262
30 **Bureau of Epidemiology**. Annual Epidemiological Surveillance Report: Trichinosis. Department of Disease Control, Ministry of Public Health, Thailand, 1995-2005
31 **Morakote N**, Khamboonruang C, Siriprasert V, Suphawitayanukul S, Marcanantachoti S, Thamasonthi W. The value of enzyme-linked immunosorbent assay (ELISA) for diagnosis of human trichinosis. *Trop Med Parasitol* 1991; **42**: 172-174
32 **Morakote N**, Sukhavat K, Khamboonruang C, Siriprasert V, Suphawitayanukul S, Thamasonthi W. Persistence of IgG, IgM, and IgE antibodies in human trichinosis. *Trop Med Parasitol* 1992; **43**: 167-169
33 **Limsuwan S**, Siriprasert V. A clinical study on trichinosis in Changwat Phayao, Thailand. *Southeast Asian J Trop Med Public Health* 1994; **25**: 305-308
34 **Chumkasian P**. Trichinosis in Thailand. Annual Epidemiological Surveillance Report, Bangkok, Office of permanent secretary, MOPH, Thailand, 1999
35 **Takahashi Y**, Mingyuan L, Waikagul J. Epidemiology of trichinellosis in Asia and the Pacific Rim. *Vet Parasitol* 2000; **93**: 227-239
36 **Ittiprasert W**, Butraporn P, Kitikoon V, Klongkamnuankarn K, Pholsena K, Vanisaveth V, Sakolvaree Y, Chongsa-nguan M, Tapchaisri P, Mahakunkijcharoen Y, Kurazono H, Hayashi H, Chaicumpa W. Differential diagnosis of schistosomiasis mekongi and trichinellosis in human. *Parasitol Int* 2000; **49**: 209-218
37 **Khamboonruang C**, Nateewatana N. Trichinosis: A recent outbreak in Northern Thailand. *Southeast Asian J Trop Med Public Health* 1975; **6**: 74-78
38 **Dissamarn R**, Chai-ananda P. The present position of trichinosis in thailand. *Bull Off Int Epizoot* 1965; **63**: 217-219
39 **Wiwatanaworapant T**. Trichinosis: a case report. *Siriraj Hosp Gaz* 1980; **32**: 220-222

40 **Chalermchaikit T**, Nawarat A, Muangyai M, Brahmasa R, Chumkasian P. Epidemiological surveillance of trichinosis outbreak in Petchaboon province. *Thai J Vet Med* 1982; **12**: 1-23
41 **Oonsombat P**, Attanatho V, Trishnananda M. Human trichinosis: the fourteenth outbreak in Thailand. *Siriraj Hospital Gazette* 1982; **34**: 69-72
42 **Pinanong M**, Tongma S, Hongphinyo V. Human trichinosis: an outbreak at Petchaboon province. *Royal Thai Air Force Med Gaz* 1985; **31**: 87-94
43 **Khamboonruang C**. The present status of trichinellosis in Thailand. *Southeast Asian J Trop Med Public Health* 1991; **22** Suppl: 312-315
44 **Charkrit S**. Study on clinical manifestations of trichinosis in Payao province. *Com Dis J* 1998; **24**: 242-247
45 **Doege TC**, Thienprasit P, Headington JT, Pongprot B, Tarawanich S. Trichinosis and raw bear meat in Thailand. *Lancet* 1969; **1**: 459-461
46 **Suriyanon V**, Klunklin K. Human trichinosis: analysis of cases during the tenth outbreak in North Thailand. *Southeast Asian J Trop Med Public Health* 1972; **3**: 390-396
47 **Dissamarn R**, Indrakamhang P. Trichinosis in Thailand during 1962-1983. *Int J Zoonoses* 1985; **12**: 257-266
48 **Srikitjakarn L**, Korakovit M, Toboran P, Srutaravong V, Bettermann G, Lingelback W. *Trichinella spiralis* in dog meat for human consumption in Sakon Nakon province. *Thai Vet Med Assoc* 1981; **32**: 271-277
49 **Chitchang S**, Vongmek V, Leelasupasri S. Trichinosis for dog meat at Changwat Chaiyaphum. *Royal Thai Army Med J* 1985; **38**: 305-309
50 **Railliet A**. *Trichinella spiralis*: In Chapter 21; Parasitic Diseases. Despommier DD, Gwadz RW, Hotez PJ and Knirsch CA. 4th ed. New York: Apple Trees Productions, 2000; 125-132
51 **Lindsay DS**, Zarlenga DS, Gamble HR, al-Yaman F, Smith PC, Blagburn BL. Isolation and characterization of Trichinella pseudospiralis Garkavi, 1972 from a black vulture (Coragyps atratus). *J Parasitol* 1995; **81**: 920-923

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COLORECTAL CANCER

Identification of specific genes and pathways involved in NSAIDs-induced apoptosis of human colon cancer cells

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Abstract

AIM: To study whether indomethacin (IND), a nonselective cyclooxygenase (COX) inhibitor or NS-398 (NS), a COX-2-selective inhibitor, induces apoptosis in human colon cancer cells and which apoptosis-related genes and pathways are involved.

METHODS: Human colon cancer Caco-2 cells were treated with either: placebo, IND (0.05-0.5 mmol/L) or NS (0.01-0.2 mmol/L) for 1, 5 and 18 h. We then studied: (1) Cell death by the TUNEL method, (2) mRNA expression of 96 apoptosis-related genes using DNA microarray, (3) expression of selected apoptosis related proteins by Western blotting.

RESULTS: Both IND and NS induced apoptosis in 30%-50% of Caco-2 cells in a dose dependent manner. IND (0.1 mmol/L for 1 h) significantly up-regulated pro-apoptotic genes in four families: (1) TNF receptor and ligand, (2) Caspase, (3) Bcl-2 and (4) Caspase recruiting domain. NS treatment up-regulated similar pro-apoptotic genes as IND. In addition, IND also down-regulated anti-apoptotic genes of the IAP family.

CONCLUSION: (1) Both non-selective and COX-2-selective NSAIDs induce apoptosis in colon cancer cells in a dose dependent manner. (2) Both NSAIDs induce apoptosis by activating two main apoptotic pathways: the death receptor pathway (involving TNF-R) and the mitochondrial pathway. (3) IND induces apoptosis by up-regulating pro-apoptotic genes and down-regulating anti-apoptotic genes, while NS only up-regulates pro-apoptotic genes. (4) Induction of apoptosis in colon cancer cells by NSAIDs may explain in part, their inhibitory action on colon cancer growth.

Key words: Nonsteroidal anti-inflammatory drugs; Colon cancer; Apoptosis; Cyclooxygenase; cDNA microarray

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INTRODUCTION

Colorectal cancer (CRC) is the second most lethal cancer in the USA. Numerous studies suggest that nonsteroidal anti-inflammatory drugs (NSAIDs) have antineoplastic effects. Epidemiological studies have shown a reduction in the risk of death from colorectal cancer associated with the use of NSAIDs[1]. Perhaps the most compelling evidence for the chemopreventive role of NSAIDs is provided by the clinical studies in patients with familial adenomatous polyposis (FAP), an inherited predisposition for CRC[2-6]. In these studies, the NSAID sulindac and the selective cyclooxygenase-2 inhibitor (COX-2) celecoxib, reduced both the number and the size of colorectal adenomas in FAP patients. Similarly, NSAIDs prevent tumor formation in a variety of animal models of CRC[7, 8].

How exactly do NSAIDs exert their effects to prevent tumorigenesis? A large body of evidence suggests that NSAIDs exert their chemopreventive actions by: (1) inhibition of cell growth and proliferation and (2) induction of apoptosis[9]. The mechanisms by which NSAIDs achieve these processes are still incompletely understood. One widely studied mechanism is *via* the COX-2 dependent pathways.

NSAIDs are known as the inhibitors of the cyclooxygenase (COX-1 and COX-2) enzymes, which are involved in the conversion of arachidonic acid to eicosanoids. COX-1 is expressed constitutively in a variety of tissues and plays a role in platelet aggregation and gastric cytoprotection. COX-2, is inducible particularly during inflammation in cells such as endothelial cells, macrophages and intestinal epithelial cells[10]. Interestingly,

COX-2 expression has been shown to be dramatically increased in colon cancer cells compared to normal colonic mucosa and is associated with a several-fold increase in concentration of prostaglandin E_2 (PGE_2)[11-13]. Studies from our lab demonstrated that COX-2 generated PGE_2 promotes colon cancer cell growth by transactivating epidermal growth factor receptor[14]. The increased expression of COX-2 also seems to prevent cancer cells from undergoing programmed cell death, at the same time, promoting angiogenesis, enhancing metastatic potential and modulating cell proliferation. Direct evidence for the role of COX-2 in colon cancer tumorigenesis has been reported in many studies. Using APC knockout mice, which develop polyps in their intestinal tracts due to the mutation of the APC gene, Oshima and colleagues were able to show a significant reduction of intestinal polyps when either one or both of COX-2 alleles were deleted[15,16]. Furthermore, the COX-2 selective inhibitor NS-398, has been reported to suppress tumor growth in different cancer cell lines, to induce apoptosis in human colon cancer cells and to have anti-angiogenic effects[9,17-19].

However, recent data suggest that COX-2 may not be the sole target for the action of NSAIDs. The anti-neoplastic effects of NSAIDs are also seen in tumor cells that do not express COX[20]. Similarly, NSAID derivatives such as sulindac sulfone, which lacks the ability to inhibit COX, is shown to inhibit colon cancer growth[21]. Furthermore, NSAIDs affect other mechanisms that regulate cell proliferation and death. For example, NSAIDs regulate PPARδ, the NFκB pathway and the lipoxygenase pathway, all of which play a critical role in the regulation of cell survival and death[22-24].

In order to better understand the complex mechanisms underlying the cancer chemopreventive properties of NSAIDs, it is important to understand the signaling pathways and the genes that directly lead to the inhibition of proliferation and/or the induction of apoptosis in colon cancer cells. With the recent development of DNA microarray technology, identification of genes and pathways can be easily achieved by using microarray chips that contain the genes of interest.

This study was aimed to provide in depth insight into the pro-apoptotic actions of Indomethacin (IND), a nonselective COX inhibitor and NS-398 (NS), a COX-2 selective inhibitor on colon cancer Caco-2 cells, and identify specific pathways and genes involved.

MATERIALS AND METHODS

Cell culture

Human colon cancer (Caco-2) cells (ATCC, Rockville, MD, USA) were grown in DMEM/F12 medium supplied with 20% fetal bovine serum and antibiotics at 37℃ in a humidified incubator containing 50 mL/L of CO_2.

Treatment of cells with Indomethacin and NS-398

A 100 mmol/L stock solution IND (Sigma Chemical, St. Louis, MO, USA) was freshly prepared by dissolving the compound in 0.2 mol/L Na_2CO_3 and 1 mol/L NaH_2PO_4, and kept at 37℃ before treatment. A 50 mmol/L stock solution of NS (Cayman Chemical, Ann Arbor, MI) was prepared by dissolving the compound in dimethyl sulfoxide. Caco-2 cells were plated in dishes (100 mm × 20 mm) at a density of 1×10^6 and kept in serum-free medium for 24 h before treatment. The cells were subsequently treated with IND (0.05 mmol/L, 0.1 mmol/L and 0.5 mmol/L) or NS (0.01 mmol/L, 0.1 mmol/L and 0.2 mmol/L) for 1, 5 and 18 h at 37℃.

Cell death detection assay

Cells were plated in 8-well chamber slides and grown until 50%-75% confluent. They were subsequently treated with IND and NS in the same way as described above. Cell death was evaluated by terminal deoxynucleotide transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) using a commercial kit (Roche Diagnostics, Indianapolis, IN, USA, Cat No. 1 684 817). Briefly, cell monolayers were air-dried and fixed with 4% paraformaldehyde for 1 h at room temperature. 3% H_2O_2 was then used to block endogenous peroxidase. The TUNEL reaction mixture was applied to the cells and incubated for 1 h. Following incubation with anti-fluorescein antibody conjugated with horseradish peroxidase (HRP), diaminobenzidine substrate was then used as developing the agent. Cells treated with culture medium without NSAIDs served as controls. Apoptosis was evaluated by counting the number of apoptotic and total cells in five random fields per chamber in each slide. Apoptotic index was expressed as percentage of apoptotic cells in total number of counted cells.

cDNA microarray study

Serum starved Caco-2 cells were treated with IND or NS in the same way as described above. Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA, USA, Cat No. 15596-026). Equal amount of RNA from each treatment was used as templates for cDNA synthesis. Radiolabeled probes were synthesized using ^{32}P dCTP following the manufacturer's protocol provided with the microarray study kit (GEArray Q series, SuperArray Inc. Bethesda, MD, USA). The denatured cDNA probes were added to the arrays containing the genes to be studied. Each of the arrays contains 96 cDNA fragments from genes associated with a specific apoptotic pathway. After overnight hybridization at 60℃ with continuous agitation, the arrays were washed for 4 × 15 min with each washing solution (2 × SSC, 1% SDS, then 0.1 × SSC, 0.5% SDS). The arrays were then developed using X-ray films. Quantification of the images was accomplished by the ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). Results were expressed in fold change.

Western blotting

Serum starved Caco-2 cells were treated with IND or NS in the same way as described above. Total protein was isolated in a lysis buffer containing 20 mmol/L Tris-HCl pH 7.5, 1 mmol/L EDTA, 300 mmol/L NaCl, 0.2 mmol/L Sodium vanadate, 1% Triton x-100 and 1% NP-40. Equal amount of protein lysate from each treatment was separated by SDA-PAGE and then transferred electrophoretically to a nitrocellulose membrane. The blots were probed with the following antibodies: APAF1

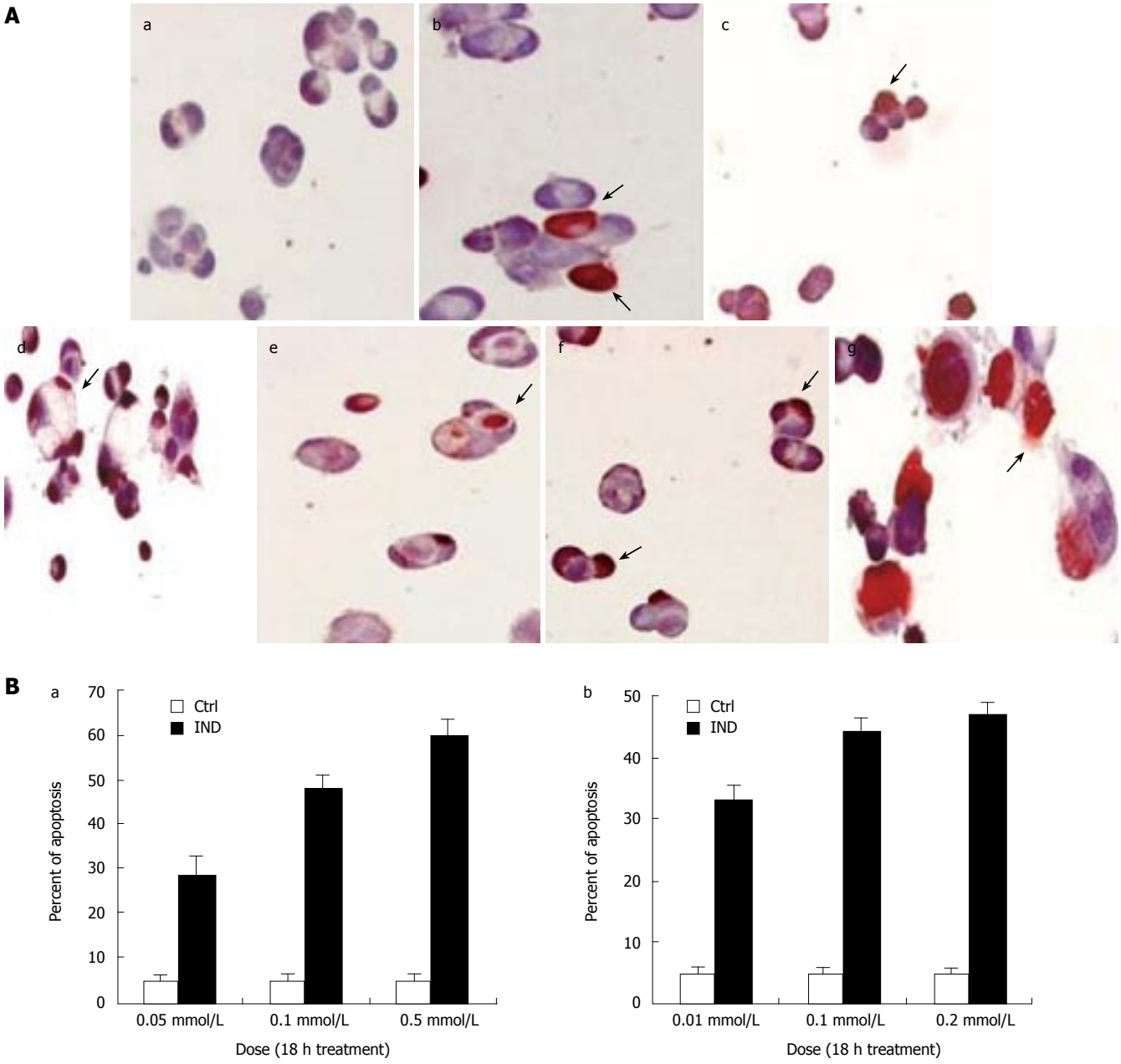

Figure 1 **A**: TUNEL staining of Caco-2 cells. IND and NS treatment cause apoptosis in a dose dependent manner after 18 h. (a) Control; (b) IND 0.05 mmol/L; (c) IND 0.1 mmol/L; (d) IND 0.5 mmol/L; (e) NS 0.01 mmol/L; (f) NS 0.1 mmol/L; (g) NS 0.2 mmol/L. Experiments performed in triplicates; **B**: Apoptosis in Caco-2 cells is dose dependent after treatment with (a) IND and (b) NS for 18 h. Experiments performed in triplicates.

(apoptotic protease activating factor), BAK (BCL2-antagonist/killer 1), BAX (BCL2-associated X protein), DFFA (DNA fragment factor-45), XIAP (X-linked IAP) and Survivin. The signal was visualized by the chemiluminescence method using ECL detection reagents (Amersham Life Sciences, Arlington Heights, IL). Signals were quantified by densitometry scanning.

Statistical analysis

Results from the TUNEL study are expressed as the mean ± the standard error. Student's *t* test was used to determine statistical difference between controls and treatments. A P value of < 0.05 was considered statistically significant. Comparisons of data between multiple groups were performed with ANOVA.

RESULTS

Apoptotic cell death detection

Using TUNEL method, we found a significant increase in apoptotic cell death in Caco-2 cells treated with both the nonselective COX inhibitor IND and the COX-2-selective inhibitor NS as compared to placebo. The IND and NS-induced apoptotic cell death was dose dependent (Figure 1A and B). Treatment with IND at 0.05 mmol/L, 0.1 mmol/L and 0.5 mmol/L resulted in 28% ± 5%, 48% ± 3% and 60% ± 4% of apoptosis respectively (all $P < 0.001$ *vs* control). On the other hand, treatment with NS at 0.01 mmol/L, 0.1 mmol/L and 0.2 mmol/L induced apoptosis in 33% ± 3%, 44% ± 2% and 47% ± 1% respectively (all $P < 0.001$ *vs* control). In addition, at 0.1mmol/L concentration, IND induced apoptosis in 33% ± 4%, 31%

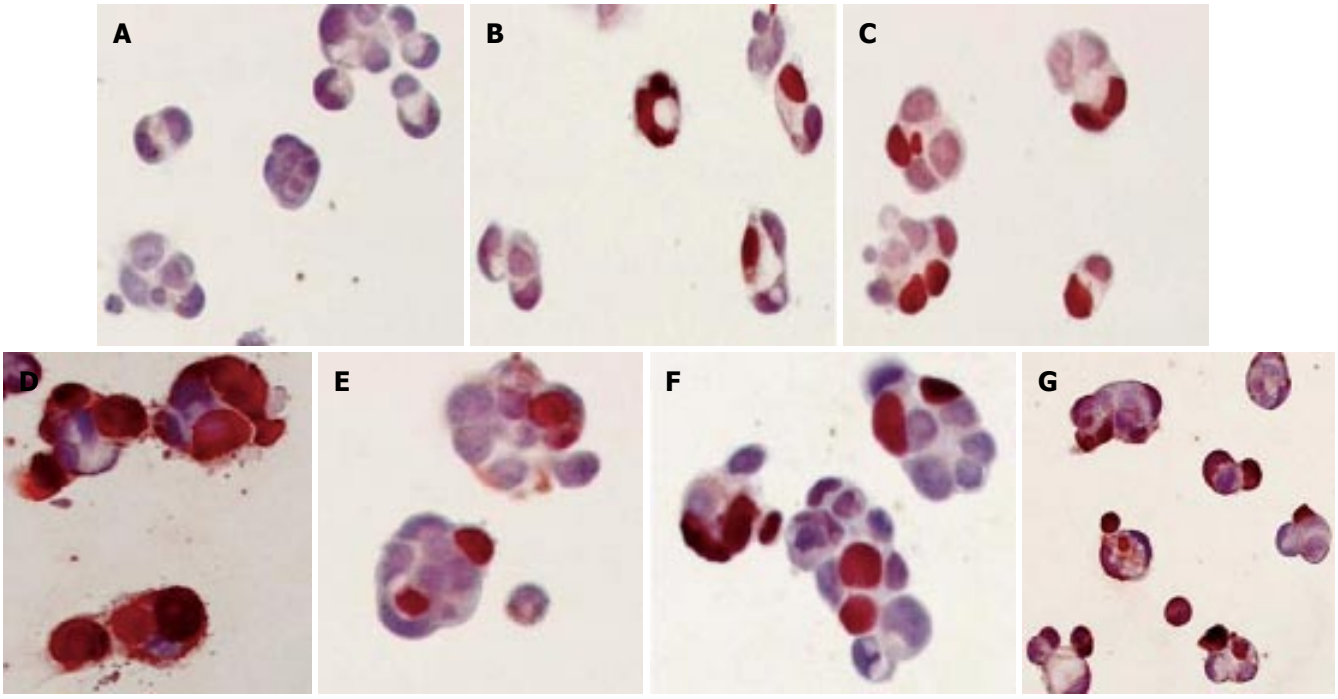

Figure 2 TUNEL staining of Caco-2 cells treated with IND (0.1 mmol/L) and NS (0.1 mmol/L) for 1, 5 and 18 h. (**A**) Control; (**B**) IND for 1 h; (**C**) IND for 5 h; (**D**) IND for 18 h; (**E**) NS for 1 h; (**F**) NS for 5 h; (**G**) NS for 18 h.

± 2% and 48% ± 5% (all $P < 0.001$ *vs* control) of cells and NS induced apoptosis in 29% ± 3%, 26% ± 4% and 46% ± 6% (all $P < 0.001$ *vs* control) of cells at 1, 5 and 18 h, respectively (graph not shown).

IND and NS treatment induced morphological changes consistent with apoptosis (cell shrinkage, condensation of chromatin, cytoplasmic budding, apoptotic bodies), which occurred as early as 1 h post-treatment and became extensively evident in 5 and 18 h (Figure 2).

cDNA microarray study of 96 apoptosis related genes

IND treatment (0.1 mmol/L for 1 h) caused a significant up-regulation of pro-apoptotic genes in mainly four families: (I) TNF receptor and ligand (TRAIL-R4-A by 27-fold, TNFSF11 by 3-fold); (II) Caspase (CASP4 by 14-fold, CASP6 by 19-fold); (III) BCL-2 (BCL2L11/bimL by 6-fold, Blk by 10-fold); (IV) Caspase recruiting domain (Apaf-1 by 3-fold, NOD/CARD4 by 14-fold).

In addition, IND significantly up-regulated other genes such as DNA fragmentation factor 45/DFFA (by 48-fold) and CRADD (by 24-fold). In addition to the genes up-regulated by IND, NS up-regulated some other genes within these families such as CASP13 (7-fold), Asc (5-fold), TRAIL receptor 2 (3-fold) and Bak (3-fold). The transcriptional up-regulation of these pro-apoptotic genes occurred as early as 1 h after drug exposure. We also found that IND, but not NS, down-regulated most of the anti-apoptotic genes in the IAP (inhibitor of apoptosis protein) family such as IAP2, XIAP (9-fold), survivin (7-fold) and Bruce (3-fold) (Figure 3). Significant up-regulation and down-regulation of apoptotic-related genes in our study is determined by a 3 or more fold change from control.

Protein analysis

The protein expression of several significantly up-regulated or down-regulated genes is shown in Figure 4. Both IND and NS treatments up-regulated the pro-apoptotic protein Apaf-1 significantly by 9, 12, and 4 fold at 1, 5, and 18 h, respectively (IND); by 96%, 160% and 102% at 1, 5 and 18 h, respectively (NS) (all $P < 0.01$ *vs* control). In addition, both of these drugs also increased Bak protein expression. However, IND treatment also down-regulated anti-apoptotic proteins such as XIAP by 40% and 22% at 1 and 5 h respectively ($P < 0.05$ *vs* control), while NS treatment did not alter it significantly.

DISCUSSION

The cure for colorectal cancer remains a challenge despite all currently available medical and surgical treatment modalities. The cure rate is particularly low in advanced disease, making chemoprevention an attractive alternative. In recent years, the NSAIDs have attracted a great interest as a potential candidate for the chemoprevention of colorectal cancer. There is now a great body of evidence supporting the role of NSAIDs in tumor chemoprevention by the induction of apoptosis. However, little is known about the exact mechanisms of NSAIDs action on colorectal cancer cells.

With the advent of microarray technology, thousands of genes can be assayed in a single experiment. We applied the microarray technology and focused on apoptosis related genes. By creating a profile of genes linked to a particular biological pathway, the expression of particular genes in a colon cancer cell line treated with NSAIDs, may suggest the activation of specific pathways. Furthermore, comparing the action of a nonselective COX inhibitor *vs* a COX-2-selective inhibitor can reveal mechanistic differences between these two NSAIDs in the induction of apoptotic cell death.

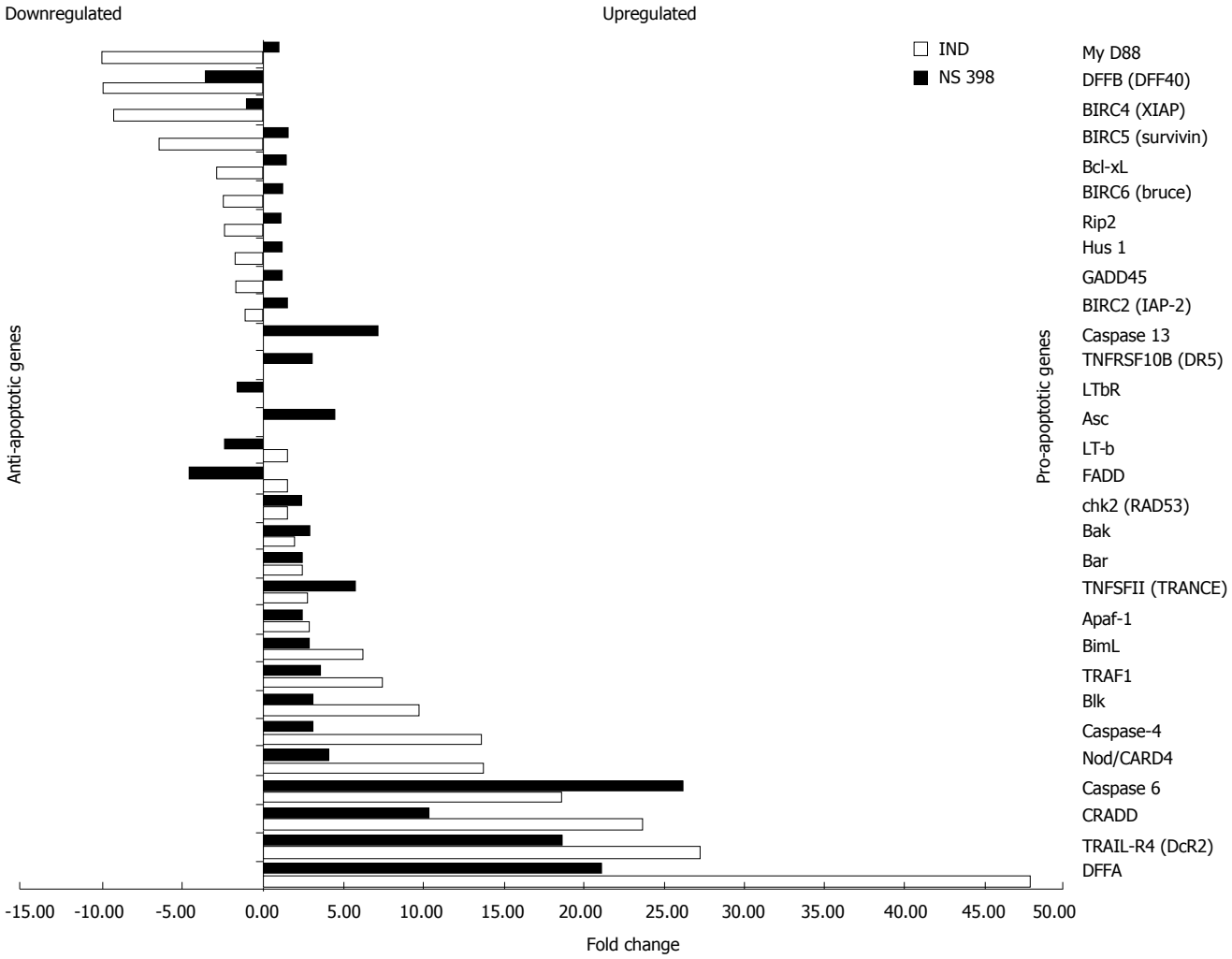

Figure 3 Genes up-regulated and down-regulated by IND and NS treatment identified by cDNA microarray. Up-regulation or down-regulation is expressed as a fold change. Down-regulated genes are shown on the top of the graph in a decreasing order toward mid graph, while up-regulated genes in an increasing order toward the bottom of the graph. Anti-apoptotic genes lie mostly in the upper half while pro-apoptotic genes lie mainly in the bottom half.

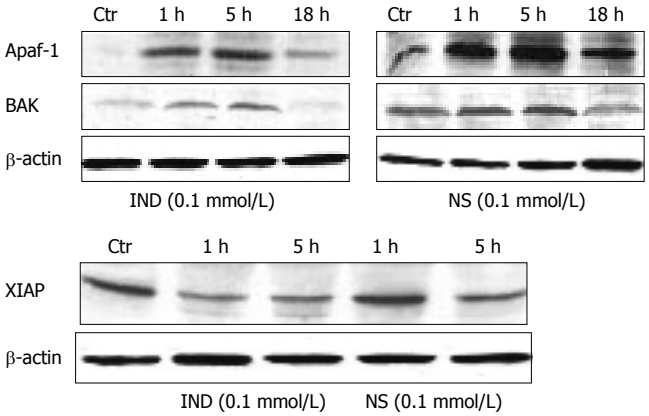

Figure 4 Protein expression by Western blotting of pro-apoptotic genes Apaf-1 and Bak (upper panel), expression of XIAP (lower panel). Blots are representative of triplicate experiments.

Our results suggest that both the nonselective COX inhibitor indomethacin and the COX-2-selective inhibitor NS-398 trigger apoptosis in Caco-2 cells through at least two major well known pathways, an extrinsic, death receptor-transmembrane pathway and an intrinsic, mitochondrial pathway.

The transmembrane pathway of apoptosis involves the tumor necrosis factor (TNF) ligand and receptor superfamily. Members of this family include TNFα, Fas ligand and TRAIL (TNF-related apoptosis-inducing ligand). These ligands when coupling to their respective receptors trigger a number of intracellular events that lead to apoptotic cell death[25,26]. The final steps of apoptosis induced through this pathway depends on the activation of caspases, cysteine proteases that cleave the aspartate residues[27,28]. Several members of the TNF family of receptors contain intracellular death domains that interact with other molecules that contain death domains and with caspase recruitment domains (CARD), propagating the apoptotic cascade[29,30]. We found that both IND and NS-398 up-regulated several genes within the TNF ligand and receptor family such as TNFSF11 (Tumor necrosis factor superfamily member 11), TRAIL-R4, TRAIL-R2. We also detected up-regulation of other genes encoding downstream molecules associated with this pathway such as CRADD (CASP2 and RIPK1 domain containing adaptor with death domain), ASC (Apoptosis-associated speck-like protein containing a CARD), NOD1 (caspase recruitment domain 4), APAF (apoptotic protease activating factor). Our data strongly suggest that NSAIDs

induce apoptosis in Caco-2 cells by up-regulating genes of the TNF ligand and receptor pathway, likely mediated by TNF receptors and TRAIL receptors. This mechanism may explain in part the chemopreventive properties of NSAIDs in colorectal cancer. Several studies have shown promise in using TNFα and TRAIL as potential chemotherapeutic agents in a variety of tumors[31-34]; however, toxicity remains a concern when these agents are used systemically. Our study provides further evidence that NSAIDs likely sensitize colorectal cells to death through TNF and TRAIL receptor. This suggests NSAIDs to be potentially useful as an adjunct chemotherapeutic agent by increasing the therapeutic window of TNFα and TRAIL and decreasing their toxicity.

Our study suggests that NSAIDs also affect the mitochondrial pathway of apoptosis. This intrinsic pathway is usually triggered by chemicals, growth factor deprivation or irradiation. The major steps in this pathway involve the release of cytochrome c with subsequent increase in mitochondrial membrane permeability that is controlled by a variety of pro-apoptotic and anti-apoptotic members of the Bcl-2 family. The release of cytochrome c triggers the assembly of Apaf-1 (Apoptotic protease-activating factor) and pro-caspase 9 to form an apoptosome. The activated caspase 9 then activates other downstream molecules to initiate apoptosis[35-37]. Several members of the Bcl-2 family were shown to be up-regulated or down-regulated in Caco-2 cells treated with IND and NS. The pro-apoptotic members up-regulated by NSAIDs include Bak (Bcl2-antagonist/killer 1), Blk (B lymphoid thyrosine kinase) and Bim (Bcl2 like 11, apoptosis facilitator). Bcl-x (Bcl2-like 1), an antiapoptotic member, was down-regulated. These data suggest that NSAIDs, both COX nonselective and COX-2 selective likely induce apoptosis *via* the death receptor pathways and the mitochondrial pathway.

There was one major difference in the pattern of genes expressed in Caco-2 cells treated with IND and NS-398. While both IND and NS-398 up-regulated pro-apoptotic genes, IND also down-regulated anti-apoptotic genes, particularly the IAP (inhibitors of apoptosis proteins) family. This family has been described consisting of at least six members and can efficiently inhibit the caspases[38-40]. In our study, IND treatment of Caco-2 cells caused significantly down-regulated IAP2, XIAP, Survivin and Bruce, while treatment with NS-398 did not affect these genes. This may suggest a mechanistic difference by which non-selective COX and COX-2 specific NSAIDs induce apoptosis. This finding indicates that COX-1 may play a role in the different genetic expression of the IAP family between IND and NS treatments. Further functional studies exploring this difference may contribute to a better understanding of the chemopreventive properties of NSAIDs.

REFERENCES

1 **Thun MJ**, Namboodiri MM, Heath CW Jr. Aspirin use and reduced risk of fatal colon cancer. *N Engl J Med* 1991; **325**: 1593-1596

2 **Giardiello FM**, Hamilton SR, Krush AJ, Piantadosi S, Hylind LM, Celano P, Booker SV, Robinson CR, Offerhaus GJ. Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. *N Engl J Med* 1993; **328**: 1313-1316

3 **Giardiello FM**, Yang VW, Hylind LM, Krush AJ, Petersen GM, Trimbath JD, Piantadosi S, Garrett E, Geiman DE, Hubbard W, Offerhaus GJ, Hamilton SR. Primary chemoprevention of familial adenomatous polyposis with sulindac. *N Engl J Med* 2002; **346**: 1054-1059

4 **Steinbach G**, Lynch PM, Phillips RK, Wallace MH, Hawk E, Gordon GB, Wakabayashi N, Saunders B, Shen Y, Fujimura T, Su LK, Levin B, Godio L, Patterson S, Rodriguez-Bigas MA, Jester SL, King KL, Schumacher M, Abbruzzese J, DuBois RN, Hittelman WN, Zimmerman S, Sherman JW, Kelloff G. The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. *N Engl J Med* 2000; **342**: 1946-1952

5 **Nugent KP**, Farmer KC, Spigelman AD, Williams CB, Phillips RK. Randomized controlled trial of the effect of sulindac on duodenal and rectal polyposis and cell proliferation in patients with familial adenomatous polyposis. *Br J Surg* 1993; **80**: 1618-1619

6 **Labayle D**, Fischer D, Vielh P, Drouhin F, Pariente A, Bories C, Duhamel O, Trousset M, Attali P. Sulindac causes regression of rectal polyps in familial adenomatous polyposis. *Gastroenterology* 1991; **101**: 635-639

7 **Jacoby RF**, Marshall DJ, Newton MA, Novakovic K, Tutsch K, Cole CE, Lubet RA, Kelloff GJ, Verma A, Moser AR, Dove WF. Chemoprevention of spontaneous intestinal adenomas in the Apc Min mouse model by the nonsteroidal anti-inflammatory drug piroxicam. *Cancer Res* 1996; **56**: 710-714

8 **Beazer-Barclay Y**, Levy DB, Moser AR, Dove WF, Hamilton SR, Vogelstein B, Kinzler KW. Sulindac suppresses tumorigenesis in the Min mouse. *Carcinogenesis* 1996; **17**: 1757-1760

9 **Smith ML**, Hawcroft G, Hull MA. The effect of non-steroidal anti-inflammatory drugs on human colorectal cancer cells: evidence of different mechanisms of action. *Eur J Cancer* 2000; **36:** 664-674

10 **Jones DA**, Carlton DP, McIntyre TM, Zimmerman GA, Prescott SM. Molecular cloning of human prostaglandin endoperoxide synthase type II and demonstration of expression in response to cytokines. *J Biol Chem* 1993; **268**: 9049-9054

11 **Fujita T,** Matsui M, Takaku K, Uetake H, Ichikawa W, Taketo MM, Sugihara K. Size- and invasion-dependent increase in cyclooxygenase 2 levels in human colorectal carcinomas. *Cancer Res* 1998; **58**: 4823-4826

12 **Eberhart CE**, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, DuBois RN. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology* 1994; **107**: 1183-1188

13 **Rigas B**, Goldman IS, Levine L. Altered eicosanoid levels in human colon cancer. *J Lab Clin Med* 1993; **122**: 518-523

14 **Pai R**, Soreghan B, Szabo IL, Pavelka M, Baatar D, Tarnawski AS. Prostaglandin E2 transactivates EGF receptor: a novel mechanism for promoting colon cancer growth and gastrointestinal hypertrophy. *Nat Med* 2002; **8:** 289-293

15 **Oshima M**, Murai N, Kargman S, Arguello M, Luk P, Kwong E, Taketo MM, Evans JF. Chemoprevention of intestinal polyposis in the Apcdelta716 mouse by rofecoxib, a specific cyclooxygenase-2 inhibitor. *Cancer Res* 2001; **61:** 1733-1740

16 **Oshima M,** Dinchuk JE, Kargman SL, Oshima H, Hancock B, Kwong E, Trzaskos JM, Evans JF, Taketo MM. Suppression of intestinal polyposis in Apc delta716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). *Cell* 1996; **87**: 803-809

17 **Elder DJ**, Halton DE, Crew TE, Paraskeva C. Apoptosis induction and cyclooxygenase-2 regulation in human colorectal adenoma and carcinoma cell lines by the cyclooxygenase-2-selective non-steroidal anti-inflammatory drug NS-398. *Int J Cancer* 2000; **86**: 553-560

18 **Jones MK**, Wang H, Peskar BM, Levin E, Itani RM, Sarfeh IJ, Tarnawski AS. Inhibition of angiogenesis by nonsteroidal anti-inflammatory drugs: insight into mechanisms and implications for cancer growth and ulcer healing. *Nat Med* 1999; **5**: 1418-1423

19 **Tsuji S**, Kawano S, Sawaoka H, Takei Y, Kobayashi I, Nagano K, Fusamoto H, Kamada T. Evidences for involvement of cyclooxygenase-2 in proliferation of two gastrointestinal cancer cell lines. *Prostaglandins Leukot Essent Fatty Acids* 1996; **55**: 179-183

20 **Hanif R**, Pittas A, Feng Y, Koutsos MI, Qiao L, Staiano-Coico L, Shiff SI, Rigas B. Effects of nonsteroidal anti-inflammatory drugs on proliferation and on induction of apoptosis in colon cancer cells by a prostaglandin-independent pathway. *Biochem Pharmacol* 1996; **52**: 237-245

21 **Piazza GA**, Alberts DS, Hixson LJ, Paranka NS, Li H, Finn T, Bogert C, Guillen JM, Brendel K, Gross PH, Sperl G, Ritchie J, Burt RW, Ellsworth L, Ahnen DJ, Pamukcu R. Sulindac sulfone inhibits azoxymethane-induced colon carcinogenesis in rats without reducing prostaglandin levels. *Cancer Res* 1997; **57**: 2909-2915

22 **Shureiqi I**, Chen D, Lotan R, Yang P, Newman RA, Fischer SM, Lippman SM. 15-Lipoxygenase-1 mediates nonsteroidal anti-inflammatory drug-induced apoptosis independently of cyclooxygenase-2 in colon cancer cells. *Cancer Res* 2000; **60**: 6846-6850

23 **Yamamoto Y**, Yin MJ, Lin KM, Gaynor RB. Sulindac inhibits activation of the NF-kappaB pathway. *J Biol Chem* 1999; **274**: 27307-27314

24 **He TC**, Chan TA, Vogelstein B, Kinzler KW. PPARdelta is an APC-regulated target of nonsteroidal anti-inflammatory drugs. *Cell* 1999; **99**: 335-345

25 **Green DR**. Apoptotic pathways: paper wraps stone blunts scissors. *Cell* 2000; **102**: 1-4

26 **Strasser A**, O'Connor L, Dixit VM. Apoptosis signaling. *Annu Rev Biochem* 2000; **69**: 217-245

27 **Thornberry NA**, Lazebnik Y. Caspases: enemies within. *Science* 1998; **281**: 1312-1316

28 **Nicholson DW**, Thornberry NA. Caspases: killer proteases. *Trends Biochem Sci* 1997; **22**: 299-306

29 **Ashkenazi A**, Dixit VM. Death receptors: signaling and modulation. *Science* 1998; **281**: 1305-1308

30 **Hofmann K**, Bucher P, Tschopp J. The CARD domain: a new apoptotic signalling motif. *Trends Biochem Sci* 1997; **22**: 155-156

31 **Kim DM**, Koo SY, Jeon K, Kim MH, Lee J, Hong CY, Jeong S. Rapid induction of apoptosis by combination of flavopiridol and tumor necrosis factor (TNF)-alpha or TNF-related apoptosis-inducing ligand in human cancer cell lines. *Cancer Res* 2003; **63**: 621-626

32 **Rubinchik S**, Yu H, Woraratanadharm J, Voelkel-Johnson C, Norris JS, Dong JY. Enhanced apoptosis of glioma cell lines is achieved by co-delivering FasL-GFP and TRAIL with a complex Ad5 vector. *Cancer Gene Ther* 2003; **10**: 814-822

33 **Singh TR**, Shankar S, Chen X, Asim M, Srivastava RK. Synergistic interactions of chemotherapeutic drugs and tumor necrosis factor-related apoptosis-inducing ligand/Apo-2 ligand on apoptosis and on regression of breast carcinoma in vivo. *Cancer Res* 2003; **63**: 5390-5400

34 **Naka T**, Sugamura K, Hylander BL, Widmer MB, Rustum YM, Repasky EA. Effects of tumor necrosis factor-related apoptosis-inducing ligand alone and in combination with chemotherapeutic agents on patients' colon tumors grown in SCID mice. *Cancer Res* 2002; **62**: 5800-5806

35 **Kroemer G**, Reed JC. Mitochondrial control of cell death. *Nat Med* 2000; **6**: 513-519

36 **Green DR**, Reed JC. Mitochondria and apoptosis. *Science* 1998; **281:** 1309-1312

37 **Petit PX**, Zamzami N, Vayssiere JL, Mignotte B, Kroemer G, Castedo M. Implication of mitochondria in apoptosis. *Mol Cell Biochem* 1997; **174**: 185-188

38 **Verhagen AM**, Coulson EJ, Vaux DL. Inhibitor of apoptosis proteins and their relatives: IAPs and other BIRPs. *Genome Biol* 2001; **2:** REVIEWS3009

39 **Vucic D**, Stennicke HR, Pisabarro MT, Salvesen GS, Dixit VM. ML-IAP, a novel inhibitor of apoptosis that is preferentially expressed in human melanomas. *Curr Biol* 2000; **10**: 1359-1366

40 **Deveraux QL**, Stennicke HR, Salvesen GS, Reed JC. Endogenous inhibitors of caspases. *J Clin Immunol* 1999; **19**: 388-398

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BASIC RESEARCH

Chlamydia pneumoniae replicates in Kupffer cells in mouse model of liver infection

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Abstract

AIM: To develop an animal model of liver infection with *Chlamydia pneumoniae* (*C. pneumoniae*) in intraperitoneally infected mice for studying the presence of chlamydiae in Kupffer cells and hepatocytes.

METHODS: A total of 80 BALB/c mice were inoculated intraperitoneally with *C. pneumoniae* and sacrificed at various time points after infection. Chlamydiae were looked for in liver homogenates as well as in Kupffer cells and hepatocytes separated by liver perfusion with collagenase. *C. pneumoniae* was detected by both isolation in LLC-MK2 cells and fluorescence *in situ* hybridization (FISH). The releasing of TNFA-α by *C. pneumoniae in vitro* stimulated Kupffer cells was studied by enzyme-linked immunosorbent assay.

RESULTS: *C. pneumoniae* isolation from liver homogenates reached a plateau on d 7 after infection when 6 of 10 animals were positive, then decreased, and became negative by d 20. *C. pneumoniae* isolation from separated Kupffer cells reached a plateau on d 7 when 5 of 10 animals were positive, and became negative by d 20. The detection of *C. pneumoniae* in separated Kupffer cells by FISH, confirmed the results obtained by culture. Isolated hepatocytes were always negative. Stimulation of Kupffer cells by alive *C. pneumoniae* elicited high TNF-α levels.

CONCLUSION: A productive infection by *C. pneumoniae* may take place in Kupffer cells and *C. pneumoniae* induces a local pro-inflammatory activity. *C. pneumoniae* is therefore, able to act as antigenic stimulus when localized in the liver. One could speculate that *C. pneumoniae* infection, involving cells of the innate immunity such as Kupffer cells, could also trigger pathological immune reactions involving the liver, as observed in human patients with primary biliary cirrhosis.

Key words: *Chlamydia pneumoniae*; Liver infection; Kupffer cells; Hepatocytes; Culture-isolation; Fluorescence *in situ* hybridization; TNF-α; Primary biliary cirrhosis

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INTRODUCTION

Chlamydia pneumoniae (*C. pneumoniae*) is a common cause of respiratory infections in humans[1,2], and it is also associated with outcomes other than respiratory disease, including coronary heart disease and myocardial infarction[3,4]. Systemic disease has also been reported in which *C. pneumoniae* was detected by polymerase chain reaction in lymph nodes and/or liver and spleen[5]. In addition, recent reports suggest a possible association of *C. pneumoniae* infection in patients with primary biliary cirrhosis (PBC)[6]. This disease is characterized by the presence of anti-mitochondrial autoantibodies and is considered as an autoimmune disease, although precise etiopathogenetic mechanisms remain unknown[7]. Infectious agents have been proposed as triggers in susceptible individuals through a mechanism known as molecular mimicry[8]. It seems therefore possible that antigens from dissociated or alive microbes in Kupffer cells, in other macrophages and lysed cells can trigger immunologically-mediated disorders of the liver such as those observed in PBC[9].

In intranasally infected mouse, *C. pneumoniae* infection has been shown to spread systemically *via* infected macrophages from the initial infection site, the lung, to other organs, including the spleen and occasionally the liver[10]. Here, we report on *C. pneumoniae* infection of the liver in intra-peritoneally infected mice and the involvement of Kupffer cells.

MATERIALS AND METHODS

Animal infection

The animals used in the studies were adult (10-11 wk old) Balb/c mice (Morini, S. Polo D'Enza, Italy). Animals anaesthetised with Ketamine, were inoculated intraperitoneally with purified[11] *C. pneumoniae* elementary body (EB) suspension[11]. Infected animals received 0.1 mL of organism suspension: the inoculum preparation contained 2.0 $\times 10^7$ inclusion-forming units (IFU) of EBs. At d 2, 7, 10, and 20 after infection, anaesthetized animals were sacrificed. The protocol was approved by the ethical committee of the University of Bologna.

C. pneumoniae isolation from the liver

Ten animals were tested at each time point: i.e. at 2, 7, 10 and 20 d after infection. The liver was removed, weighed and homogenized in a mortar to obtain a 10% (wt/vol) suspension in cold sucrose phosphate-glutamic acid (SPG) buffer. Tissue suspensions were centrifuged at 300 × *g* for 10 min at 4℃ to remove coarse debris. The clarified homogenates (200 μL) were inoculated in duplicate onto LLC-MK2 cells (a continuous cell line derived from Rhesus monkey kidney tissue, used to isolate chlamydiae) seeded into plastic individual wells of a 24-well plate, incubated at 37℃ for 72 h in chlamydial growth medium (Eagle's minimal essential medium supplemented with 10% heat inactivated fetal calf serum, containing 2 mmol/L glutamine, 5 mg/L glucose and 1 ng/L cycloheximide) and then fixed in methanol. Chlamydial inclusions were visualized by immunofluorescence.

Isolation of Kupffer cells and hepatocytes

To isolate Kupffer cells and hepatocytes, animals, infected as above, were anaesthetized with Ketamine and sacrificed 2, 7, 10 and 20 d after infection: 10 animals were tested at each time. Kupffer cells and hepatocytes were harvested and separated following the procedure of Smedsrød and Pertoft[12] with minor modifications, as previously described[13,14]. Briefly, the liver was perfused with 30 mL of calcium- and magnesium-free Hanks' balanced salt solution (BSS) followed by Hanks' (BSS) containing 0.05% collagenase (type Ⅳ; Sigma) for 10 min. The liver was then excised and the cells dispersed in calcium- and magnesium-free Hanks' (BSS). The cells were then centrifuged at 50 × *g* at 4℃ for 2 min, in a Beckman J6B centrifuge (Beckman Instrument, Palo Alto, Calif.). The non-parenchymal cell-enriched supernatant was centrifuged at 800 × *g* for 10 min, the pellet resuspended in 40 mL of PBS, and portions of 10 mL were layered on top of preformed two-step Percoll gradient (the bottom cushion with a density of 1.066 g/mL and an osmolality of 310 mOsm; the overlying cushion with a density of 1037 g/mL and an osmolality of 300 mL), and centrifuged at 400 × *g* for 15 min at 4℃. Purified non-parenchymal cells enriched in Kupffer cells extended throughout the lower Percoll cushion. The pellets consisted of erythrocytes, non-parenchymal liver cells, and other small white cells. The Kupffer cells-enriched fraction was diluted in PBS and centrifuged at 800 × *g* for 10 min. The resulting pellet was resuspended in culture medium (RPMI 1640 with 10% fetal calf serum) at a concentration of 1.0×10^6 cells/mL. A 0.5-mL portion of cell suspension was added to 8-well culture plate (Lab-Tek, Nalge Nunc International, Naperville, IL. USA). Kupffer cells were selected by allowing them to adhere for 2 h at 37℃ in an atmosphere with 50 mL/L CO_2. After nonadherent cells were removed by gentle washing, adherent cells were incubated in RPMI 1640. More than 95% of adherent cells were esterase-positive. The purity of Kupffer cell preparations was also validated using the FITC labelled anti-mouse F4/80 monoclonal antibody (Tebu-bio, Magenta, Italy). Hepatocytes were obtained by differential centrifugation of the initial suspension of mouse liver cells followed by an additional purification step, consisting of sedimentation of the cells through a single cushion of Percoll (density 1.08 g/mL). The hepatocytes were seeded on fibronectin-coated slides and grown in RPMI 1640 with 10% fetal calf serum. Kupffer cells and hepatocytes were also separated from uninfected animals.

In vitro infection of Kupffer cells and hepatocytes by C. pneumoniae

When required, Kupffer cells from uninfected animals were obtained as above described and seeded into 8-well culture plate (1.5×10^5 cells/well) and cultured in RPMI 1640 medium for 24 h at 37℃ in an atmosphere with 5% CO_2. Cultures of Percoll-separated hepatocytes were likewise established from uninfected animals by seeding on fibronectin and grown in RPMI 1640 medium, in 8-well culture plate (Lab-Tek). Kupffer cells and hepatocytes were then infected with *C. pneumoniae* EBs (5×10^5 inclusion forming units/mL) and examined for inclusion formation and infectivity at various times after infection by culturing of infected preparations in LLC-MK2 cells.

Detection of viable chlamydiae

To detect the presence of viable chlamydiae in separated Kupffer cells and hepatocytes either from infected animals or from *in vitro* infected Kupffer cells and hepatocytes. Both Kupffer cells and hepatocytes were resuspended in chlamydial growth medium, sonicated and clarified by low speed centrifugation. The supernatant was then inoculated onto LLC-MK2 cells. Chlamydial inclusions were detected by immunofluorescence.

Detection of C. pneumoniae by immunofluorescence assay (IFA)

The detection of *C. pneumoniae* by IFA was performed by fixing separated Kupffer cells and hepatocytes or LLC-MK2 cells with methanol for 10 min at room temperature. Inclusions were then visualized using fluorescein-conjugated *C. pneumoniae* species-specific (Chlamydia-cell, Cellabs PTY LTD Brookvale, Australia) monoclonal antibody for 35 min at room temperature. Slides were observed under a Zeiss UV microscope.

Detection of C. pneumoniae 16S RNA by fluorescence in situ hybridization (FISH)

The *C. pneumoniae* 16S rRNA detection by FISH was performed by fixing mouse Kupffer cells and hepatocytes within the Lab-Tek culture plates with 2% paraformaldehyde for 30 min at 4℃ and prior to FISH, cells were dehydrated with increasing concentrations of ethanol (50%, 80% and 100%). The *Chlamydia*-specific probes used in this

Table 1 Isolation of *C. pneumoniae* from liver homogenates of 40 intraperitoneally infected mice at various days after infection

Days after infection	*n* (pos)/*n* (tested)
2	0/10
7	6/10
10	4/10
20	0/10

Table 2 Detection of *C. pneumoniae* in isolated Kupffer cells and hepatocytes by FISH, IFA and culture, performed in 40 intraperitoneally infected mice at various days after infection

Days after infection	*C. pneumoniae* by FISH and IFA in:		*C. pneumoniae* isolation from:	
	Kupffer cells *n* (pos)/*n* (tested)	Hepatocytes *n* (pos)/*n* (tested)	Kupffer cells *n* (pos)/*n* (tested)	Hepatocytes *n* (pos)/*n* (tested)
2	0/10	0/10	0/10	0/10
7	6/10	0/10	5/10	0/10
10	4/10	0/10	4/10	0/10
20	0/10	0/10	0/10	0/10

study have been previously described by Poppert *et al*[15] and deposited in ProbeBase (http://www.microbial-ecology.de/probebase/index.html). Cpn-0974 (5'-AAGTCCAGGTAAGGTCCT-3') was the species-specific 16S rRNA-targeted oligonucleotide probe that was 5'end labelled with Cy3 fluorochrome (Tib Molbiol, s.r.l., Genova, Italy) giving a red-orange signal. For Kupffer cells and hepatocytes hybridization, a 10 μL aliquot of the hybridization buffer (0.9 mol/L NACl, 20 mol/L Tris-HCl pH 8, 0.01% SDS and from 0% to 30% formamide) containing 5 pmol of each fluorescent probe was applied to each well of Lab Tek culture plate. After 1 h of incubation in a moist chamber at 46℃ in the dark, the slides were washed for 15 min in pre-heated washing buffer containing 20 mmol/L Tris-HCl pH 8, from 0.9 mol/L to 0.15 mol/L NaCl (depending on formamide concentration) and 0.01% SDS. The slides were air dried and mounted with Citifluor AF1 (Citifluor Ltd, London, United Kingdom). The slides were viewed under an epi-fluorescence microscope (Eclipse E600, Nikon) equipped with a super high pressure mercury lamp and Plan Fluor DLL 10 ×, 40 ×, 100 × objectives. Epi-fluorescence filter G-2A was used to analyse Cy3 signal at a magnification of 1000 ×. Photomicrographs were taken using a DXM-1200 digital camera (Nikon, Japan) and image processing was performed with ACT-1 for DXM-1200 software.

TNF-α production by Kupffer cells

To evaluate the TNF-α induction in Kupffer cells infected by *C. pneumoniae in vitro,* Kupffer cells, purified as above reported, were added (5×10^5) to each well of an 8-well culture plate (Lab-Tek). After 2 h at 37℃, non-adherent cells were removed by washing three to five times with RPMI 1640 supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine. Cells were stimulated for 6 h with either LPS (10 mg/L) (Sigma) or viable *C. pneumoniae* at a ratio of 100 EBs/cell. The supernatants were then harvested and tested by enzyme-linked immunosorbent assay (ELISA) kit for TNF-α (Bender MedSystems GmbH, Wien, Austria), according to the manufacturer's protocol. When required, preincubation of material with polymyxin B (10 mg/L) (Sigma) was used to abrogate the effect of LPS.

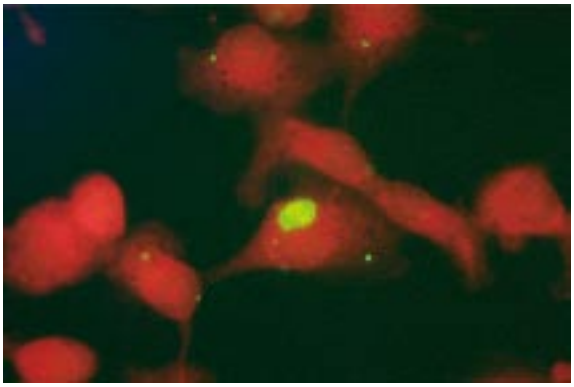

Figure 1 IFA for the detection of *C. pneumoniae* antigens in separated Kupffer cells. 1000 x. Kupffer cells separated from intraperitoneally infected mice sacrificed 7 d after infection were tested by immunofluorescence assay (IFA) with specific fluorescein-conjugated monoclonal antibodies. A positive Kupffer cell is clearly evident for the presence of an apple-green intracytoplasmic bacterial inclusion. The inclusion represents an intracytoplasmic bacterial microcolony.

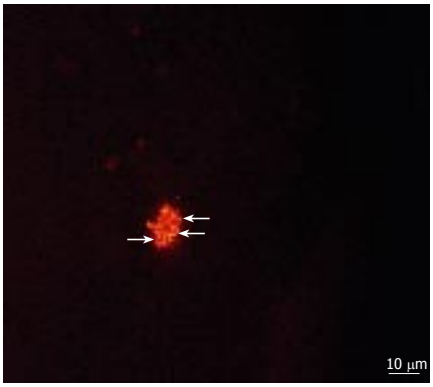

Figure 2 FISH for *C. pneumoniae* 16S rRNA in separated Kupffer cells. *In situ* hybridization was performed on Kupffer cells separated from intraperitoneally infected mice, sacrificed 7 d after infection. An infected Kupffer cell is evidenced with Cpn-0974 specific probe: the granular inclusion stained red.

RESULTS

Four groups of infected animals (10 for each group) were sacrificed 2, 7, 10 and 20 d following intraperitoneal infection by *C. pneumoniae* and isolation of viable chlamydiae was performed from liver homogenates. The peak of *C. pneumoniae* isolation from the liver was obtained on d 7 after infection, thereafter the infectivity decreased, all the animals became negative by d 20 of infection (Table 1).

In order to verify whether *C. pneumoniae* was able to infect *in vivo* Kupffer cells and/or hepatocytes, purified liver cells were tested by IFA, to detect chlamydial antigens and by FISH to detect chlamydial rRNA. Four further groups of infected animals (10 for each group) were sacrificed 2, 7, 10, and 20 d after infection, respectively, then isolated Kupffer cells and hepatocytes were analysed. Positive Kupffer cells were observed in preparations obtained on d 7 and 10 (Table 2), both by IFA and FISH, thus confirming the results obtained by culture. Kupffer cells purified 20 d after infections were negative. Positive cells showed

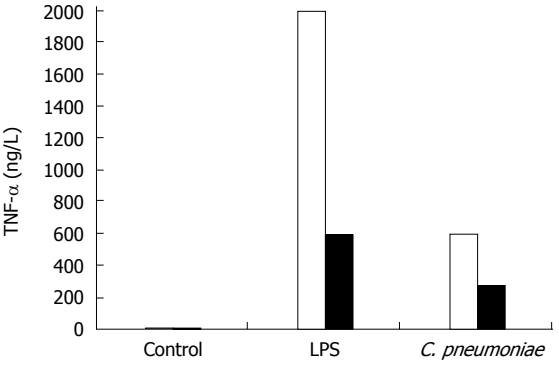

Figure 3 TNF-α release by Kupffer cells following *in vitro* exposure to alive *C. pneumoniae*. Kupffer cells were stimulated for 6 h with alive *C. pneumoniae*. Control LPS was used at 10 mg/L. Stimulation was performed with (black) or without (white) polymyxin B at concentration of 10 mg/L.

cytoplasmic apple-green inclusions by IFA (Figure 1) and red granular inclusions by FISH (Figure 2). The number of positive cells was 10/100 in preparations obtained 7 d after infection, and 5/100 in preparations obtained 10 d after infection. The preparations from animals sacrificed 20 d after infection were negative. The passage of the content of Kupffer cells obtained from IFA- and FISH-positive animals induced chlamydial inclusion formations in LLC-MK2 cells. Hepatocytes were always negative by IFA and FISH and by culture in LLC-MK2 cells.

The *in vitro* infection of purified mouse Kupffer cells and hepatocytes by *C. pneumoniae* was followed by FISH and by culture: Kupffer cells were positive for the presence of inclusions detectable by 12 h of infection. Sub-culturing Kupffer cells in LLC-MK2 mono-layers three days after infection allowed re-isolation of *C. pneumoniae*. Hepatocytes infected *in vitro* by *C. pneumoniae* and studied by IFA and FISH was negative 12, 24, and 48 h after infection. Sub-culturing hepatocytes in LLC-MK2 cells three days after infection did not allow re-isolation of *C. pneumoniae,* demonstrating that *C. pneumoniae* is unable to infect hepatocytes.

In vitro stimulation of Kupffer cells by alive *C. pneumoniae* elicited cellular responses resulting in the production of TNF-α (Figure 3). Pretreatment of chlamydiae with polymyxin B, which binds and inactivates LPS[16], substantially diminished the ability of bacteria to stimulate Kupffer cells (Figure 3).

DISCUSSION

Chlamydiae are obligate intracellular bacteria parasitizing eukaryotic cells. Chlamydiae replicate in the cytoplasm of infected cells within an inclusion that does not fuse with lysosomes[17]. Within the genus *Chlamydia,* various species preferentially infect different target cells and cause a variety of diseases all ultimately due to inflammatory responses. In particular, *C. pneumoniae* has been associated with respiratory human infections and with the development of atherosclerosis and cardiovascular disease[18-21]. *C. pneumoniae* has been also associated with sarcoidosis and it was detected in 4 of 38 liver biopsies from autopsy specimens of patients with sarcoidosis[22]. In a rabbit model of *C. pneumoniae* infection, three of 10 infected rabbits had evidence of *C. pneumoniae* elementary bodies in their livers by immunocytochemical staining and *C. pneumoniae* was cultured in two of them[23]. In a hamster model of *C. pneumoniae* infection, similarly *C. pneumoniae* was isolated from the liver of the infected animals[24]. These studies altogether indicate that *C. pneumoniae* can potentially persist in liver tissues.

More recently, Abdulkarim *et al*[6] reported the presence of *C. pneumoniae* antigens and rRNA in liver tissue of patients with end-stage primary biliary cirrhosis, suggesting a potential role of *C. pneumoniae* in the etiology and pathogenesis of the disease, in contrast with serological data by Liu *et al*[25] that do not support the concept that *Chlamydia pneumoniae* can be a triggering or causative agent in PBC.

To get further insight into a possible role of *C. pneumoniae* in liver related pathologies, evidence was to be produced as to the possible localization and/or replication of *C. pneumoniae* in parenchymal and not parenchymal liver cells, mainly in Kupffer cells, where chlamydiae could act as a trigger for cellular immunologically-mediated alterations. Kupffer cells constitute the largest population of fixed tissue macrophages found in the body, and blood-clearance and elimination of bacteria taken up by the liver are widely attributed to Kupffer cells. Sometimes micro-organisms can survive or even multiply in Kupffer cells, as observed in *Leishmania* and S*almonella typhi* infections in mice and humans[26,27]. In this study, we have described a mouse model of *C. pneumoniae* infection where the organism demonstrated the ability to infect and to multiply in Kupffer cells both *in vivo* and *in vitro*, and to trigger the release of the proinflammatory cytokine TNF-α, by these macrophages. In our animal model, mice eliminated *C. pneumoniae* by d 20 of infection and a chronic infection was not established. However, it is well known that persistence, for as yet unpredictable reasons, is a frequent case of human chlamydial infections[28].

PBC is a chronic liver inflammatory disease whose etiology and pathogenesis remain still unknown. A wide range of data suggest an autoimmune pathogenesis for the disease[29,30], mostly based on the presence of anti-mitochondrial autoantibodies and autoreactive T cells directed against autoantigens. Despite the fact that the autoimmune reaction is directed against ubiquitous mitochondrial autoantigens, the ensuing damage involves primarily biliary epithelial cells[7]. Infectious agents have been proposed as triggers in susceptible individuals through a mechanism known as molecular mimicry[8]. However, over the past years several studies failed to demonstrate a specific chronic microbial[8,31], viral[32,33] or bacterial[34-36] infection. Recently, it has been proposed that an aberrant innate immune response to infections has the potential to initiate the development of autoimmunity[37]. The ability of *C. pneumoniae* to survive and to replicate in Kupffer cells, as documented in this study, demonstrates the ability of this bacterium to escape, under certain conditions, the defence mechanisms of natural immunity. This characteristic can favour, as suggested by Liu *et al*[25], the production of highly immunogenic substrates and/or provide an inflammatory microenvironment to enhance the self-reactivity of pathogenic T lymphocytes, or can activate pre-existing autoreactive cellular repertoire.

REFERENCES

1 **Grayston JT**, Aldous MB, Easton A, Wang SP, Kuo CC, Campbell LA, Altman J. Evidence that Chlamydia pneumoniae causes pneumonia and bronchitis. *J Infect Dis* 1993; **168**: 1231-1235

2 **Marrie TJ**, Peeling RW, Fine MJ, Singer DE, Coley CM, Kapoor WN. Ambulatory patients with community-acquired pneumonia: the frequency of atypical agents and clinical course. *Am J Med* 1996; **101**: 508-515

3 **Saikku P**, Leinonen M, Tenkanen L, Linnanmaki E, Ekman MR, Manninen V, Manttari M, Frick MH, Huttunen JK. Chronic Chlamydia pneumoniae infection as a risk factor for coronary heart disease in the Helsinki Heart Study. *Ann Intern Med* 1992; **116**: 273-278

4 **Melnick SL**, Shahar E, Folsom AR, Grayston JT, Sorlie PD, Wang SP, Szklo M. Past infection by Chlamydia pneumoniae strain TWAR and asymptomatic carotid atherosclerosis. Atherosclerosis Risk in Communities (ARIC) Study Investigators. *Am J Med* 1993; **95**: 499-504

5 **Grayston JT**. Chlamydia pneumoniae (TWAR) infections in children. *Pediatr Infect Dis J* 1994; **13**: 675-684; quiz 685

6 **Abdulkarim AS**, Petrovic LM, Kim WR, Angulo P, Lloyd RV, Lindor KD. Primary biliary cirrhosis: an infectious disease caused by Chlamydia pneumoniae? *J Hepatol* 2004; **40**: 380-384

7 **Kaplan MM**, Gershwin ME. Primary biliary cirrhosis. *N Engl J Med* 2005; **353**: 1261-1273

8 **Haydon GH**, Neuberger J. PBC: an infectious disease? *Gut* 2000; **47**: 586-588

9 **Ljungh A**, Nilsson HO, Wadstrom T, Stenram U, Willen R. Microbes as trigger for primary biliary cirrhosis and primary sclerosing cholangitis. *Dig Liver Dis* 2005; **37**: 803-804

10 **Yang ZP**, Kuo CC, Grayston JT. Systemic dissemination of Chlamydia pneumoniae following intranasal inoculation in mice. *J Infect Dis* 1995; **171**: 736-738

11 **Montigiani S**, Falugi F, Scarselli M, Finco O, Petracca R, Galli G, Mariani M, Manetti R, Agnusdei M, Cevenini R, Donati M, Nogarotto R, Norais N, Garaguso I, Nuti S, Saletti G, Rosa D, Ratti G, Grandi G. Genomic approach for analysis of surface proteins in Chlamydia pneumoniae. *Infect Immun* 2002; **70**: 368-379

12 **Smedsrod B**, Pertoft H. Preparation of pure hepatocytes and reticuloendothelial cells in high yield from a single rat liver by means of Percoll centrifugation and selective adherence. *J Leukoc Biol* 1985; **38**: 213-230

13 **Sambri V**, Aldini R, Massaria F, Montagnani M, Casanova S, Cevenini R. Uptake and killing of Lyme disease and relapsing fever borreliae in the perfused rat liver and by isolated Kupffer cells. *Infect Immun* 1996; **64**: 1858-1861

14 **Marangoni A**, Aldini R, Sambri V, Montagnani M, Ballardini G, Storni E, Cevenini R. Uptake and killing of Leptospira interrogans and Borrelia burgdorferi, spirochetes pathogenic to humans, by reticuloendothelial cells in perfused rat liver. *Infect Immun* 2000; **68**: 5408-5411

15 **Poppert S**, Essig A, Marre R, Wagner M, Horn M. Detection and differentiation of chlamydiae by fluorescence in situ hybridization. *Appl Environ Microbiol* 2002; **68**: 4081-4089

16 **Jacobs DM**. Structural features of binding of lipopolysaccharides to murine lymphocytes. *Rev Infect Dis* 1984; **6**: 501-505

17 **Fields KA**, Hackstadt T. The chlamydial inclusion: escape from the endocytic pathway. *Annu Rev Cell Dev Biol* 2002; **18**: 221-245

18 **Siscovick DS**, Schwartz SM, Caps M, Wang SP, Grayston JT. Chlamydia pneumoniae and atherosclerotic risk in populations: the role of seroepidemiology. *J Infect Dis* 2000; **181** Suppl 3: S417-S420

19 **Siscovick DS**, Schwartz SM, Corey L, Grayston JT, Ashley R, Wang SP, Psaty BM, Tracy RP, Kuller LH, Kronmal RA. Chlamydia pneumoniae, herpes simplex virus type 1, and cytomegalovirus and incident myocardial infarction and coronary heart disease death in older adults: the Cardiovascular Health Study. *Circulation* 2000; **102**: 2335-2340

20 **Kuo CC**, Grayston JT, Campbell LA, Goo YA, Wissler RW, Benditt EP. Chlamydia pneumoniae (TWAR) in coronary arteries of young adults (15-34 years old). *Proc Natl Acad Sci USA* 1995; **92**: 6911-6914

21 **Kuo CC**, Shor A, Campbell LA, Fukushi H, Patton DL, Grayston JT. Demonstration of Chlamydia pneumoniae in atherosclerotic lesions of coronary arteries. *J Infect Dis* 1993; **167**: 841-849

22 **Puolakkainen M**, Campbell LA, Kuo CC, Leinonen M, Gronhagen-Riska C, Saikku P. Serological response to Chlamydia pneumoniae in patients with sarcoidosis. *J Infect* 1996; **33**: 199-205

23 **Fong IW**, Chiu B, Viira E, Fong MW, Jang D, Mahony J. Rabbit model for Chlamydia pneumoniae infection. *J Clin Microbiol* 1997; **35**: 48-52

24 **Sambri V**, Donati M, Storni E, Di Leo K, Agnusdei M, Petracca R, Finco O, Grandi G, Ratti G, Cevenini R. Experimental infection by Chlamydia pneumoniae in the hamster. *Vaccine* 2004; **22**: 1131-1137

25 **Liu HY**, Deng AM, Zhang J, Zhou Y, Yao DK, Tu XQ, Fan LY, Zhong RQ. Correlation of Chlamydia pneumoniae infection with primary biliary cirrhosis. *World J Gastroenterol* 2005; **11**: 4108-4110

26 **Vazquez-Torres A**, Vallance BA, Bergman MA, Finlay BB, Cookson BT, Jones-Carson J, Fang FC. Toll-like receptor 4 dependence of innate and adaptive immunity to Salmonella: importance of the Kupffer cell network. *J Immunol* 2004; **172**: 6202-6208

27 **el Hag IA**, Hashim FA, el Toum IA, Homeida M, el Kalifa M, el Hassan AM. Liver morphology and function in visceral leishmaniasis (Kala-azar). *J Clin Pathol* 1994; **47**: 547-551

28 **Morrison RP**, Caldwell HD. Immunity to murine chlamydial genital infection. *Infect Immun* 2002; **70**: 2741-2751

29 **Bogdanos DP**, Baum H, Vergani D. Antimitochondrial and other autoantibodies. *Clin Liver Dis* 2003; **7**: 759-777, vi

30 **Ishibashi H**, Nakamura M, Shimoda S, Gershwin ME. T cell immunity and primary biliary cirrhosis. *Autoimmun Rev* 2003; **2**: 19-24

31 **Tanaka A**, Prindiville TP, Gish R, Solnick JV, Coppel RL, Keeffe EB, Ansari A, Gershwin ME. Are infectious agents involved in primary biliary cirrhosis? A PCR approach. *J Hepatol* 1999; **31**: 664-671

32 **Mason AL**, Xu L, Guo L, Munoz S, Jaspan JB, Bryer-Ash M, Cao Y, Sander DM, Shoenfeld Y, Ahmed A, Van de Water J, Gershwin ME, Garry RF. Detection of retroviral antibodies in primary biliary cirrhosis and other idiopathic biliary disorders. *Lancet* 1998; **351**: 1620-1624

33 **Morshed SA**, Nishioka M, Saito I, Komiyama K, Moro I. Increased expression of Epstein-Barr virus in primary biliary cirrhosis patients. *Gastroenterol Jpn* 1992; **27**: 751-758

34 **Harada K**, Tsuneyama K, Sudo Y, Masuda S, Nakanuma Y. Molecular identification of bacterial 16S ribosomal RNA gene in liver tissue of primary biliary cirrhosis: is Propionibacterium acnes involved in granuloma formation? *Hepatology* 2001; **33**: 530-536

35 **Hopf U**, Moller B, Stemerowicz R, Lobeck H, Rodloff A, Freudenberg M, Galanos C, Huhn D. Relation between Escherichia coli R(rough)-forms in gut, lipid A in liver, and primary biliary cirrhosis. *Lancet* 1989; **2**: 1419-1422

36 **O'Donohue J**, Fidler H, Garcia-Barcelo M, Nouri-Aria K, Williams R, McFadden J. Mycobacterial DNA not detected in liver sections from patients with primary biliary cirrhosis. *J Hepatol* 1998; **28**: 433-438

37 **Mao TK**, Lian ZX, Selmi C, Ichiki Y, Ashwood P, Ansari AA, Coppel RL, Shimoda S, Ishibashi H, Gershwin ME. Altered monocyte responses to defined TLR ligands in patients with primary biliary cirrhosis. *Hepatology* 2005; **42**: 802-808

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BASIC RESEARCH

Peroxisome proliferator-activated receptor γ agonist reduces the severity of post-ERCP pancreatitis in rats

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Abstract

AIM: To determine the effects of prophylactic peroxisome proliferator-activated receptor (PPARγ) agonist administration in an experimental model of post-endoscopic retrograde cholangiopancreatography (post-ERCP) acute pancreatitis.

METHODS: Post-ERCP pancreatitis was induced in male Wistar rats by infusion of contrast medium into the pancreatic duct. In additional group, rosiglitazone, a PPARγ agonist, was administered 1 h before infusion of contrast medium. Plasma and pancreas samples were obtained 6 h after the infusion.

RESULTS: Infusion of contrast medium into the pancreatic duct resulted in an inflammatory process characterized by increased lipase levels in plasma, and edema and myeloperoxidase activity (MPO) in pancreas. This result correlated with the activation of nuclear factor κB (NFκB) and the inducible NO synthase (iNOS) expression in pancreatic cells. Rosiglitazone reduced the increase in lipase and the level of edema and the increase in myeloperoxidase as well as the activation of NFκB and iNOS expression.

CONCLUSION: A single oral dose of rosiglitazone, given 1 h before post-ERCP pancreatitis induction is effective in reducing the severity of the subsequent inflammatory process. The protective effect of rosiglitazone was associated with NFκB inhibition and the blockage of leukocyte infiltration in pancreas.

Key words: Peroxisome proliferator-activated receptor γ; Pancreatitis; Endoscopic retrograde cholangio pancreatography; Inflammation; Nuclear factor κB

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INTRODUCTION

Acute pancreatitis is one of the major and serious complications after diagnostic or therapeutic endoscopic retrograde cholangiopancreatography (ERCP). Despite the technical improvements of recent years and the experience of endoscopists, the incidence has not decreased and it ranges from 1% to 10% of patients[1]. The most severe forms of pancreatitis, with pancreatic necrosis, multi-organ failure and even death, occurs in 0.3%-0.6% of patients, but a silent increase in serum pancreatic enzymes could be observed in up to 70% of patients[2]. The triggering mechanism of the inflammatory response remains unclear and different pharmacological agents has been tested to prevent post-ERCP pancreatitis including anti-inflammatory steroids, somatostatin analogs, heparin, protease inhibitors and anti-inflammatory cytokines[3-6]. Only few drugs showed some efficacy to prevent post-ERCP pancreatitis like recently nitrate therapy by decreasing the pancreatic ductal pressure or diclofenac by lowering inflammatory process[7,8]. Therefore, the availability of effective drugs and strategy of chemoprevention remain as unsettled points in the pharmacological prophylaxis of post-ERCP pancreatitis[5-8], leading to the apparition of endoscopic procedure such as pancreatic sphincterotomy to try to decrease the risk of post-ERCP pancreatitis[9].

Recent evidences indicate an important role for the peroxisome proliferator-activated receptors (PPARs) in the regulation of both inflammation and lipid metabolism[10]. In particular, it has been reported, using an experimental model of cerulein-induced pancreatitis, that the pancreatic inflammation and tissue injury was markedly reduced by the administration of PPARγ agonists[11]. PPARγ is a member of the nuclear hormone receptor superfamily originally reported to be expressed at high levels in

adipose tissue and to play a critical role in adipocyte differentiation, glucose metabolism and lipid storage. In an experimental model of intestinal ischemia-reperfusion, a more severe injury was observed in PPARγ-deficient mice and protection against local and remote tissue injury in mice treated with a PPARγ-activating ligand[12]. Then, it has been demonstrated that PPARγ ligands can inhibit the inflammatory response by decreasing IL-6, IL-1β, TNFα and the inducible NO synthase (iNOS) by interfering with nuclear factor κB (NFκB) and AP1[13,14]. The aim of this study was to investigate the efficacy of prophylactic PPARγ agonist treatment in reducing the pancreatic damage in an experimental post-ERCP acute pancreatitis model.

MATERIALS AND METHODS

Reagents

Reagents for SDS-PAGE and nitrocellulose membranes were from Amersham Pharmacia (Buckinghamshire, England). Antibodies against p65, PPARγ and Histone H1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), antibody against iNOS was obtained from BD Transduction (Heildelberg, Germany), antibody against β-actin and the secondary antibody linked to horseradish peroxidase were from Sigma Chemicals (St Louis, MO). The following reagents were obtained from Sigma Chemicals (St Louis, MO): $NaVO_3$, NaF, Nonidet P40, ethidium bromide, Hexadecyltrimethylammonium bromide, Tetramethylbenzidine, DMSO, H_2O_2.

Post-ERCP pancreatitis model

Male Wistar rats (250-300 g) were used for all experiments. Animals were housed in light-dark cycle regulated, air conditioned (23℃) and air humity (60%) animal quarters, given free access to drinking water and standard food pellets until 12 h prior to the experiment, at which point food was withdrawn. Animal care was in compliance with the European Community (Directive 86/609/EEC) for the use of experimental animals and the institutional committee of animal care and research approved it. Rats were anaesthetized with ip injection of sodium pentobarbital (10 μL/kg). The biliopancreatic duct was cannulated through the duodenum and the hepatic duct was closed by a small bulldog clamp. Post-ERCP pancreatitis was induced by retrograde infusion into the biliopancreatic duct of low osmolarity contrast medium Meglumine/Sodium Ioxaglate (Hexabrix 320) in a volume of 10 μL/kg using a Harvard '22' infusion pump (Harvard Instruments, Edenbridge, UK). Control animals were subjected to anesthesia and laparotomy[15].

Experimental design

In the first set of experiments, we evaluated the severity of pancreatic damage and tissue inflammation after the infusion of contrast medium into the pancreatic duct. For this purpose, rats (n = 6 for each group) were sacrificed at 0, 3, 6 and 24 h after infusion and samples of pancreatic tissue and plasma were obtained, immediately frozen and maintained at -80℃ until assayed.

In a second series of experiments, a PPARγ agonist (Rosiglitazone, AVANDIA® GlaxoSmithKline, Brentford, UK) was administered (10 mg/kg intragastric bolus) 1 h before infusion of contrast medium (n = 8 for each group)[11]. Samples of plasma and pancreas were obtained 6 h after infusion.

Edema

The extent of pancreas edema was assayed by measuring tissue water content. Freshly obtained samples of pancreas were weighted on aluminum foil, dried for 24 h at 95℃ and reweighed. The difference between wet and dry tissue weight was calculated and expressed as a tissue wet: dry mass ratio.

Myeloperoxidase activity

Neutrophil infiltration was assessed by measuring myeloperoxidase (MPO) activity. Myeloperoxidase was measured photometrically with 3,3',5,5' -tetramethylbenzidine as a substrate[16]. Samples were macerated with 5 g/L hexadecyltrimethylammoniu m bromide in 50 mmol/L phosphate buffer pH 6.0. Homogenates where then disrupted for 30 s using a Labsonic (B.Braun) sonicator at 20% power and submitted to three cycle of snap freezing in dry ice and thawing before a final 30 s sonication. Samples were incubated at 60℃ for 2 h and then spun down at 4000 g for 12 min. Supernatants were collected for myeloperoxidase assay. Enzyme activity was assessed photometrically at 630 nm. The assay mixture consisted of 20 μL supernatant, 10 μL tetramethylbenzidine (final concentration 1.6 mmol/L) dissolved in DMSO and 70 μL H_2O_2 (final concentration 3.0 mmol/L) diluted in 80 mmol/L phosphate buffer pH 5.4. An enzyme unit is defined as the amount of enzyme that produces an increase of 1 absorbance unit per minute.

NFκB and PPARγ DNA binding

Binding of NFκB p65 subunit to the NFκB binding consensus sequence 5'-GGGACTTTCC-3' and binding of PPARγ to the PPRE binding consensus sequence 5' -AACTAGGTCAAAGGTCA-3' were measured with the ELISA-based TransAM kits (Active Motif, Carlsbad, CA) using tissue nuclear extracts. This assay is performed in 96-well plates coated with an oligonucleotide containing the binding consensus sequence. The active forms in nuclear extracts can be detected using specific Abs for epitopes that are accessible only when the subunits are activated and bound to its target DNA. Specificities were checked by measuring the ability of soluble wild-type oligonucleotides to inhibit binding.

Western blot

Pancreatic tissue was lysed by using the Nuclear Extract Kit from Active Motif (Carlsbad, CA) following the manufacturer conditions for preparation of cytoplasmatic and nuclear extracts. SDS-PAGE was performed using 100 g/L or 120 g/L acrylamide gels. Proteins were electrotransfered to nitrocellulose membrane and probed with primary Ab (anti-p65, 1/1000; anti-PPARγ, 1/200; anti-iNOS, 1/400; anti-β-actin, 1/400; anti-Histone H1, 1/500). The membranes were incubated with corresponding horseradish peroxidase-linked secondary Ab, washed and subsequently incubated with ECL reagents

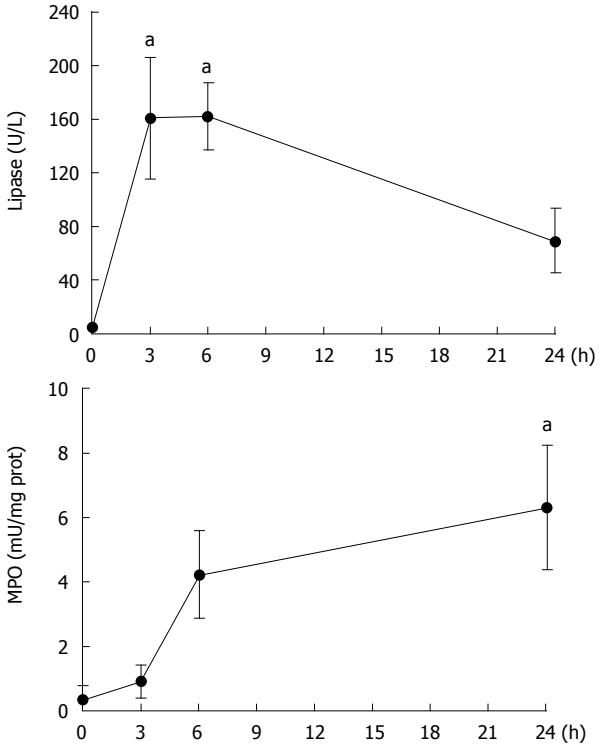

Figure 1 Plasma lipase and pancreas MPO activity after retrograde infusion of contrast media. ($^aP < 0.05$ *vs* $t = 0$).

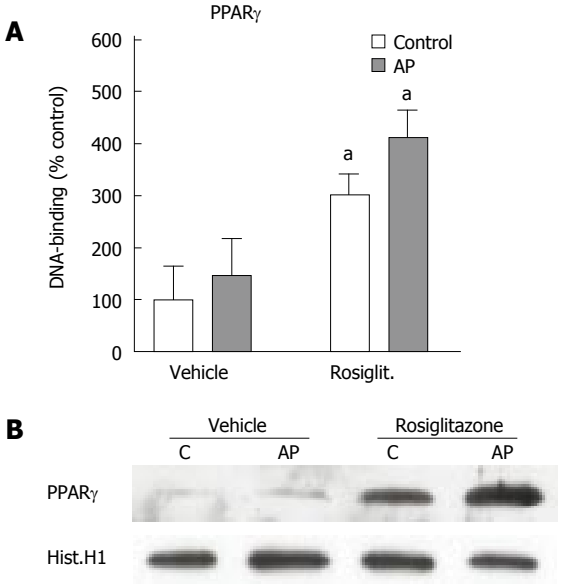

Figure 2 PPARγ activation. **A**: PPARγ DNA binding activity of pancreas nuclear extracts expressed as % of control activity. No significant differences were observed on PPARγ binding to DNA after pancreatitis induction. By contrast, PPARγ binding to DNA is strongly induced by rosiglitazone in both control and pancreatitis groups. $^aP < 0.05$ Rosiglitazone-treated *vs* vehicle-treated groups. **B:** Western blot of nuclear PPARγ confirmed the nuclear translocation of PPARγ protein after rosiglitazone administration in both control and pancreatitis groups. Western blot was representative of three different experiments.

from Amersham Pharmacia (Buckinghamshire, England) before exposure to high performance chemiluminescence films. Gels were calibrated using Bio-Rad standard proteins (Hercules, CA) with markers covering a 7-240 kDa range.

Protein measurement

Total protein concentration in homogenates was determined using a commercial kit from BioRad (Munich, Germany).

Lipase

Plasma lipase was determined by using commercial kits from Randox (Antrim, UK), according to the supplier's specifications.

Histological study

Pancreatic tissue samples were taken and fixed in 40 g/L neutral buffered formaldehyde solution, paraplast-embedded, cut into 5 μm sections and stained with hematoxilyn-eosin for light microscopy. Two different observers evaluated randomly ten fields from each animal and cell infiltration was recorded blindly on photomicrographs.

Statistical analysis

Data have been expressed as mean ± SE. Means of different groups were compared using a one-way analysis of variance. Tukey's multiple comparison test was performed for evaluation of significant differences between groups. Differences were assumed to be significant when $P < 0.05$.

RESULTS

ERCP-induced pancreatic damage

The evolution of pancreatic damage was evaluated by measuring plasma lipase and tissue MPO activity at different time points after pancreas infusion of contrast medium (Figure 1). A rapid and significant increase was observed in plasma lipase activity that achieved a peak between 3 and 6 h after surgery. By contrast, MPO activity was not increased until 6 h after infusion and remained increased until the end of experiment (24 h). Since in this model the inflammation required 6 h to be established, we selected this time point for the rest of experiments.

Treatment with rosiglitazone induced pancreatic activation of PPARγ

To evaluate the efficacy of rosiglitazone to induce PPARγ activation in the pancreatic tissue, we measured, on nuclear extracts, the levels of DNA binding activity to an immobilized oligonucleotide containing the PPRE sequence. Binding activity was significantly increased in rosiglitazone-treated animals (Figure 2A). By contrast, in non-treated animals, ERCP-induced pancreatitis was not associated with changes in pancreatic PPARγ activity. These results were confirmed by western blot analysis of the translocation of PPARγ into the nuclear fraction upon rosiglitazone treatment (Figure 2B).

Rosiglitazone reduced the ERCP-induced pancreatic damage

Both tissue edema and plasma lipase activity showed

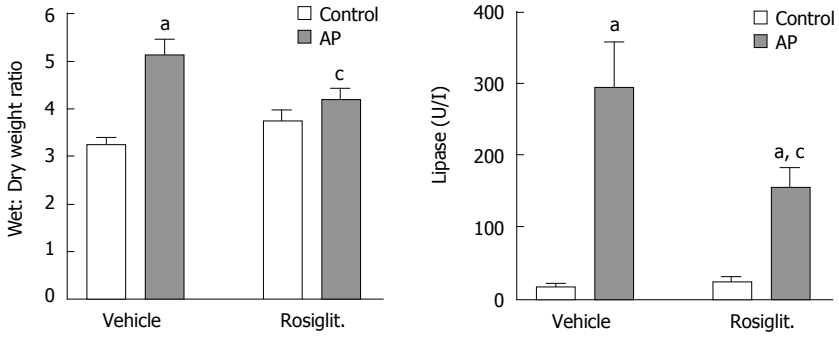

Figure 3 Pancreatic tissue edema and plasma lipase activity 6 h after contrast medium infusion. Retrograde administration of contrast medium induced increases in edema and lipase activity. These increases were partially prevented by pre-treatment of rosiglitazone. [a]*P* < 0.05 AP *vs* their corresponding control; [c]*P* < 0.05 rosiglitazone-treated AP group *vs* vehicle-treated AP group.

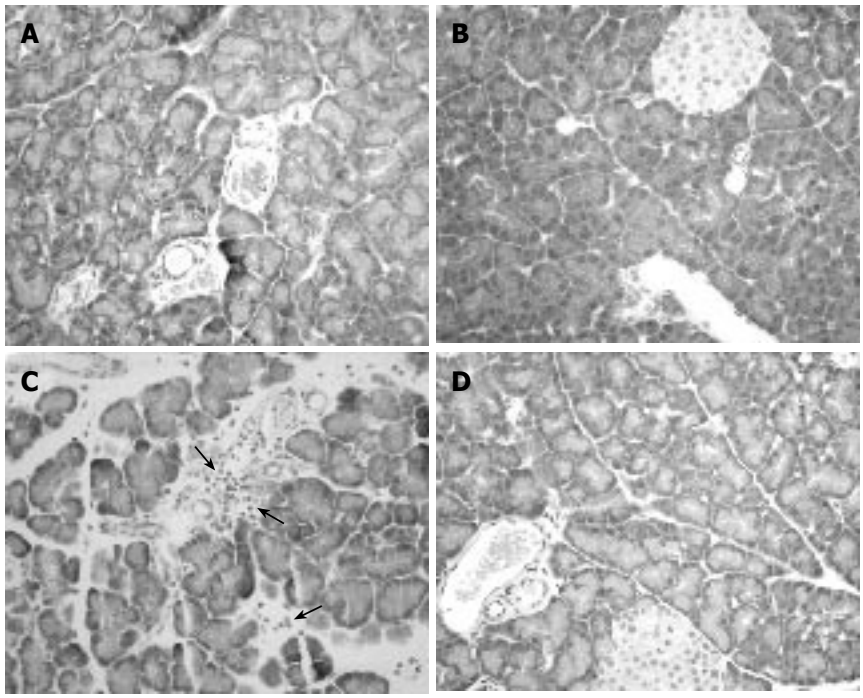

Figure 4 Histological examination of the pancreas (x 200). **A:** Control pancreas showed normal acinar structure; **B:** No morphological changes were observed after rosiglitazone administration in control animals; **C:** Experimental ERCP-induced pancreatic damage reflected in interlobular and interacinar edema and areas of leukocyte infiltration (arrows). Acinar necrosis was not observed; **D:** Rosiglitazone pre-treatment before contrast medium infusion resulted in a reduced edema and absence of leukocyte cell infiltration.

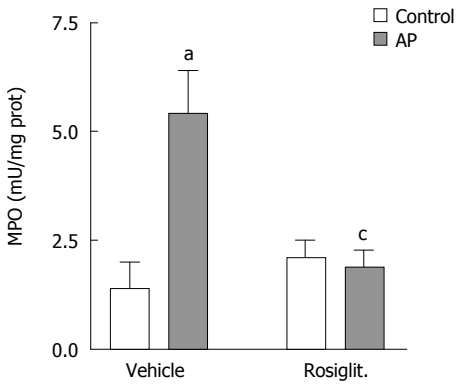

Figure 5 Myeloperoxidase activity in pancreas. MPO activity was significantly increased in post-ERCP induced pancreatitis in comparison with vehicle. Rosiglitazone treatment prevented this increase. [a]*P* < 0.05 AP *vs* control; [c]*P* < 0.05 rosiglitazone-treated AP group *vs* Vehicle-treated AP group.

increased levels 6 h after contrast media infusion (Figure 3). Pre-treatment with rosiglitazone significantly reduced these increases, but not to control levels. Histological findings also showed a clear reduction on pancreatic interlobular edema (Figure 4). No acinar necrosis was observed after contrast-media infusion.

ERCP-induced inflammatory response was inhibited by rosiglitazone

When measuring the MPO activity, we observed that the increase induced by contrast medium infusion was completely abrogated by pre-treatment with rosiglitazone (Figure 5). This result was confirmed by histological results. In ERCP group, areas of intense cell infiltration with extravasation of leukocytes to the interacinar space were observed (Figure 4C). Treatment with rosiglitazone completely prevented the infiltration of leukocytes (Figure 4D).

Rosiglitazone prevented the ERCP-induced NFkB-activation

In order to evaluate the possible involvement of NFκB on this anti-inflammatory effect, we measured the levels of p65 DNA binding activity in pancreatic nuclear extracts. Results indicated that infusion of contrast media into the pancreatic duct induced a significant activation of NFκB (Figure 6A). This increase was completely prevented by pre-treatment of rosiglitazone. The effect of the PPAR-γ agonist on NFκB was confirmed by detecting, by western blot, the presence of p65 subunit of NFκB into the nuclear fraction (Figure 6B). In the ERCP group, p65

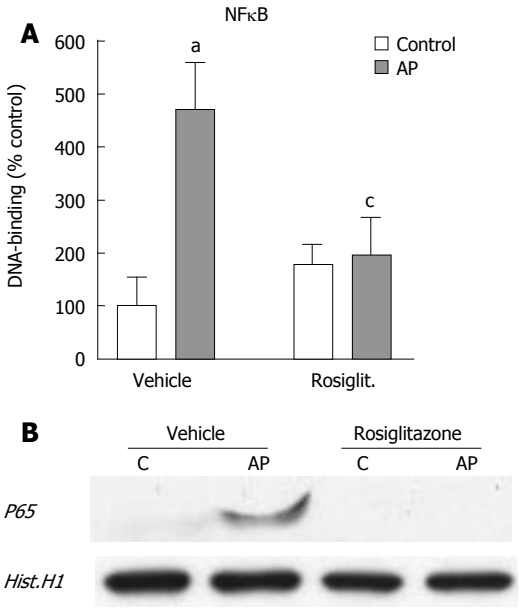

Figure 6 NFκB activation. **A**: NFκB DNA binding activity of pancreas nuclear extracts expressed as % of control p65 activity. Post-ERCP induced pancreatitis resulted in increased levels of p65 binding activity. This increase was inhibited by rosiglitazone pre-treatment. $^{a}P < 0.05$ AP *vs* Control; $^{c}P < 0.05$ rosiglitazone-treated AP group *vs* vehicle-treated AP group; **B:** Western blot of nuclear p65 confirmed the nuclear translocation of p65 protein during ERCP-induced pancreatitis. This nuclear translocation was not observed after rosiglitazone administration. Western blot was representative of three different experiments.

translocated into the nucleus and this translocation was prevented by pre-treatment of rosiglitazone.

Rosiglitazone prevented ERCP-induced expression of iNOS

Finally, similar result was found when determining the expression of iNOS, an NFκB-dependent enzyme (Figure 7). In ERCP group, iNOS was strongly induced, and pre-treatment with rosiglitazone prevented this increase. Interestingly, the enzyme, that is undetectable in control animals showed a weak expression in control animals treated with rosiglitazone.

DISCUSSION

Since pancreatitis represents the most common complication after ERCP, different drugs, including IL-10, gabexate mesylate, heparin or somatostatin, nitrate derivates or diclofenac have been tested to reduce the incidence and severity of post ERCP-pancreatitis. Many of these studies, despite the use of randomized procedures were criticized because of non reproducible results[3]. In addition, the multifactorial etiology and pathophysiology of post-ERCP pancreatitis needs to be taken into account. Simplified procedures and the absence of adverse effects are required to deliver prophylactic treatment for post-ERCP pancreatitis. Our study shows that administration of a PPARγ agonist in a single oral dose before starting ERCP decreases the severity of the inflammatory reaction triggered by this procedure. Evidence has been accumulated indicating that PPARγ plays a role modulating the inflammation. Several studies have demonstrated that the use of PPARγ ligands inhibits the intensity of the

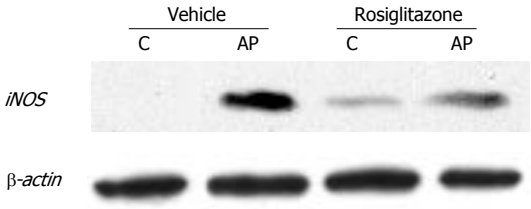

Figure 7 Inducible NO synthase (iNOS) expression. Western blot of cytoplasmatic iNOS showed increased expression of this protein during post-ERCP induced pancreatitis. This increase was reduced by rosiglitazone pre-treatment. Results are representative of three separate experiments.

inflammatory response in different processes including colitis[17], adjuvant-induced arthritis[14], and cerulein-induced pancreatitis[11]. In vitro, the expression of inflammatory mediators such as TNFα, IL-1β, IL-6, iNOS or MMP-9[13,18] could be inhibited by PPARγ ligands. These findings have raised the possibility that these agents could be useful for the treatment of the inflammatory disorders.

Our results indicate that administration of rosiglitazone before experimental ERCP completely prevented the inflammatory response in the pancreas, reflected in a reduced MPO activity and the lack of leukocytes infiltrate observed in pancreas. By contrast, increases in lipase plasma activity and edema were only partially prevented by rosiglitazone. This fact was not unexpected, since rosiglitazone prevents the activation of the inflammatory response, but has no effect on the mechanical damage related with changes in osmolarity or increased intraductal pressure. Pancreatic damage results from both mechanical and inflammatory processes associated with intraductal activation of pancreatic proenzymes. Nitrate derivates or pancreatic stenting have been proposed to increase pancreatic out-flow, diclofenac or IL-10 following inflammatory response and somatostatin to decrease the intraductal concentration of pancreatic proenzymes[5-9]. Rosiglitazone was supposed to act only on the inflammatory-related increases in lipase and edema.

It is known that the activation of PPARγ results in a reduction of the inflammatory response due to its inhibitory effect on main inflammatory signal transduction pathways, in particular, NFκB. It has been reported, in several experimental models of pancreatitis, that pancreatic damage was associated with increased nuclear translocation of NFκB dimers p65/p50 that trigger the transcription and generation of a broad spectrum of inflammatory mediators by pancreatic cells[19]. These mediators, including cytokines, chemokines and adhesion molecules, generate a microenvironment that promotes the recruitment and activation of inflammatory cells and contributes to increasing the extension of the pancreatic damage. Consequently, we have evaluated the involvement of NFκB in the observed anti-inflammatory effect of PPARγ in post-ERCP pancreatitis. For this purpose we have measured the nuclear translocation and DNA binding of p65, the key component of NFκB. The results indicate that nuclear translocation of p65 was significantly increased after experimental ERCP and this increase was completely prevented by pre-treatment of rosiglitazone. This inhibition could explain the lack of inflammatory

infiltrate observed in pancreas, since NFκB activation is a requisite for the generation of the main pro-inflammatory mediators involved in pancreatitis.

Similar results were observed when measuring the expression of iNOS in pancreatic tissue. This enzyme was induced mainly in activated leukocytes in order to generate nitric oxide at a cytotoxic concentration as a part of the bactericidal mechanism of these cells. The presence of iNOS in pancreas in post-ERCP pancreatitis confirms that an intense inflammatory process was triggered. Since the synthesis of this enzyme is strongly dependent on NFκB, it is not a surprise that rosiglitazone treatment downregulates the expression of iNOS. On the other hand, the reduced levels of iNOS could also reflect the lack of cell infiltration that occurs under these conditions.

The blockage of NFκB activation and the resulting inhibition in the NFκB-dependent mediators is of importance not only for the local inflammation, but also in order to prevent the release into the circulatory bloodstream of pro-inflammatory cytokines that could trigger a systemic inflammatory response. Although this occurs in a reduced percentage of patients, the severity of the process justifies the use of prophylactic measures to prevent it despite the failure or the weakness of many previous series.

In conclusion, despite the general limitations of all the animal models relative to clinical setting and the multifactorial etiology of post-ERCP pancreatitis, these results suggest that rosiglitazone and other PPARγ agonists are potential new therapeutic agents for the prevention of post-ERCP-induced acute pancreatitis. It could be administered in an oral dose to the patients, shortly before the ERCP. However, further studies are needed to determine the proper dose and time-point of administration in human patients.

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REFERENCES

1 **Barthet M**, Lesavre N, Desjeux A, Gasmi M, Berthezene P, Berdah S, Viviand X, Grimaud JC. Complications of endoscopic sphincterotomy: results from a single tertiary referral center. *Endoscopy* 2002; **34**: 991-997

2 **Freeman ML**, DiSario JA, Nelson DB, Fennerty MB, Lee JG, Bjorkman DJ, Overby CS, Aas J, Ryan ME, Bochna GS, Shaw MJ, Snady HW, Erickson RV, Moore JP, Roel JP. Risk factors for post-ERCP pancreatitis: a prospective, multicenter study. *Gastrointest Endosc* 2001; **54**: 425-434

3 **Andriulli A**, Leandro G, Niro G, Mangia A, Festa V, Gambassi G, Villani MR, Facciorusso D, Conoscitore P, Spirito F, De Maio G. Pharmacologic treatment can prevent pancreatic injury after ERCP: a meta-analysis. *Gastrointest Endosc* 2000; **51**: 1-7

4 **Hackert T**, Werner J, Gebhard MM, Klar E. Effects of heparin in experimental models of acute pancreatitis and post-ERCP pancreatitis. *Surgery* 2004; **135**: 131-138

5 **Deviere J**, Le Moine O, Van Laethem JL, Eisendrath P, Ghilain A, Severs N, Cohard M. Interleukin 10 reduces the incidence of pancreatitis after therapeutic endoscopic retrograde cholan giopancreatography. *Gastroenterology* 2001; **120**: 498-505

6 **Arvanitidis D**, Anagnostopoulos GK, Giannopoulos D, Pantes A, Agaritsi R, Margantinis G, Tsiakos S, Sakorafas G, Kostopoulos P. Can somatostatin prevent post-ERCP pancreatitis? Results of a randomized controlled trial. *J Gastroenterol Hepatol* 2004; **19**: 278-282

7 **Moreto M**, Zaballa M, Casado I, Merino O, Rueda M, Ramirez K, Urcelay R, Baranda A. Transdermal glyceryl trinitrate for prevention of post-ERCP pancreatitis: A randomized double-blind trial. *Gastrointest Endosc* 2003; **57**: 1-7

8 **Murray B**, Carter R, Imrie C, Evans S, O'Suilleabhain C. Diclofenac reduces the incidence of acute pancreatitis after endoscopic retrograde cholangiopancreatography. *Gastroenterology* 2003; **124**: 1786-1791

9 **Freeman ML**, Guda NM. Prevention of post-ERCP pancreatitis: a comprehensive review. *Gastrointest Endosc* 2004; **59**: 845-864

10 **Clark RB**. The role of PPARs in inflammation and immunity. *J Leukoc Biol* 2002; **71**: 388-400

11 **Cuzzocrea S**, Pisano B, Dugo L, Ianaro A, Britti D, Patel NS, Di Paola R, Genovese T, Di Rosa M, Caputi AP, Thiemermann C. Rosiglitazone, a ligand of the peroxisome proliferator-activated receptor-gamma, reduces acute pancreatitis induced by cerulein. *Intensive Care Med* 2004; **30**: 951-956

12 **Nakajima A**, Wada K, Miki H, Kubota N, Nakajima N, Terauchi Y, Ohnishi S, Saubermann LJ, Kadowaki T, Blumberg RS, Nagai R, Matsuhashi N. Endogenous PPAR gamma mediates anti-inflammatory activity in murine ischemia-reperfusion injury. *Gastroenterology* 2001; **120**: 460-469

13 **Ricote M**, Li AC, Willson TM, Kelly CJ, Glass CK. The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. *Nature* 1998; **391**: 79-82

14 **Shiojiri T**, Wada K, Nakajima A, Katayama K, Shibuya A, Kudo C, Kadowaki T, Mayumi T, Yura Y, Kamisaki Y. PPAR gamma ligands inhibit nitrotyrosine formation and inflammatory mediator expressions in adjuvant-induced rheumatoid arthritis mice. *Eur J Pharmacol* 2002; **448**: 231-238

15 **He ZJ**, Winston JH, Yusuf TE, Micci MA, Elfert A, Xiao SY, Pasricha PJ. Intraductal administration of an NK1 receptor antagonist attenuates the inflammatory response to retrograde infusion of radiological contrast in rats: implications for the pathogenesis and prevention of ERCP-induced pancreatitis. *Pancreas* 2003; **27**: e13-e17

16 **Trush MA**, Egner PA, Kensler TW. Myeloperoxidase as a biomarker of skin irritation and inflammation. *Food Chem Toxicol* 1994; **32**: 143-147

17 **Sanchez-Hidalgo M**, Martin AR, Villegas I, Alarcon De La Lastra C. Rosiglitazone, an agonist of peroxisome proliferator-activated receptor gamma, reduces chronic colonic inflammation in rats. *Biochem Pharmacol* 2005; **69**: 1733-1744

18 **Zafiriou S**, Stanners SR, Saad S, Polhill TS, Poronnik P, Pollock CA. Pioglitazone inhibits cell growth and reduces matrix production in human kidney fibroblasts. *J Am Soc Nephrol* 2005; **16**: 638-645

19 **Vaquero E**, Gukovsky I, Zaninovic V, Gukovskaya AS, Pandol SJ. Localized pancreatic NF-kappaB activation and inflammatory response in taurocholate-induced pancreatitis. *Am J Physiol Gastrointest Liver Physiol* 2001; **280**: G1197-G1208

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BASIC RESEARCH

Attenuation of dextran sodium sulphate induced colitis in matrix metalloproteinase-9 deficient mice

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Abstract

AIM: To study whether matrix metalloproteinase-9 (MMP-9) is a key factor in epithelial damage in the dextran sodium sulphate (DSS) model of colitis in mice.

METHODS: MMP-9-deficient and wild-type (wt) mice were given 5% DSS in drinking water for 5 d followed by recovery up to 7 d. On d 5 and 12 after induction of colitis, gelatinases, MMP-2 and MMP-9, were measured in homogenates of colonic tissue by zymography and Western blot, whereas tissue inhibitor of metalloproteinases (TIMPs) were measured by reverse zymography. The gelatinolytic activity was also determined in supernatants of polymorphonuclear leukocytes (PMN) isolated from mice blood. Moreover, intestinal epithelial cells were stimulated with TNF-α to study whether these cells were able to produce MMPs. Finally, colonic mucosal lesions were measured by microscopic examination.

RESULTS: On d 5 of colitis, the activity of MMP-9 was increased in homogenates of colonic tissues (0.24 ± 0.1 *vs* 21.3 ± 6.4, $P < 0.05$) and PMN from peripheral blood in wt (0.5 ± 0.1 *vs* 10.4 ± 0.7, $P < 0.05$), but not in MMP-9-deficient animals. The MMP-9 activity was also up-regulated by TNF-α in epithelial intestinal cells (2.5 ± 0.5 *vs* 14.7 ± 3.0, $P < 0.05$). Although colitis also led to increase of TIMP-1 activity, the MMP-9/TIMP-1 balance remained elevated. Finally, in the MMP-9-deficient colitic mice both the extent and severity of intestinal epithelial injury were significantly attenuated when compared with wt mice.

CONCLUSION: We conclude that DSS induced colitis is markedly attenuated in animals lacking MMP-9. This suggests that intestinal injury induced by DSS is modulated by MMP-9 and that inhibition of this gelatinase may reduce inflammation.

Key words: Matrix metalloproteinases; MMP-9-deficient; Dextran sodium sulphate; Inflammatory bowel disease; Experimental colitis

Santana A, Medina C, Paz-Cabrera MC, Díaz-Gonzalez F, Farré E, Salas A, Radomski MW, Quintero E. Attenuation of dextran sodium sulphate induced colitis in matrix metalloproteinase-9 deficient mice. *World J Gastroenterol* 2006; 12(40): 6464-6472

http://www.wjgnet.com/1007-9327/12/6464.asp

INTRODUCTION

Matrix metalloproteinases (MMPs) comprise a group of zinc and calcium-dependent endopeptidases that exhibit differential proteolytic activity against extracellular matrix (ECM) proteins. Based on substrate specificity, MMPs have been classically divided into collagenases, gelatinases (MMP-9 and MMP-2) and stromelysins. These proteinases are secreted as latent enzymes that require proteolytic cleavage for activation. However, a subset of MMPs, known as membrane-type MMPs (MT-MMPs) are not secreted but instead remain attached to cell surfaces and activate secreted MMPs[1,2]. The MMP activity is tightly controlled by specific endogenous inhibitors of these enzymes (TIMPs), which complex with MMPs. Four TIMPs have been described until now and TIMP-1 seems to be the most important endogenous inhibitor, which binds to activated interstitial collagenase and gelatinases[3-5].

Ulcerative colitis (UC) is a chronic inflammatory disease affecting primarily the distal colon and rectum of young adults and its etiology still remains unclear. Degradation and remodeling of the ECM is increasingly implicated in the pathogenesis of several inflammatory disorders,

such as periodontal disease and rheumatoid arthritis[6-8]. In physiological conditions, MMPs act as part of the normal connective tissue turnover. However, MMPs released by diverse cells in response to several cytokines during inflammation, may lead to excess degradation of ECM and tissue injury. Previous studies have found both increased activity and expression of MMPs in colonic tissues of patients with inflammatory bowel disease (IBD)[9-13]. Moreover, MMP-9 may be a key factor responsible for accelerated breakdown of ECM in UC, because it was demonstrated that MMP-9 is abundantly expressed in patients with UC compared with controls[9]. Hence, MMP inhibition has emerged as a potential therapeutic approach in colonic inflammatory disorders[14-16]. Furthermore, we have previously demonstrated a significant up-regulation of MMP-9 expression and activity in a rat model of colitis induced by dextran sulphate sodium (DSS). In this experimental model, a synthetic MMP inhibitor (CGS-27023-A) significantly reduced the extent and severity of tissue injury[17]. However, this compound is a broad spectrum MMP inhibitor and the relative contribution of MMP-9 in this experimental model still remains unclear.

In the present investigation, we sought to determine whether MMP-9 extinction could alter intestinal damage in a mouse model of colitis induced by DSS. This model exhibits clinical and morphological features resembling human UC, including diarrhea and rectal bleeding, diffuse lesions circumscribed to the mucosa, and predominance of distal involvement of the large intestine[18]. Our findings support the conclusion that MMP-9 modulates colonic mucosal injury in this entity.

MATERIALS AND METHODS

Animals

Our studies used the homozygous MMP-9-deficient male mice with FVB background and wild-type (wt) purchased from Jackson Laboratories (Ca, USA). The animals were 8 wk to 10 wk old and weighted from 25 g to 30 g. They were maintained in a restricted-access room with controlled temperature (23℃) and light-dark cycle (12 h:12 h) and were housed in rack-mounted cages with a maximum of 10 mice per cage. Mice were allowed to drink and feed *ad libitum*. The study was approved by the local Animal Welfare Committee and conformed to the principles of laboratory animal care and use (NIH publication 86-23).

Genotyping analysis

Liver samples from selected mice were collected and genotype was verified by polymerase chain reaction (PCR) using a sense oligonucleotide primer (5'-GCATACTTGTACCGCTATGG-3') and an antisense oligonucleotide primer (5'-TAACCGGAG GTCCAAACTGG-3'). For the neomycin cassette, we also used a sense oligonucleotide primer (5´-GAAGGGACTGGCTGCTATTG-3´) and an antisense primer (5´-AATATCACGGGTAGCCAACG-3´). All procedures were performed blind with respect to genotype.

Experimental design

Distal colitis was induced by oral DSS (molecular wt 40 000; ICN Biomedicals, Aurora, OH) at 5% in tap water *ad libitum* for 5 d in wt and MMP-9 deficient mice and then switched to water and monitored for 7 additional days. At least 15 age and sex matched wt and MMP-9 deficient mice were studied at the end of 5 d with DSS treatment and at 7 d after DSS (recovery phase), whereas 15 control wt and 15 control MMP-9 deficient mice were allowed free access to drinking water. Body weight was routinely obtained every second day and mortality was recorded at any time. Mice were euthanized by cervical dislocation on d 5 and 12 after induction of colitis. With the use of sterile equipment, a mid laparotomy was performed, the colon was removed, opened longitudinally, rinsed with sterile saline, and divided into two parts by a longitudinal section. One specimen was homogenized and stored at -20℃ for MMPs, TIMPs, TNF-α and myeloperoxidase (MPO) assay. The second specimen was used for microscopic assessment of mucosal lesions.

Isolation of neutrophils

Mice were anesthetized by an intraperitoneal injection of ketamine (100 mg/kg, Park Davis, Morris Plains, NJ). Blood samples were collected from the abdominal aorta. Neutrophils were isolated using Histopaque (Sigma Ltd, England) and erythrocyte removed using hypotonic lysis with ammonium chloride. The neutrophil count was determined by using a Neubaver hemocytometer and cell viability was assessed by trypan blue exclusion. Typically, the neutrophil preparations were > 98% pure and > 94% viable. The isolated neutrophils were washed and resuspended in HBSS with calcium and magnesium at a concentration of 5×10^9 cells/L and incubated for 12 h at 37℃. The release of MMPs was determined in the supernatant by zymography[19].

Cell culture

Caco-2 cells, a human intestinal epithelial cell line, were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured in 75-cm^2 flasks in a humidified atmosphere containing 5% CO_2 at 37℃. Cells were grown in minimum essential medium supplemented with pyruvate, sodium bicarbonate, 20% fetal bovine serum, gentamycin sulfate (0.05 mg/mL), penicillin G (0.06 mg/mL) and streptomycin sulfate (0.01 mg/mL). The medium was changed every two days and cells were subcultured three times each week. When confluent, cells were detached using a Trypsin-EDTA solution. For the *in vitro* experiments, Caco-2 cells were seeded into 24-well plates (30 000 cells per well) in free serum-medium and cultured in the presence or absence of human recombinant TNF-α (1 or 10 μg/L) for 24 h. Afterwards, conditioned media was collected and stored at -20℃ for the zymography assay and Western blot analysis.

Analytical Methods

Zymography: The activity of pro-MMP-9 was measured as previously described[20,21] in homogenates of colonic

tissue, supernatants of purified neutrophils and Caco-2 conditioned media. Briefly, samples were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with copolymerized gelatin (0.2%; Sigma Chemical Co, St. Louis, Mo). After electrophoresis, the gels were washed with 2% Triton X-100 (2 times, 20 min each), and then incubated in development buffer (50 mmol/L Tris HCl, 200 mmol/L NaCl, 10 mmol/L $CaCl_2$ and 1 μmol/L $ZnCl_2$, pH = 7.5) at 37℃ overnight. Human recombinant MMP-9 and MMP-2 (Oncogene Research, Nottingham, UK), conditioned medium of HT-1080 human fibrosarcoma cells and molecular weight markers were used as standards. After incubation, gels were fixed and stained in 40% methanol, 10% acetic acid and 0.1% (wt/v) Coomassie Blue for 1 h and then de-stained. The low availability of active MMP-9 seen in the zymographies may be due to its high level of instability and the removal of active enzyme during the washing of specimens, as it has been previously suggested[22].

Reverse zymography: The TIMP-1 activity was analyzed in homogenates of colonic tissues, as previously described[23]. Briefly, samples were subjected to 15% SDS-PAGE with copolymerized 0.2% gelatin and human recombinant MMP-2 (160 μg/L). Human recombinant TIMP-1 was used as the internal standard. TIMP-1 activity was identified by inhibition of gelatinolysis when compared with the standard.

Western-blot: Western-blot analysis of MMP-9 was performed in homogenates of colonic tissue and Caco-2 conditioned media, as previously described[20]. Samples were denatured and loaded (50 μg/lane) onto 10% (vol/vol) polyacrylamide gels. Following electrophoresis, proteins were transferred onto a nitrocellulose membrane (Protran, Schleider&Schuell) and detected using a rabbit monoclonal anti-MMP-9 antibody (Chemicon, Ca, USA) at 1:1000 concentration and a chemiluminescent substrate (Pierce, Rockford, USA). To account for the inter- blot variations in MMP immunoreactivity, an internal standard (conditioned medium of HT-1080 cells, and recombinant MMP-9) was used[18]. Western Blotting with monoclonal anti-beta-actin (Sigma Chemical Co, St. Louis, Mo) was performed as an internal control.

Bands quantification: Clear bands were analyzed using a calibrated densitometer (GS-800, BioRad) and Quantity One Quantitation analysis software (BioRad, version 4). Each band was measured in terms of Optical Density Units of trace quantity (ODu) × mm.

Quantification of TNF-α by ELISA: TNF-α expression was assayed in colonic tissues. Fragments of colon (40-50 mg) from the proximal region of the rectum were collected from wt and KO animals treated as described above. Afterwards, colon tissues were placed in 500 mL ice-cold PBS containing 20 μL of a proteinase inhibitor cocktail (Roche Diagnostic GmbH, San Francisco, CA) and immediately sonicated. After 10 min in ice, cellular debris was removed by centrifugation at 10 000 g for 10 min at 4℃ and the protein content of the supernatant was assayed by Bradford's method. The specific ELISAs for mouse TNF-α used in these experiments were purchased from BD Biosciences (San Diego, CA). Murine TNF-α concentrations in tissue extracts were assayed in duplicate wells following the manufacturer's recommendations. The sensitivity of ELISA for TNF-α in our laboratory was 20 ng/L. Data were expressed as pg TNF-α/μg of total protein.

Table 1 Microscopic assessment of histologic changes in DSS colitis

A: Grade of crypt lesion	Score
Intact crypt	0
Grade 1: Loss of the basal third	1
Grade 2: Loss of two thirds	2
Grade 3: Loss of entire crypt	3
Grade 4: Erosion	4
B: Extension of each grade of crypt lesion as % of total mucosal surface	0-100
Crypt lesion score	Σ (**A** × **B**)
Grade of inflammation, epithelial regeneration or crypt distortion	Score
Absent	0
Mild	1
Moderate	2
Severe	3

Crypt lesion, acute and chronic inflammation, epithelial regeneration and crypt distortion are scored separately.

MPO assay: For the MPO activity assay[24], the colonic specimen was homogenized in 2 mL phosphate-buffered saline, using a Tissue Tearor (model 985-370, Biospec, Racine, WI), and centrifuged. The pellets were again homogenized in an equivalent volume of phosphate buffer (50 mmol/L, pH 6) containing 0.5% hexadecyltrimethylammonium bromide (Sigma) and 5 mmol/L EDTA, sonicated three times for 30 s each time (Labasonic 2000, Braun), and centrifuged. Supernatants were used for determination of tissue MPO activity by a kinetic method. One unit of enzyme activity is defined as the amount of MPO that degrades 1 mmol of peroxide per minute at 25℃.

Histological Measurement of Colonic Lesions

Colonic specimens were fixed in formalin and coded for blind microscopic measurement of mucosal lesions. Samples were embedded in paraffin using a "Swiss roll" technique, and longitudinal sections from cecum to rectum were prepared and stained with hematoxylin and eosin. This procedure allows the examination of the entire specimen from cecum to rectum in every section. Two pathologists who were unaware of the treatment measured the extent of mucosal surface involved by crypt lesions as a percentage of the total colonic surface. For each specimen, a crypt damage score was obtained by summation of the products of each grade of crypt damage times the extent of mucosal surface involved by this grade of damage (Table 1). In addition, the specimens were graded 0 to 3 for inflammation (acute + chronic), regenerative changes (hyperplastic epithelium) and crypt distortion (distorted epithelium) using the criteria described by Cooper *et al*[25]. Acute inflammation scores evaluated of the presence of polymorphonuclear neutrophils in the infiltrate, whereas chronic inflammation scores evaluate the presence of mononuclear cells.

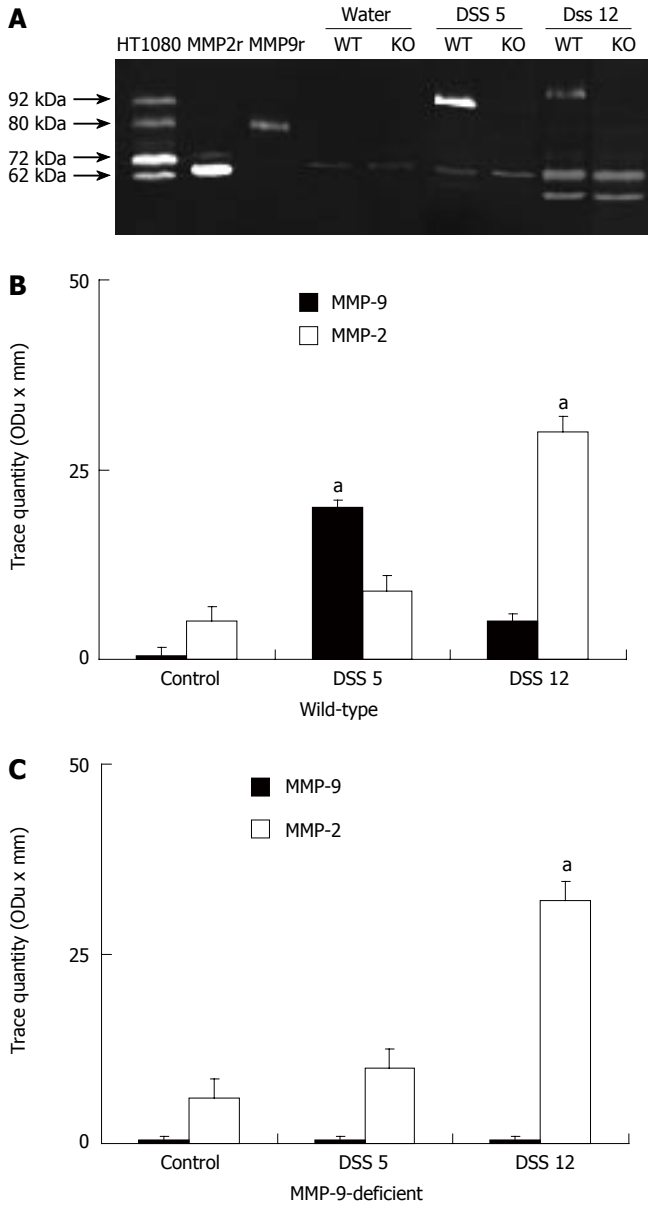

Figure 1 Gelatinase activity in colonic homogenates. **A**: Representative zymogram in wild-type (wt) and MMP-9-deficient (KO) animals on d 5 (DSS 5) and 12 (DSS 12) after induction of colitis (n = 9 in each group). Supernatant of HT1080 cells, human recombinants MMP-9 (rMMP9) and MMP-2 (rMMP2) were used as positive controls. Gelatinases with molecular weights of 62, 72 and 92 kDa corresponding to activated MMP-2, pro-MMP-2 and pro-MMP-9, respectively, were detected. Quantitative data of pro-MMP-9 and total MMP-2 (pro-MMP-2 and MMP-2) ($^aP < 0.05$ *vs* control) in wt (**B**) and MMP-9-deficient mice (**C**).

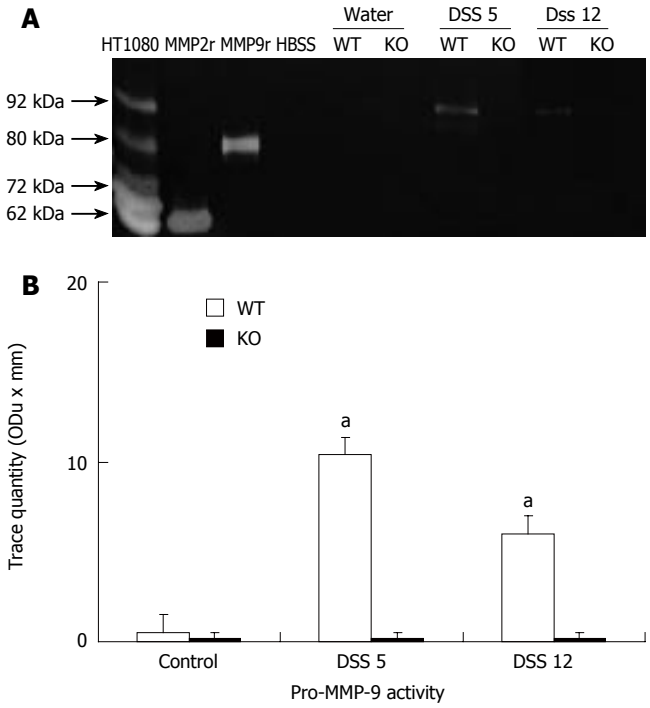

Figure 2 Gelatinase Activity in purified Neutrophils. **A**: Representative zymogram in wild-type (wt) and MMP-9-deficient (KO) animals on d 5 (DSS 5) and 12 (DSS 12) after induction of colitis (n = 15 in each group). Supernatant of HT1080 cells, human recombinants MMP-9 (rMMP9) and MMP-2 (rMMP2) were used as positive controls. HBSS was used as negative control. Pro-MMP-9 was detected in neutrophils from colitic wt animals; **B**: Quantitative data ($^aP < 0.05$ *vs* controls).

Statistical analysis

Results are presented as mean ± SE. The statistical difference between means was determined using one way analysis of variance for overall comparison and the Student-Newman-Keuls test as post-test for single comparisons. The mortality data were analyzed by Kaplan-Meier survival curves.

RESULTS

DSS Colitis

Exposure to 5% DSS in drinking water for 5 d induced diarrhea and rectal bleeding in all mice. Furthermore, DSS treatment resulted in a significant increase in MPO activity in the colonic tissues of both MMP-9 deficient mice (colitic: 2.5 ± 0.82 *vs* control: 0.3 ± 0.05 mU/mg protein, $P < 0.05$) and wt (colitic: 1.95 ± 0.67 *vs* control: 0.25 ± 0.06 mU/mg protein, $P < 0.05$). Histological examination of colonic sections revealed that administration of DSS resulted in epithelium injury and loss of normal crypt architecture, with some areas of erosion. Mixed infiltrate of neutrophils and mononuclear cells was observed in the lamina propria and submucosa. The muscularis propria was not involved. These changes were diffuse but predominated at distal parts of the colon. Histological examination revealed no changes in colons from normal control mice.

Gelatinase activity in DSS colitis

Zymographies revealed that MMP-2 but not pro-MMP-9, was a dominant gelatinase in colonic homogenates from non-colitic animals in both wt and deficient animals (Figure 1A). However, DSS treatment resulted in a significant up-regulation of pro-MMP-9, but not MMP-2 activity in colitic wt animals in comparison to deficient animals on d 5 after the induction of colitis (Figure 1A and B). On d 12, pro-MMP-9 activity was still up-regulated in wt animals, although to a less extent than in d 5. In addition, there was an up-regulation of MMP-2 in both groups (Figure 1A and B). In peripheral neutrophils isolated from wt animals pro-MMP-9 activity was also markedly enhanced on d 5 and, to a lesser extent, on d 12 following the induction of colitis, but not from MMP-9 deficient mice (Figure 2A and B). By contrast, no MMP-2 activity was shown in neutrophils isolated from any animal group in the presence or absence of DSS treatment.

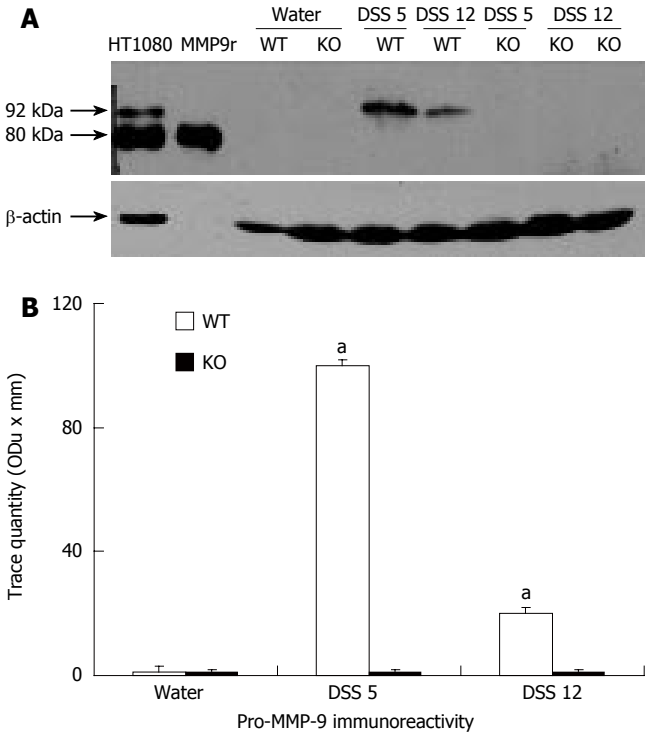

Figure 3 Pro-MMP-9 immunoreactivity in colonic homogenates. **A**: Representative immunoblot in wild-type (wt) and MMP-9-deficient (KO) (n = 9 in each group) animals on d 5 (DSS 5) and 12 (DSS 12) after colitis induction. Supernatant of HT1080 cells and human recombinants MMP-9 (rMMP9) were used as positive controls. A band of 92 kDa corresponding to pro-MMP-9 was detected in colitic samples. **B**: Quantitative analysis ($^aP < 0.05$ *vs* control).

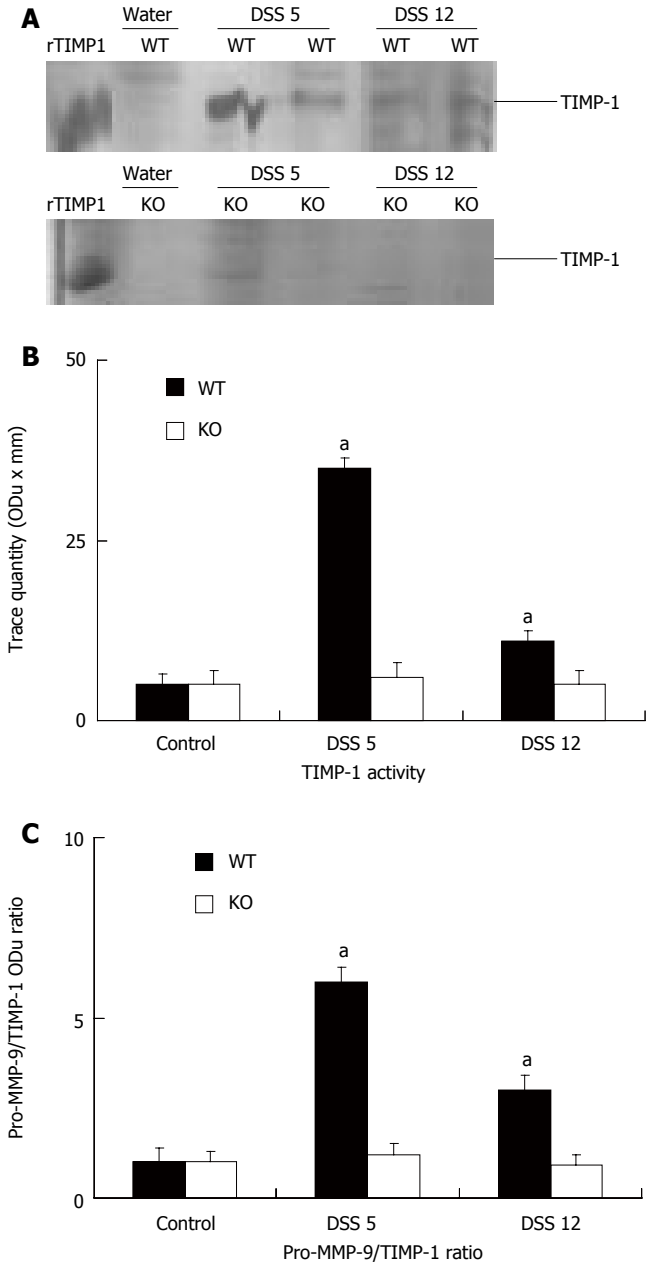

Figure 4 TIMP-1 activity in DSS-induced colitis: **A**: Representative reverse zymograms in homogenates of colonic tissue in wild-type (wt) and MMP-9-deficient (KO) animals on d 5 (DSS 5) and 12 (DSS 12) after induction of colitis; **B**: Quantitative data of TIMP-1 activity ($^aP < 0.05$ *vs* control) (n = 5 in each group); **C**: wt mice had a pro-MMP-9/TIMP-1 ratio significantly ($^aP < 0.05$) higher than controls on d 5 (DSS 5) and 12 (DSS 12) following the induction of colitis. The pro-MMP9/TIMP-1 ratio was not modified by DSS-induced colitis in KO mice.

DSS Colitis Enhanced MMP-9 Immunoreactivity

As shown by the immunoreactive band migrating at 92 kDa, a significant up-regulation of pro-MMP-9 was observed in wt, but not in controls or MMP-9 deficient mice, on d 5 and 12 following the induction of colitis (Figure 3).

TIMP-1 Activity in DSS Colitis

Colitis resulted in a significant up-regulation of TIMP-1 in wt animals receiving DSS compared to controls on d 5 and this increase remained elevated on d 12 after induction of colitis (Figure 4A and B). In wt mice the pro-MMP-9/TIMP-1 ratio was significantly higher ($P < 0.05$) than in control mice on d 5 and 12 after the induction of colitis. In addition, the pro-MMP-9/TIMP-1 ratio was not altered by colitis in MMP-9-deficient mice (Figure 4C).

TNF-α Content in DSS Colitis

Figure 5 shows that wt and MMP-9-deficient animals exposed to DSS for during 5 d showed a significant increase in TNF-α concentration as compared with non-colitic animals. In addition, the TNF-α level remained elevated in wt animals on d 12 after colitis induction, whereas returned to base line levels in MMP-9 deficient animals.

Effect of TNF-α Treatment

Since in DSS-induced colitis the predominant lesions are circumscribed to the mucosa, we measured the MMP activity in intestinal epithelial (Caco-2) cells in response to TNF-α. Zymographies showed that, under basal conditions, MMP-2 was the predominant gelatinase in Caco-2 cells. Interestingly, TNF-α treatment resulted in a dose-dependent significant increase of pro-MMP-9 activity whereas MMP-2 activity remained unchanged (Figure 6A and C). In addition, Western blot analysis corroborated that TNF-α treatment significantly increased pro-MMP-9 immunoreactivity in intestinal epithelial cells (Figure 6B and D).

Impact of MMP-9-silencing on DSS-colitis outcome

Both wt and MMP-9-deficient mice developed diarrhea and rectal bleeding with DSS. Percent loss of body weight is shown in Figure 7A. Neither deficient nor wt mice showed major loss of body weight during DSS treatment.

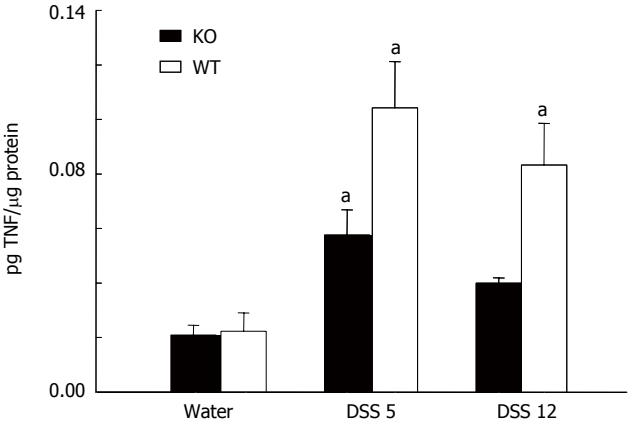

Figure 5 TNF-α content in colonic tissue. Concentration of TNF-α protein in homogenates of colonic tissue from wild-type (wt) (n = 5) and deficient (KO) (n = 5) animals on d 5 (DSS 5) and 12 (DSS 12) after colitis induction ($^aP < 0.05$ *vs* control).

However, during the recovery phase, wt mice showed significant weight loss. In addition, the mortality was significantly higher in wt than in MMP-9 KO mice (Figure 7B). The mean of severity of crypt damage, inflammation, epithelial regeneration and crypt distortion scores are summarized in Table 2. MMP-9 deficient mice showed a significant decrease in the crypt damage scores when compared with wt mice on d 5 and 12 after colitis induction. Moreover, deficient mice showed significantly lower scores of acute and chronic inflammation on d 12. Epithelial regeneration and crypt distortion scores were almost absent on d 5, and no differences were found between both groups at any time.

DISCUSSION

The etiology and the cascade of the events resulting in intestinal injury of UC patients still remain unclear. There is growing evidence that MMPs are implicated in tissue remodeling and destruction associated with several inflammatory conditions[6-8], including IBD[9-13]. Moreover, increased gelatinase activity has been shown in diverse animal experimental models of colitis[17,26] Our study revealed that MMP-9-deficient animals showed improved survival and indices of epithelial injury, suggesting that MMP-9 is a key factor in tissue damage in DSS-induced colitis.

The destructive effect of MMPs in the gut has been shown by several *in vitro* studies[27-29]. In addition, previous reports have found increased levels of MMP-9 in colonic tissues of patients with IBD. For instance, Bailey *et al*[30] have shown an increased MMP-9 protein in patients with IBD by immunohistochemical techniques. Furthermore, Baugh *et al* have found that MMP-9 activity measured by zymography was the most abundant MMP expressed in intestinal tissues of patients with UC compared with normal controls[9]. Moreover, Tartlon *et al*[26] have also shown increased gelatinase activity in a transmural experimental model of colitis induced in immunodeficient mice. Finally, we have previously found that MMP-9 is up-regulated in intestinal tissues from rats with DSS-induced

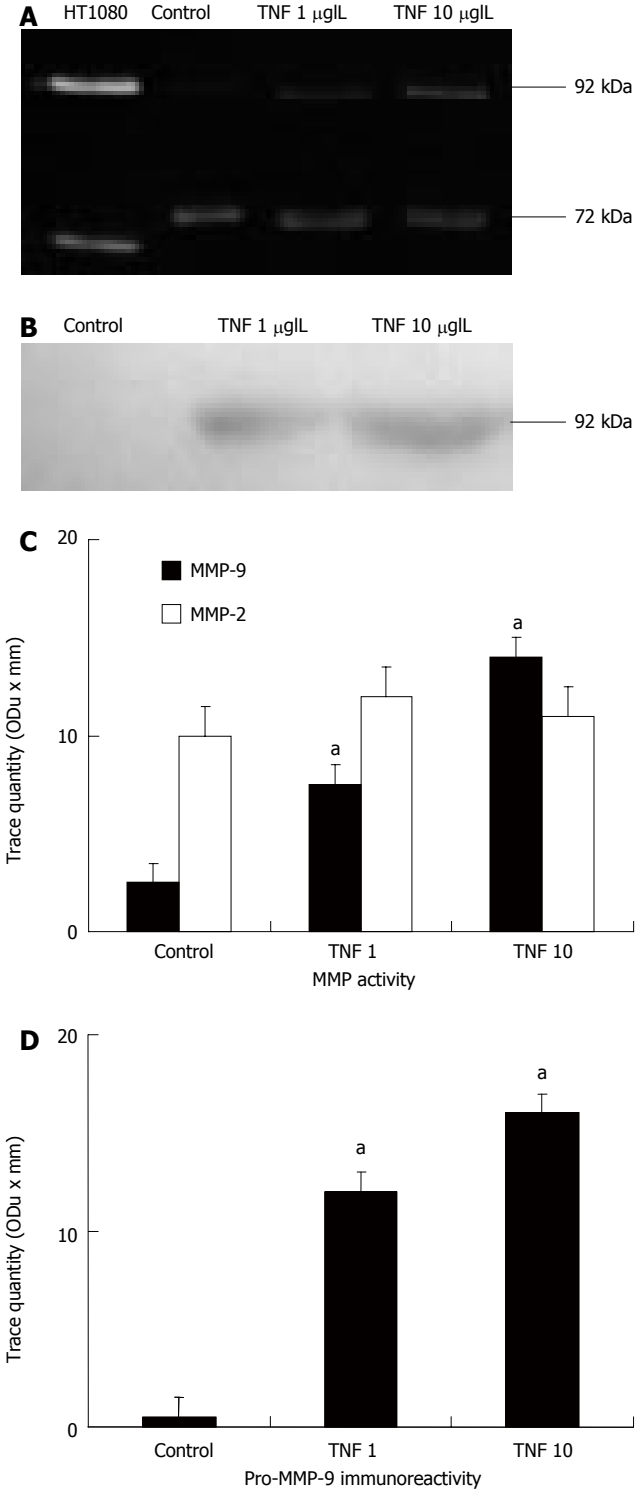

Figure 6 Activity of gelatinases in Caco-2 cells in absence (control) or presence of TNF-α (1 μg/L or 10 μg/L). **A**: Representative zymogram of gelatinases with molecular weights of 72 and 92 kDa corresponding to pro-MMP-2 and pro-MMP-9, respectively, were detected. Supernatants of HT1080 cells were used as controls. **B**: Representative immunoblot of pro-MMP-9 (92 kDa) protein in Caco-2 cells as above. **C**: Quantitative zymographic analysis ($^aP < 0.05$ *vs* control). **D**: Quantitative western-blot analysis ($^aP < 0.05$ *vs* control) (n = 3 in each group).

colitis[17]. Our present study shows that DSS treatment induced an increased activity and expression of pro-MMP-9 in wt mice compared to the MMP-9 deficient group. It could be surprising that DSS did not induce the active form of MMP-9, however, the low availability

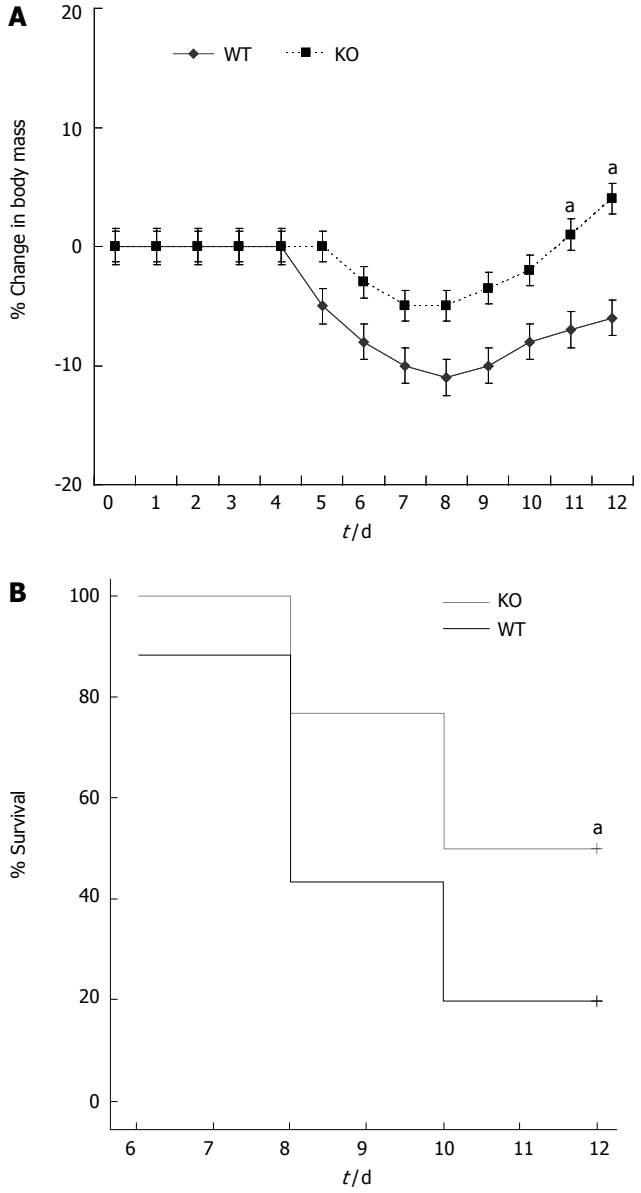

Figure 7 **A**: Percentage of body weight change during DSS-induced colitis in wild-type (WT) and deficient (KO) animals (n = 30 in each group). Values are mean ± SE of percentage body weight change in each animal relative to weight at the start of DSS treatment. WT animals showed more weight loss than deficient mice ($^aP < 0.05$). **B**: Survival curve of both colitic groups. The mortality was significantly higher in wt animals compared to deficient mice ($^aP = 0.015$).

of this enzyme may be due to the removal of the active form of MMP-9 during the washing of specimens, as it has been previously suggested[22]. Our data also show that colitis induced by DSS was enhanced in wt animals. In fact, histological evidence of less mucosal damage was observed in MMP-9 deficient animals on d 5 and 12 after induction of colitis. In addition, the higher weight loss and mortality of wt mice compared with deficient animals supports this hypothesis. In our experiments the indices of DSS-induced colitic injury correlated well with pro-MMP-9, but not with MMP-2 up-regulation. By contrast, whereas MMP-2 activity was elevated during the recovery phase of colitis, the activity and expression of pro-MMP-9 decreased on d 12 after colitis induction. These data suggest that MMP-9 is mainly implicated in mucosal injury at the early stage of colitis, whereas MMP-2 seems to be linked to mucosal

Table 2 Histologic scores on d 5 and 12 after Induction of colitis by DSS

	D 5		D 12	
	WT	KO	WT	KO
Crypt lesion	40.0 ± 4.0	21.0 ± 5.0[a]	18.0 ± 2.4	9.5 ± 2.4[a]
Acute inflammation	1.0 ± 0.2	0.9 ± 0.1	1.8 ± 0.2	0.5 ± 0.15[a]
Chronic inflammation	0.5 ± 0.1	0.6 ± 0.1	1.5 ± 0.10	0.4 ± 0.1[a]
Epithelial regeneration	0.2 ± 0.05	0.1 ± 0.02	1.2 ± 0.15	0.95 ± 0.2
Crypt distortion	0	0	0.6 ± 0.10	0.5 ± 0.2

[a]$P < 0.05$ *vs* WT.

repair, although MMP-2 deficient animals should be tested to confirm this hypothesis. Therefore, further investigation is warranted.

In many inflammatory conditions, the excess degradation of ECM may result from an imbalance between MMP and TIMP activity[31]. Since TIMP-1 seems to be the major inhibitor of gelatinase activity[3] we studied the activity of this inhibitor in DSS-induced colitis. We found that TIMP-1 was increased in colitic wt animals on d 5 after induction of colitis in comparison with non-colitic mice. However, our results showed an imbalance between pro-MMP-9 and TIMP-1 activity in colitic wt animals in favour of MMP-9, suggesting that the increase of TIMP-1 was insufficient in preventing pro-MMP-9 from exerting its biologic effects in DSS-induced colitis. Interestingly, in MMP-9 deficient animals DSS treatment did not increase TIMP-1 activity, which could suggest an adapted regulatory response of this endogenous inhibitor to the levels of pro-MMP-9. Our results are consistent with previous studies in human IBD where an imbalance between MMPs and their endogenous inhibitors has been shown[10,12].

We next studied the cellular origin of MMP-9 in DSS-induced colitis. MMP-9 released from neutrophils could be an important factor for transmigration and proteolysis of ECM in several inflammatory conditions contributing to tissue injury[19,32]. We found that pro-MMP-9 is expressed by neutrophils only in colitic wt animals. However, DSS treatment did not induce any significant difference in MPO activity and inflammation score (markers of neutrophilic infiltration) between wt and MMP-9 deficient animals on d 5. This is consistent with the fact that inflammation is thought to be a secondary event that follows epithelial damage in this experimental model of colitis[25]. Therefore, it seems reasonable that other cells may also generate MMP-9 contributing to crypt damage during the initial phase of DSS-induced colitis. Intestinal epithelium plays an important role in the immunomodulatory response of the intestinal mucosa[33,34]. Indeed, the loss of intestinal epithelium integrity could lead to an interaction between the luminal antigenic stimuli with the mucosal immune system, resulting in intestinal inflammation[35-37]. We used a human intestinal epithelial cell line to confirm whether MMP-9 and MMP-2 are up-regulated by inflammatory stimuli, as previously described[38]. Consistent with the colonic tissue samples, we found that TNF-α treatment only resulted in up-regulation of pro-MMP-9 activity in intestinal epithelial cells. These data therefore suggest that MMP-9 could

be released from inflamed intestinal epithelial cells with the subsequent loss of mucosal integrity, thus facilitating the penetration of inflammatory cells such as PMN into inflamed tissue in the late inflammatory phase of DSS-induced colitis. Our results are consistent with a recent study where epithelial MMP-9 has been shown as an important factor for tissue damage using the Caco-2 intestinal cell line, since MMP-9 inhibited cell attachment and wound healing in an *in vitro* model[39]. In addition, epithelial protection in MMP-9-deficient mice during the early phase of colitis could also account for a mitigated inflammatory response in the late phase of this experimental model. In fact, only on d 12 after DSS administration MMP-9-deficient animals showed lower inflammation scores than wt animals. These results are consistent with our previous study, where MMP inhibition with CGS-27023-A treatment in rats with DSS-induced colitis significantly reduced the inflammation scores only during the recovery phase of the colitis[17].

In conclusion, this study presents direct evidence that DSS-induced colitis is attenuated in mice deficient in MMP-9. The reduction in inflammatory indices and pathological scoring suggests a link between MMP-9 and DSS-induced colitis that might help elucidate the physiopathology of UC. These results suggest that selective inhibition of this gelatinase could be a novel therapeutic strategy for patients with UC.

REFERENCES

1 **Nelson AR**, Fingleton B, Rothenberg ML, Matrisian LM. Matrix metalloproteinases: biologic activity and clinical implications. *J Clin Oncol* 2000; **18**: 1135-1149
2 **Sternlicht MD**, Werb Z. How matrix metalloproteinases regulate cell behavior. *Annu Rev Cell Dev Biol* 2001; **17**: 463-516
3 **Carmichael DF**, Sommer A, Thompson RC, Anderson DC, Smith CG, Welgus HG, Stricklin GP. Primary structure and cDNA cloning of human fibroblast collagenase inhibitor. *Proc Natl Acad Sci USA* 1986; **83**: 2407-2411
4 **Greene J**, Wang M, Liu YE, Raymond LA, Rosen C, Shi YE. Molecular cloning and characterization of human tissue inhibitor of metalloproteinase 4. *J Biol Chem* 1996; **271**: 30375-30380
5 **Leco KJ**, Khokha R, Pavloff N, Hawkes SP, Edwards DR. Tissue inhibitor of metalloproteinases-3 (TIMP-3) is an extracellular matrix-associated protein with a distinctive pattern of expression in mouse cells and tissues. *J Biol Chem* 1994; **269**: 9352-9360
6 **Ruwanpura SM**, Noguchi K, Ishikawa I. Prostaglandin E2 regulates interleukin-1beta-induced matrix metalloproteinase-3 production in human gingival fibroblasts. *J Dent Res* 2004; **83**: 260-265
7 **Kim H**, Kim WJ, Jeon ST, Koh EM, Cha HS, Ahn KS, Lee WH. Cyclophilin A may contribute to the inflammatory processes in rheumatoid arthritis through induction of matrix degrading enzymes and inflammatory cytokines from macrophages. *Clin Immunol* 2005; **116**: 217-224
8 **Ishikawa T**, Nishigaki F, Miyata S, Hirayama Y, Minoura K, Imanishi J, Neya M, Mizutani T, Imamura Y, Ohkubo Y, Mutoh S. Prevention of progressive joint destruction in adjuvant induced arthritis in rats by a novel matrix metalloproteinase inhibitor, FR217840. *Eur J Pharmacol* 2005; **508**: 239-247
9 **Baugh MD**, Perry MJ, Hollander AP, Davies DR, Cross SS, Lobo AJ, Taylor CJ, Evans GS. Matrix metalloproteinase levels are elevated in inflammatory bowel disease. *Gastroenterology* 1999; **117**: 814-822
10 **Heuschkel RB**, MacDonald TT, Monteleone G, Bajaj-Elliott M, Smith JA, Pender SL. Imbalance of stromelysin-1 and TIMP-1 in the mucosal lesions of children with inflammatory bowel disease. *Gut* 2000; **47**: 57-62
11 **Stallmach A**, Chan CC, Ecker KW, Feifel G, Herbst H, Schuppan D, Zeitz M. Comparable expression of matrix metalloproteinases 1 and 2 in pouchitis and ulcerative colitis. *Gut* 2000; **47**: 415-422
12 **von Lampe B**, Barthel B, Coupland SE, Riecken EO, Rosewicz S. Differential expression of matrix metalloproteinases and their tissue inhibitors in colon mucosa of patients with inflammatory bowel disease. *Gut* 2000; **47**: 63-73
13 **Kirkegaard T**, Hansen A, Bruun E, Brynskov J. Expression and localisation of matrix metalloproteinases and their natural inhibitors in fistulae of patients with Crohn's disease. *Gut* 2004; **53**: 701-709
14 **Medina C**, Videla S, Radomski A, Radomski M, Antolin M, Guarner F, Vilaseca J, Salas A, Malagelada JR. Therapeutic effect of phenantroline in two rat models of inflammatory bowel disease. *Scand J Gastroenterol* 2001; **36**: 1314-1319
15 **Medina C**, Santana A, Llopis M, Paz-Cabrera MC, Antolin M, Mourelle M, Guarner F, Vilaseca J, Gonzalez C, Salas A, Quintero E, Malagelada JR. Induction of colonic transmural inflammation by Bacteroides fragilis: implication of matrix metalloproteinases. *Inflamm Bowel Dis* 2005; **11**: 99-105
16 **Sykes AP**, Bhogal R, Brampton C, Chander C, Whelan C, Parsons ME, Bird J. The effect of an inhibitor of matrix metalloproteinases on colonic inflammation in a trinitrobenzenesulphonic acid rat model of inflammatory bowel disease. *Aliment Pharmacol Ther* 1999; **13**: 1535-1542
17 **Medina C**, Videla S, Radomski A, Radomski MW, Antolin M, Guarner F, Vilaseca J, Salas A, Malagelada JR. Increased activity and expression of matrix metalloproteinase-9 in a rat model of distal colitis. *Am J Physiol Gastrointest Liver Physiol* 2003; **284**: G116-G122
18 **Okayasu I**, Hatakeyama S, Yamada M, Ohkusa T, Inagaki Y, Nakaya R. A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. *Gastroenterology* 1990; **98**: 694-702
19 **Keck T**, Balcom JH 4th, Fernandez-del Castillo C, Antoniu BA, Warshaw AL. Matrix metalloproteinase-9 promotes neutrophil migration and alveolar capillary leakage in pancreatitis-associated lung injury in the rat. *Gastroenterology* 2002; **122**: 188-201
20 **Fernandez-Patron C**, Martinez-Cuesta MA, Salas E, Sawicki G, Wozniak M, Radomski MW, Davidge ST. Differential regulation of platelet aggregation by matrix metalloproteinases-9 and -2. *Thromb Haemost* 1999; **82**: 1730-1735
21 **Radomski A**, Sawicki G, Olson DM, Radomski MW. The role of nitric oxide and metalloproteinases in the pathogenesis of hyperoxia-induced lung injury in newborn rats. *Br J Pharmacol* 1998; **125**: 1455-1462
22 **Deleve LD**, Wang X, Tsai J, Kanel G, Strasberg S, Tokes ZA. Sinusoidal obstruction syndrome (veno-occlusive disease) in the rat is prevented by matrix metalloproteinase inhibition. *Gastroenterology* 2003; **125**: 882-890
23 **Oliver GW**, Leferson JD, Stetler-Stevenson WG, Kleiner DE. Quantitative reverse zymography: analysis of picogram amounts of metalloproteinase inhibitors using gelatinase A and B reverse zymograms. *Anal Biochem* 1997; **244**: 161-166
24 **Schierwagen C**, Bylund-Fellenius AC, Lundberg C. Improved method for quantification of tissue PMN accumulation measured by myeloperoxidase activity. *J Pharmacol Methods* 1990; **23**: 179-186
25 **Cooper HS**, Murthy SN, Shah RS, Sedergran DJ. Clinicopathologic study of dextran sulfate sodium experimental murine colitis. *Lab Invest* 1993; **69**: 238-249
26 **Tarlton JF**, Whiting CV, Tunmore D, Bregenholt S, Reimann J, Claesson MH, Bland PW. The role of up-regulated serine proteases and matrix metalloproteinases in the pathogenesis of a murine model of colitis. *Am J Pathol* 2000; **157**: 1927-1935
27 **Pender SL**, Tickle SP, Docherty AJ, Howie D, Wathen NC, MacDonald TT. A major role for matrix metalloproteinases in

T cell injury in the gut. *J Immunol* 1997; **158**: 1582-1590

28 **Pender SL**, Fell JM, Chamow SM, Ashkenazi A, MacDonald TT. A p55 TNF receptor immunoadhesin prevents T cell-mediated intestinal injury by inhibiting matrix metalloproteinase production. *J Immunol* 1998; **160**: 4098-4103

29 **Pender SL**, Breese EJ, Gunther U, Howie D, Wathen NC, Schuppan D, MacDonald TT. Suppression of T cell-mediated injury in human gut by interleukin 10: role of matrix metalloproteinases. *Gastroenterology* 1998; **115**: 573-583

30 **Bailey CJ**, Hembry RM, Alexander A, Irving MH, Grant ME, Shuttleworth CA. Distribution of the matrix metalloproteinases stromelysin, gelatinases A and B, and collagenase in Crohn's disease and normal intestine. *J Clin Pathol* 1994; **47**: 113-116

31 **Chakraborti S**, Mandal M, Das S, Mandal A, Chakraborti T. Regulation of matrix metalloproteinases: an overview. *Mol Cell Biochem* 2003; **253**: 269-285

32 **Delclaux C**, Delacourt C, D'Ortho MP, Boyer V, Lafuma C, Harf A. Role of gelatinase B and elastase in human polymorphonuclear neutrophil migration across basement membrane. *Am J Respir Cell Mol Biol* 1996; **14**: 288-295

33 **Reinecker HC**, Podolsky DK. Human intestinal epithelial cells express functional cytokine receptors sharing the common gamma c chain of the interleukin 2 receptor. *Proc Natl Acad Sci USA* 1995; **92**: 8353-8357

34 **Jung HC**, Eckmann L, Yang SK, Panja A, Fierer J, Morzycka-Wroblewska E, Kagnoff MF. A distinct array of proinflammatory cytokines is expressed in human colon epithelial cells in response to bacterial invasion. *J Clin Invest* 1995; **95**: 55-65

35 **Garcia-Lafuente A**, Antolin M, Guarner F, Crespo E, Salas A, Forcada P, Laguarda M, Gavalda J, Baena JA, Vilaseca J, Malagelada JR. Incrimination of anaerobic bacteria in the induction of experimental colitis. *Am J Physiol* 1997; **272**: G10-G15

36 **Qian BF**, Tonkonogy SL, Hoentjen F, Dieleman LA, Sartor RB. Dysregulated luminal bacterial antigen-specific T-cell responses and antigen-presenting cell function in HLA-B27 transgenic rats with chronic colitis. *Immunology* 2005; **116**: 112-121

37 **Rath HC**, Schultz M, Freitag R, Dieleman LA, Li F, Linde HJ, Scholmerich J, Sartor RB. Different subsets of enteric bacteria induce and perpetuate experimental colitis in rats and mice. *Infect Immun* 2001; **69**: 2277-2285

38 **Su CG**, Wen X, Bailey ST, Jiang W, Rangwala SM, Keilbaugh SA, Flanigan A, Murthy S, Lazar MA, Wu GD. A novel therapy for colitis utilizing PPAR-gamma ligands to inhibit the epithelial inflammatory response. *J Clin Invest* 1999; **104**: 383-389

39 **Castaneda FE**, Walia B, Vijay-Kumar M, Patel NR, Roser S, Kolachala VL, Rojas M, Wang L, Oprea G, Garg P, Gewirtz AT, Roman J, Merlin D, Sitaraman SV. Targeted deletion of metalloproteinase 9 attenuates experimental colitis in mice: central role of epithelial-derived MMP. *Gastroenterology* 2005; **129**: 1991-2008

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BASIC RESEARCH

Gene expression profiles of hepatic cell-type specific marker genes in progression of liver fibrosis

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Abstract

AIM: To determine the gene expression profile data for the whole liver during development of dimethylnitrosamine (DMN)-induced hepatic fibrosis.

METHODS: Marker genes were identified for different types of hepatic cells, including hepatic stellate cells (HSCs), Kupffer cells (including other inflammatory cells), and hepatocytes, using independent temporal DNA microarray data obtained from isolated hepatic cells.

RESULTS: The cell-type analysis of gene expression gave several key results and led to formation of three hypotheses: (1) changes in the expression of HSC-specific marker genes during fibrosis were similar to gene expression data in *in vitro* cultured HSCs, suggesting a major role of the self-activating characteristics of HSCs in formation of fibrosis; (2) expression of mast cell-specific marker genes reached a peak during liver fibrosis, suggesting a possible role of mast cells in formation of fibrosis; and (3) abnormal expression of hepatocyte-specific marker genes was found across several metabolic pathways during fibrosis, including sulfur-containing amino acid metabolism, fatty acid metabolism, and drug metabolism, suggesting a mechanistic relationship between these abnormalities and symptoms of liver fibrosis.

CONCLUSION: Analysis of marker genes for specific hepatic cell types can identify the key aspects of fibrogenesis. Sequential activation of inflammatory cells and the self-supporting properties of HSCs play an important role in development of fibrosis.

Key words: Liver fibrosis; Gene expression; Microarray; Dimethylnitrosamine; Marker genes; Hepatic stellate cell; Kupffer cell; Hepatocytes; Metabolic pathway

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INTRODUCTION

The pathological relationship between chronic inflammation and formation of fibrosis has been established in various organs, including the liver, kidney, lung and pancreas. Although liver fibrosis has been studied extensively, the underlying mechanisms remain unclear and drugs to prevent and treat fibrosis are only partially effective. DNA microarray technology offers an approach to this kind of complex problems, and microarray analyses of the whole liver have been reported for liver fibrosis[1,2]. However, these analyses did not address the behavior of individual hepatic cell-types and the interactions of hepatic cells during fibrosis. Therefore, in the current study we identified hepatic cell-specific marker genes that could be used to understand the *in vivo* behavior of each type of hepatic cells during fibrogenesis.

About 70%-80% of hepatic cells are parenchymal hepatocytes, while the non-parenchymal cells are mainly composed of Kupffer cells, hepatic stellate cells (HSC) and sinusoidal endothelial cells (SECs)[3,4]. Kupffer cells are the resident monocytes in liver, and act in phagocytosis of foreign substances such as microorganisms, as well as management of inflammatory processes. Kupffer cells and infiltrated monocytes and lymphocytes are considered to trigger inflammation in the early phase of hepatitis and then maintain chronic inflammation. HSCs control hepatic and cardiovascular contraction, and produce extracellular

matrix (ECM) components and cytokines for repair of organs. HSCs are also believed to have a central role in hepatic fibrosis formation. Hepatocytes fulfill the main functions of the liver, including regulation of nutrition, production of major serum proteins, and elimination of unnecessary materials to maintain homeostasis of the whole body[4].

In the current study, we first examined gene expression profiles of hepatic cells that were isolated at different time points during liver fibrosis. Marker genes specific for different types of hepatic cells were obtained by comparing these profiles and using information from previous studies. DNA microarray data for the whole liver were then interpreted during liver fibrogenesis using the hepatic cell-type specific marker genes. Our results suggest that new pathological properties and intracellular events are associated with each cell type during liver fibrogenesis, and provide candidates for diagnostic markers of liver fibrosis.

MATERIALS AND METHODS

Animals and experimental protocols

Male Sprague-Dawley rats (Charles River Japan, Yokohama, Japan) weighing 160-190 g were housed with unrestricted access to food (CRF-1, Oriental Yeast, Tokyo, Japan) and water in air-conditioned animal quarters with a 12 h light/dark cycle (light between 07:00 and 19:00 h). Hepatic fibrosis was induced by intraperitoneal injection of 0.5% dimethylnitrosamine (DMN; Wako Pure Chemical Industries, Osaka, Japan) at 2 mL/kg of body weight for three consecutive days each week for four weeks. Blood samples were drawn from the inferior vena cava on d 0, 4, 7, 14, 21 and 28, respectively. Liver specimens obtained on these days were dissected and immediately frozen in liquid nitrogen. Fibrosis was confirmed by haematoxylin and eosin (HE) staining of the liver tissue. The hydroxyproline content of liver specimens was determined as previously described by Horie *et al*[5] . Plasma alanine aminotransferase (ATL) and aspartate aminotransferase (AST) were measured using commercial kits (Fuji Film, Tokyo, Japan), and hyaluronic acid levels were determined using a commercial ELISA kit (Fujirebio, Tokyo, Japan). The animal facilities and protocol were reviewed and approved by the Institutional Animal Care and Use Committee of Ajinomoto Co., Inc.

Preparation of HSCs, inflammatory cells (including Kupffer cells) and hepatocytes

HSCs were isolated from rat liver using the pronase-collagenase digestion method as previously reported[6]. Kupffer cell fraction was prepared with an elutriator, using essentially the same method as previously described[6], and hepatocytes were isolated as previously described[7].

Selection of marker genes for hepatic cells

Hepatic cells (HSCs, Kupffer cell fraction and hepatocytes) were isolated from the liver on d 0, 4, 7, 14, 21 and 28 during liver fibrogenesis. The isolated hepatic cells at each time point were subjected to DNA microarray analysis. If the maximum or minimum expression of a gene in hepatocytes was ten times higher or lower than that of the same gene in HSCs and the Kupffer cell fraction, the up- or down-regulated gene was defined as a hepatocyte-specific marker gene. If the maximum or minimum expression of a gene in HSCs was ten times higher or lower than that of the same gene in hepatocytes and three times higher or lower than the same gene in the Kupffer cell fraction, this gene was defined as a HSC-specific marker gene. Similarly, if the maximum or minimum expression of a gene in the Kupffer cell fraction was ten times higher or lower than that of the same gene in hepatocytes and three times higher or lower than the same gene in HSCs, the gene was defined as an inflammatory cell-specific marker gene. An explanation of the use of the term 'inflammatory cell', rather than 'Kupffer cell', was given in the Results section. The ratios used to determine cellular specificity were based on previous reports[8,9]: the number of hepatocytes was about ten times higher than that of the inflammatory cell fraction or HSCs, while the number of Kupffer cells was similar to that of HSCs. Use of a high ratio improved the definition of cellular specificity, but also decreased the number of marker genes. However, we found that these ratios were the most appropriate for identification of a set of genes for analysis of liver fibrogenesis.

Microarray analysis

RNAs both from frozen liver tissues and from each isolated cell type were prepared using Isogen reagent (Nippon Gene), and the quality and quantity of each RNA sample were assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies, Inc.). RNA samples were reverse-transcribed with a poly (dT) oligonucleotide attached to a T7 promoter and copied into dsDNA (Invitrogen). *In vitro* RNA transcription was performed to incorporate biotin-labeled ribonucleotides into the cRNA transcripts using a RNA transcript labeling kit (Enzo Biochem). Some of the RNA (15 g) was utilized for hybridization to a rat genome U34A array (Affymetrix) and a quality assay using test 3 array probe chips was performed according to the manufacturer' s protocol. After hybridization and subsequent washing using the Affymetrix Fluidics Station 400, fluorescence signals amplified with streptavidin phycoerythrin were measured using the Affymetrix scanner, and the results were analyzed using the MicroArray suite software. In the whole liver analysis, two rats and two arrays were used at each time point (d 4, 7, 14, 21 and 28). In the cell-type-specific analysis, one rat which was selected by ALT/AST score and one array were used at each time point (d 4, 7, 14, 21 and 28).

Statistical analysis

Clustering of K-means was performed using the TIGR MeV (MultiExperiment Viewer)[10]. Gene expression profiles in the chronic phase were clustered into 10 patterns using K-means analysis. Clustered genes with a tendency to temporally decrease (clusters 1, 3 and 10) or increase (clusters 7 and 9) were selected as gene markers that had a strong relationship with fibrogenesis.

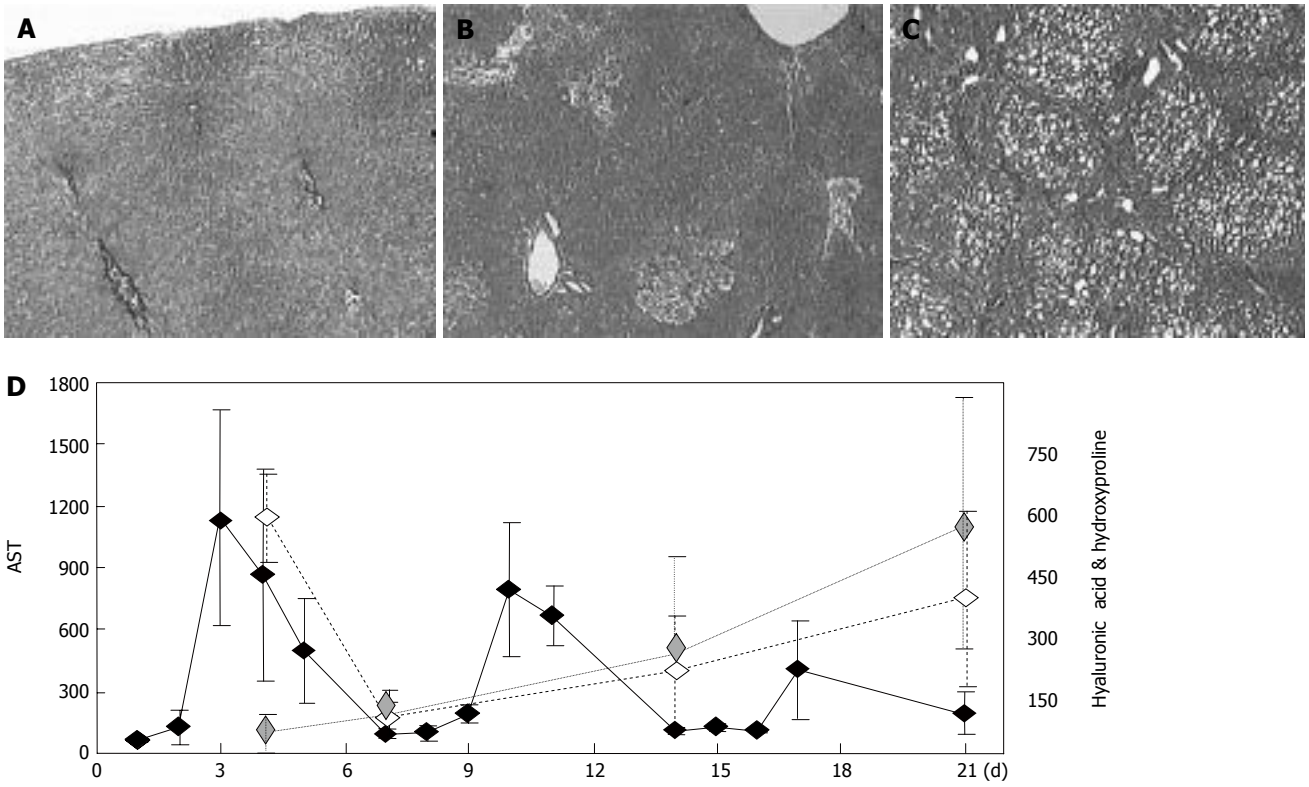

Figure 1 Histological and biochemical analyses of fibrogenesis. **A**-**C**: Histological staining (HE staining) of control liver sections (**A**) and sections obtained on d 4 (**B**) and d 21 (**C**), respectively, after DMN administration; **D**: Biochemical analysis of fibrogenesis showing plasma AST levels (IU/L, solid line), plasma hyaluronic acid levels (ng/mL, dashed line), and liver hydroxyproline levels (ng/mL, dotted line). The x-axis shows the days of fibrogenesis, and each value on the graph is shown as the mean ± SE, $n = 5$.

RESULTS

Time course of gene expression profiles for the whole liver during fibrogenesis

Administration of DMN for three days induced an inflammatory reaction in liver cells simulating the active phase in hepatitis, and the subsequent lack of administration of DMN for four days was used to simulate the remission phase in hepatitis. Repetition of this cycle led to fibrosis in three or four weeks, as shown in Figure 1. AST (GOT) and ALT (GPT) increased on the days of DMN administration and decreased on the days during which DMN was not administered (Figure 1), but both AST and the hyaluronic acid content in serum, a marker of fibrosis[11], gradually increased on days without DMN administration. The inflammatory reaction in periods without DMN administration was weaker than that in periods with DMN-administration, but gradually increased in intensity and response. Based on the behavior shown in Figure 1, gene expression profiles on d 4 (just after a period of DMN administration) were defined as representative of the acute phase response, and those on d 7, 14, 21 and 28 (just before a period of DMN administration) were defined as representative of the chronic phase response.

Marker genes indicating fibrotic activity of HSCs

HSCs were isolated from liver at each time point over the time course of development of DMN-induced fibrosis. Marker genes expressed mainly in HSCs were selected from a DNA microarray analysis, as described in the Materials and Methods, and the selected HSC-specific marker genes are listed in Figure 2. In addition, HSCs isolated from normal rats were cultured *in vitro* for 7 d and DNA microarray analysis of these cells was performed on d 0, 4, and 7, respectively. Marker genes identified in isolated HSCs in the DMN-induced fibrosis model showed the same behavior in the *in vitro* culture, supporting the HSC specificity of the selected marker genes. The behavior of HSC-specific marker genes *in vivo* during fibrogenesis was analyzed using DNA microarray data for the whole liver at each time point during development of DMN-induced fibrosis. These data could indicate the actual behavior of the marker genes *in vivo*, since the data from isolated HSCs might contain some bias due to isolation stimuli. The behavior of HSC-specific marker genes is shown in Figure 2, and supplemental background data are provided in Figure 3. The genes were separated into 2 groups as shown in Figure 2. Group 1 contained marker genes that were linearly up-regulated during fibrogenesis and in the acute inflammation phase, whereas group 2 contained marker genes that were linearly up-regulated during fibrogenesis but not in the acute inflammation phase. Both groups could be further separated into 2 subgroups. Group 1-1 included marker genes that were linearly up-regulated during fibrogenesis and remained in an up-regulated state on d 28, and group 1-2 included marker genes that were also linearly up-regulated during fibrogenesis but then decreased in expression on d 28. Group 2 was similarly separated into groups 2-1 and 2-2. In summary, two groups of HSC-specific marker genes were identified, one in which the genes responded to inflammatory stimuli and the other in which the genes did not respond to such stimuli. Furthermore,

Table 1 Marker genes for hepatic stellate cells (HSCs)

Group	Probe ID	Annotation	Symbol	GenBank	Whole liver						*In vitro* cultured HSC		
					d 0	4	7	14	21	28	d 0	3	7
1	M24067_at	Serine proteinase inhibitor clade E member 1/plasminogen activator inhibitor-1 (PAI-1)	PAI1	M24067	*10.6*	9.3	*1.6*	2.1	*1.8*	**2.1**	*6.0*	29.5	14.8
	M23566exon_s_at	alpha-2-macroglobulin	A2M	M23566	*191.8*	2.3	*0.6*	1.1	*1.0*	**2.3**	*182.9*	2.8	0.8
	M55534mRNA_s_at	Crystallin alpha polypeptide 2/ alpha-crystallin B chain	CRYAB	M55534	13.1	8.9	1.1	1.9	3.2	**3.8**	369.6	3.0	1.5
	Z12298cds_s_at	Decorin	Dcn	Z12298	17.9	2.8	2.5	2.9	3.8	**5.6**	470.1	2.5	2.8
	rc_AA891527_at	Four and a half LIM domains 2	Fhl2	AA891527	*12.7*	3.5	2.7	3.8	5.8	**7.9**	396.3	2.8	2.6
	S57478cds_s_at	Annexin A1/lipocortin I	Anxa1	S57478	14.4	2.6	1.7	1.9	1.8	**2.3**	188.3	3.4	2.5
	D50093_s_at	Prion protein (RaPrP gene for prion protein)	Prnp	D50093	*2.2*	46.0	7.3	19.1	31.4	**37.3**	489.8	2.1	1.8
	rc_AI231472_s_at	Collagen, type 1, alpha 1	Col1a1	AI231472	113.0	1.7	1.0	2.7	3.1	**4.2**	153.1	3.2	5.1
	rc_AA900769_s_at	Vascular alpha-actin/actin, alpha-2, smooth muscle, aorta	ACTA2	AA900769	*24.0*	8.5	1.8	10.1	15.4	**22.2**	56.4	12.1	12.5
	L00382cds_at	Skeletal muscle beta-tropomyosin and fibroblast tropomyosin 1, alternative/Tropomyosin 2	TPM2	L00382	*3.4*	*3.2*	*2.0*	4.1	5.4	**7.4**	1.6	69.0	62.3
	U57362_at	Procollagen, type XII, alpha 1 (collagen XII alpha 1)	Col12a1	U573262	6.3	1.8	2.0	1.9	2.6	**2.8**	47.1	3.1	2.8
	M14656_at	Secreted phosphoprotein 1/ Sialoprotein	Spp1	M14656	7.4	17.8	2.0	2.1	2.4	**20.8**	28.7	34.2	31.9
	rc_AI172064_at	Lectin, galactose binding, soluble 1	Lgals1	AI172064	61.6	2.3	1.2	2.6	**4.3**	3.0	224.3	4.9	3.8
	rc_AA894345_at	Phosphoprotein enriched in astrocytes 15 (predicted)	"Pea15 _predicted"	AA894345	20.5	2.2	1.2	2.0	**2.3**	2.1	112.0	2.1	1.8
	M83107_g_at	Transgelin (SM22-alpha)	Tagln	M83107	*29.3*	3.1	1.6	3.4	**5.1**	**4.6**	*81.7*	14.7	10.0
2	L03294_g_at	Lipoprotein lipase	Lpl	L03294	22.5	1.2	1.6	1.5	2.3	**4.5**	76.9	3.5	7.8
	rc_AI012030_at	Matrix Gla protein	Mgp	AI012030	120.4	1.0	0.9	1.5	2.6	**5.3**	236.2	2.5	4.3
	M80829_at	Troponin T2, cardiac	Tnnt2	M80829	*15.7*	*1.2*	*0.7*	*1.1*	1.8	**2.6**	14.9	18.4	15.5
	M22400_at	Glypican 3 /developmentally regulated intestinal protein (OCI-5)	GPC3	M22400	*3.7*	*0.9*	*2.4*	*3.9*	*7.4*	**10.0**	*8.6*	*0.6*	*1.0*
	D00680_at	Glutathione peroxidase 3/plasma glutathione peroxidase precursor	Gpx3	D00680	*20.5*	1.4	*1.5*	2.7	3.4	**3.9**	104.3	5.1	4.3
	rc_AA800844_s_at	Similar to Loxl protein /LoxL1=lysyl oxidase-like 1	LoxL1	AA800844	*29.3*	1.5	1.5	4.8	7.5	**8.5**	166.0	3.5	5.3
	S77494_s_at	Lysyl oxidase	Lox	S77494	27.5	0.8	0.8	1.9	2.4	**3.0**	26.3	29.1	38.0
	AF030358_g_at	Small inducible cytokine subfamily D, number1/chemokine CX3C motif, ligand 1	CX3CL1	AF030358	*4.6*	*1.0*	2.2	2.3	3.0	**4.0**	24.0	*0.6*	1.5
	X84039_at	Lumican	Lum	X84039	8.1	1.0	2.1	2.1	**2.3**	1.1	52.8	2.3	3.2
	U09540_g_at	Cytochrome P450, family 1, subfamily b, polypeptide 1	Cyp1b1	U09540	*8.1*	*1.0*	*1.3*	1.2	**1.9**	1.6	*29.9*	5.0	6.2

Expression profiles of the whole liver and *in vitro* cultured isolated HSCs were obtained using a rat genome U34A array (Affymetrix). Gene markers for HSCs are listed. For analysis of HSCs in the whole liver, expression intensities are given for d 0, and expression intensity data for d 4, 7, 14, 21 and 28 are shown as ratios to the d 0 expression data. The classification of group1 (2) corresponds to the presence (absence) of up-regulated peak in the acute inflammation phase. For analysis of in vitro cultured HSCs, expression levels for d 0 are also shown, and data for d 4 and 7 are similarly shown as ratios to the expression level on d 0. Italicized values indicate an "absent" call by the Affymetrix software. Bold text indicates the highest ratio in the chronic phase (d 7, 14, 21 and 28). □ between 0.667 and 1.5; ■ ⩾ 1.5; ■ ⩽ 0.667.

HSC-specific marker genes were found that could identify biological changes in HSCs in the late phase of fibrosis.

Marker genes indicating inflammatory activity in immune cell populations

The Kupffer cell fraction was separated from other hepatic cells at each time point during the course of fibrosis development, and marker genes expressed mainly in the Kupffer cell fraction were selected from the DNA microarray analysis. These marker genes are shown in Figure 4, and supplemental background data are provided in Figure 5. Marker genes in the Kupffer cell fraction indicated the presence of other hematopoietic cells, such as mast cells, lymphocytes, erythrocytes and their progenitors in this fraction, as shown in Figures 4 and 5. Since the source cell population for these marker genes could not be confirmed, a particular hematopoietic cell was postulated to be the source of each marker gene based on previous reports as shown in supplemental Table 1. These data indicated that the method used for isolation of Kupffer cells was not appropriate in fibrotic liver, although it was effective for isolation of normal liver cells. However, despite this

Table 2 Marker genes in hepatic stellate cells (HSC)

Group	Probe ID	Annotation	Symbol	Gen Bank	Whole liver						Isolated HSC						*In vitro* cultured HSC		
					d 0	4	7	14	21	28	d 0	4	7	14	21	28	d 0	3	7
Group 1	M24067	Serine proteinase inhibitor clade E member 1/ plasminogen activator inhibitor-1 (PAI-1)	PAI1	M24067	*10.6*	9.3	*1.6*	2.1	*1.8*	**2.1**	565.8	2.6	1.4	2.5	2.2	1.8	*6.0*	29.5	14.8
	M23566	alpha-2-macroglobulin	A2M	M23566	*191.8*	2.3	*0.6*	1.1	*1.0*	**2.3**	*34.3*	*0.5*	*4.1*	7.9	9.4	47.0	*182.9*	2.8	0.8
	M55534	Crystallin alpha polypeptide 2/ alpha-crystallin B chain	CRYAB	M55534	13.1	8.9	1.1	1.9	3.2	**3.8**	619.5	1.9	1.0	1.0	1.5	1.2	369.6	3.0	1.5
	Z12298cds_s_at	Decorin	Dcn	Z12298	17.9	2.8	2.5	2.9	3.8	**5.6**	384.5	2.5	1.6	2.3	2.4	3.5	470.1	2.5	2.8
	rc_AA891527_at	Four and a half LIM domains 2	Fhl2	AA891527	*12.7*	3.5	2.7	3.8	5.8	**7.9**	260.8	2.9	1.5	1.3	2.0	3.7	396.3	2.8	2.6
	S57478cds_s_at	Annexin A1/ lipocortin I	Anxa1	S57478	14.4	2.6	1.7	1.9	1.8	**2.3**	464.2	1.6	0.9	1.3	1.8	2.5	188.3	3.4	2.5
	D50093	Prion protein (RaPrP gene for prion protein)	Prnp	D50093	*2.2*	46.0	7.3	19.1	31.4	**37.3**	361.6	4.3	0.9	1.0	2.9	3.7	489.8	2.1	1.8
	rc_AI231472_s_at	Collagen, type 1, alpha 1	Col1a1	AI231472	113.0	1.7	1.0	2.7	3.1	**4.2**	890.1	0.5	0.8	2.5	2.9	3.8	153.1	3.2	5.1
	rc_AA900769_s_at	Vascular alpha-actin/actin, alpha-2, smooth muscle, aorta	ACTA2	AA900769	*24.0*	8.5	1.8	10.1	15.4	**22.2**	68.0	21.8	18.0	10.8	20.3	15.2	56.4	12.1	12.5
	L00382cds_at	Skeletal muscle beta-tropomyosin and fibroblast tropomyosin 1, alternative/ Tropomyosin 2	TPM2	L00382	*3.4*	*3.2*	*2.0*	4.1	5.4	**7.4**	34.9	2.9	2.3	1.6	6.0	10.2	1.6	69.0	62.3
	U57362_at	Procollagen, type XII, alpha 1 (collagen XII alpha 1)	Col12a1	U57326	6.3	1.8	2.0	1.9	2.6	**2.8**	73.4	2.2	1.1	1.2	2.0	2.9	47.1	3.1	2.8
	M14656_at	Secreted phosphoprotein 1/Sialoprotein	Spp1	M14656	7.4	17.8	2.0	2.1	2.4	**20.8**	8.5	25.1	12.1	11.1	18.6	204.3	28.7	34.2	31.9
	rc_AI172064_at	Lectin, galactose binding, soluble 1	Lgals1	AI172064	61.6	2.3	1.2	2.6	4.3	**3.0**	980.7	2.0	1.3	1.6	1.9	1.6	224.3	4.9	3.8
	AA894345	Similar to MAT1 gene			20.5	2.2	1.2	2.0	2.3	**2.1**	172.0	2.0	0.9	0.9	1.0	0.8	112.0	2.1	1.8
	M83107_g_at	Transgelin (SM22-alpha)	Tagln	M83107	*29.3*	3.1	1.6	3.4	5.1	**4.6**	275.9	8.2	5.3	4.2	7.5	7.2	*81.7*	14.7	10.0
Group 2	L03294_g_at	Lipoprotein lipase	Lpl	L03294	22.5	1.2	1.6	1.5	2.3	**4.5**	130.8	0.5	1.0	1.5	2.2	4.4	76.9	3.5	7.8
	rc_AI012030_at	Matrix Gla protein	Mgp	AI012030	120.4	1.0	0.9	1.5	2.6	**5.3**	1246.7	0.3	0.5	0.6	1.6	2.7	236.2	2.5	4.3
	M80829_at	Troponin T2, cardiac	Tnnt2	M80829	*15.7*	*1.2*	*0.7*	*1.1*	1.8	**2.6**	*17.3*	*0.8*	2.2	3.3	7.7	17.5	14.9	18.4	15.5
	M22400	Glypican 3/ developmentally regulated intestinal protein (OCI-5)	GPC3	M22400	*3.7*	*0.9*	*2.4*	*3.9*	*7.4*	**10.0**	61.9	0.4	0.9	1.5	3.1	9.9	*8.6*	*0.6*	*1.0*
	D00680_at	Glutathione peroxidase 3/ plasma glutathione peroxidase precursor	Gpx3	D00680	*20.5*	1.4	*1.5*	2.7	3.4	**3.9**	134.5	0.7	1.8	3.9	9.2	9.2	104.3	5.1	4.3
	rc_AA800844_s_at	Similar to Loxl protein/ LoxL1=lysyl oxidase-like 1	LoxL1	AA800844	*29.3*	1.5	1.5	4.8	7.5	**8.5**	436.8	0.8	1.4	2.1	3.0	2.5	166.0	3.5	5.3
	S77494_s_at	Lysyl oxidase	Lox	S77494	27.5	0.8	0.8	1.9	2.4	**3.0**	183.1	1.0	3.2	3.4	4.3	3.1	26.3	29.1	38.0

Group	Probe ID	Annotation	Symbol	Gen Bank	Whole liver						Isolated HSC						*In vitro* cultured HSC		
					d 0	4	7	14	21	28	d 0	4	7	14	21	28	d 0	3	7
	AF030358 g at	Small inducible cytokine subfamily D, number1/ chemokine CX3C motif, ligand 1	CX3CL1	AF030358	*4.6*	*1.0*	2.2	2.3	3.0	**4.0**							24.0	*0.6*	1.5
	X84039_at	Lumican	Lum	X84039	8.1	1.0	2.1	2.1	**2.3**	1.1	91.5	0.3	0.8	2.4	1.5	0.3	52.8	2.3	3.2
	U09540_g_at	Cytochrome P450, family 1, subfamily b, polypeptide 1	Cyp1b1	U09540	*8.1*	*1.0*	*1.3*	1.2	**1.9**	1.6	26.5	4.9	0.8	3.9	10.2	14.6	*29.9*	5.0	6.2
Group 3	M31038	Non-RT1 class Ib/ MHC class I non-RT1.A alpha-1-chain		M31038	42.2	1.8	4.0	**6.5**	3.2	3.9	38.3	2.0	10.1	5.0	8.9	0.8	40.7	0.7	0.8
	U44948	Cysteine-and glycine rich-protein 2/smooth muscle cell LIM protein (SmLIM)	CSRP2	U44948	41.9	2.2	1.2	**1.6**	1.1	1.2	406.7	2.6	0.4	0.4	0.5	0.4	202.0	2.9	1.7
	X02601 at	Matrix metalloproteinase 3		X0260	8.6	1.3	**2.0**	1.0	0.9	1.3							597.2	1.1	0.1
Group 4	M15880_at	Neuropeptide Y	Npy	M15880	49.2	4.2	1.0	1.2	1.3	0.8	63.1	2.0	0.7	0.2	1.7	*0.3*	*6.2*	*0.8*	*6.6*
	U50736	Cardiac adriamycin-responsive protein	CARP	U50736	14.3	1.9	1.0	1.0	1.2	1.1	33.7	8.1	2.6	1.6	3.0	4.0	63.6	2.4	1.0

Expression profiles of the whole liver, of isolated HSCs during fibrogenesis, and of in vitro cultured isolated HSCs were obtained using a rat Genome U34A Array (Affymetrix). Marker genes for HSCs are listed. Expression intensities are shown for d 0, and expression intensity data for d 4, 7, 14, 21 and 28 are displayed as ratios to the d 0 expression levels for the analysis of whole liver and isolated HSCs. For in vitro cultured HSCs, the d 0 data are similarly shown as expression intensities, and data on d 4 and 7 are shown as ratios to the expression levels on d 0. Italics indicate an "absent" call by the Affymetrix software, and bold text indicates the highest ratio in the chronic phase (d 7, 14, 21 and 28). □ between 0.667 and 1.5; ■ ⩾ 1.5; ■ ⩽ 0.667.

drawback, the marker genes could be used to study the behavior of inflammatory cells during fibrogenesis, and these genes were therefore defined as inflammatory cell-specific marker genes. The behavior of the inflammatory cell-specific marker genes *in vivo* during fibrogenesis was analyzed using DNA microarray data for the whole liver at each time point during DMN-induced fibrogenesis of marker genes, as summarized in Figures 4 and 5.

The acute phase response was followed by an immunological response, based on the increase in expression of Kupffer cell (or macrophage) markers such as Lyz, Gzmb, and Il1b, as well as surface markers of T cells, such as T-cell receptor, Il2rb, Cd8, Cd76, and Cd45. The up-regulated expression of these genes seemed to indicate activation of Kupffer cells and T lymphocytes, as well as activation of the interaction between these cells, around d 7. Temporary up-regulation of mast cell markers such as chemokines and mast cell proteases indicated the invasion and/or activation of mast cells around d 14, which is of interest since mast cells are known not only to cause acute inflammation, but also to have a role in the induction of chronic inflammation[12-14]. However, whether activation of mast cells is essential for liver fibrosis is unknown. Peak expression of B cell markers such as immunoglobulin occurred on d 21 or d 28, and therefore activation or invasion of B cells seemed to reach its peak in the late phase. Overall, these results showed that inflammatory cell-specific marker genes could be used to monitor the transition of active inflammatory cell populations in fibrosis, and this sequential activation or invasion of inflammatory cells might be related to the stage of fibrotic progression.

Marker genes indicating damage to hepatocytes

Hepatocytes were separated from other hepatic cells at each time point during the course of DMN-induced fibrogenesis, and marker genes expressed mainly in hepatocytes were selected by DNA microarray analysis. The behavior of hepatocyte-specific marker genes *in vivo* during fibrogenesis was analyzed using DNA microarray data for the whole liver at each time point during fibrosis development. Marker genes were categorized based on their functions, as shown in Figure 6. Many abnormally expressed genes were identified and temporal analysis revealed groups of genes showing consistent variation in expression. Gene expression profiles in the chronic phase (d 7, 14, 21 and 28) were clustered into 10 patterns using K-means analysis. Clustered genes with a tendency to temporally decrease (clusters 1, 3 and 10) or increase (clusters 7 and 9) were selected, and then genes were further selected based on a strong correlation coefficient ($\geqslant$ 0.7 or $\leqslant$ -0.7) with fibrosis stage (that is, d 7, 14, 21 and 28). The time courses of expression ratios are shown in Figure 7 as supplemental data. The 41 down-regulated and 19 up-regulated genes that were finally selected are shown in Table 2. These genes appeared to have a strong relationship with progression of fibrosis, and might also share common regulatory expression mechanisms.

Down-regulation of the expression of Cdo1 and Csad showed a strong relationship with fibrotic stage, while expression of Gsta2 was simultaneously up-regulated. Down-regulation of other metabolic enzymes in sulfur-containing metabolic pathways is also shown in Figure 6. These data suggested a broad range of abnormalities in sulfur-containing amino acid metabolic pathways in

Table 3 Marker genes for hematopoietic cells in the Kupffer cell fraction

Group	Probe ID	Annotation	Symbol	GenBank	Whole liver						Cell type	Predicted classification	Reference
					D 0	4	7	14	21	28			
1	M34097_at	Granzyme B/natural killer (NK) cell protease 1 (RNKP-1)	Gzmb	M34097	21.4	1.2	**2.1**	1.1	0.9	0.8	M/L	Kupffer cell, T cell	5
	rc_AA892775_at	Lysozyme	Lyz	AA892775	220.8	9.1	**6.4**	3.4	5.3	**6.4**	M	Kupffer cell	6
	Y12009_at	Chemokine, cc motif, receptor 5	Ccr5	Y12009	11.7	1.8	**2.6**	1.4	1.4	1.2	M/L	T cell	7
	E13732cds_at	Macrophage inflammatory protein-1 alpha receptor/ chemokine, CCmotif, receptor 1/RANTES receptor	Ccr1	E13732	9.4	3.8	**2.3**	1.5	2.2	**2.3**	M/L	T cell	8
	X13044_g_at	CD74 antigen/invariant polpypeptide of major histocompatibility class Ⅱ antigen-associated	Cd74	X13044	131.7	1.9	**13.9**	7.8	7.7	5.6	L	B cell	9, 10
	X04139_s_at	Protein kinase C, beta 1	Prkcb1	X04139	14.5	1.5	**3.7**	2.4	2.2	1.7	M		11
	X03369_s_at	Similar to tubulin, beta	TUBB	X03369	18.6	0.9	**2.0**	1.8	1.1	1.4	M		12
	M98820_at	Interleukin 1 beta	Il1b	M98820	*19.4*	1.6	**3.6**	1.6	1.1	1.1	M	Kupffer cell	13
	U87627_at	Solute carrier family16 (monocarboxylate transporter), member 3	Slc16a3	U87627	24.8	1.9	**1.5**	0.8	1.2	0.9	M/L		14
	D00403_g_at	Interleukin 1 alpha	Il1a	D00403	27.6	0.6	**1.7**	0.8	0.5	0.5	M		15
	rc_AI639534_at	Properdin factor, complement/ Factor P PROPERDIN P FACTOR, COMPLEMENT; PFC	Pfc	AI639534	68.5	1.2	**2.1**	1.6	1.1	0.8	M		16
	X03015_at	CD8 antigen, alpha chain	Cd8a	X03015	3.3	8.5	**15.7**	5.0	9.1	3.8	L	T cell	OMIM
	M18854_at	Similar to T-cell receptor beta-chain/T-cell receptor active beta-chain C-region	---	M18854	*22.7*	*2.0*	**3.7**	2.6	2.6	2.5	L	T cell	
	rc_AA892506_at	Coronin 1A	Coro1a	AA892506	*30.5*	3.8	**4.1**	3.7	3.2	3.6	M/L		OMIM
	M55050_at	Interleukin 2 receptor, beta chain	Il2rb	M55050	48.5	0.9	**1.5**	0.9	0.9	0.8	L	T cell	OMIM
	M30691_at	Ly6-C antigen gene/CD56	Ly6c	M30691	30.2	3.6	**5.0**	1.6	1.7	1.1	L	T cell, NK cell	17
	rc_AA891302_at	Similar to Ser/Thr kinase (BL44)	---	AA891302	*6.9*	*3.2*	***5.4***	4.5	*3.9*	4.1	L	B cell	1
	M10072mRNA_s_at	protein tyrosine phosphatase, receptor type,C/CD45	Ptprc	M10072	8.2	5.9	**4.3**	3.1	3.3	2.7	M		OMIM
	S74141_s_at	Hemopoietic cell kinase/hck tyrosine kinase	Hck	S74141	*42.8*	2.3	**3.5**	2.3	2.5	2.1	M		OMIM
	X52196cds_at	Arachidonate 5-lipoxygenase activating protein	Alox5ap	X52196	34.5	2.3	***2.1***	1.7	1.7	1.8	M	Kupffer cell	2
	U93306_at	Kinase insert domain protein receptor	Kdr	U93306	31.3	0.5	**1.6**	1.2	0.8	0.6	M		18
	U55192_at	Inositol polyphosphate-5-phosphatase D	Inpp5d	U55192	*11.6*	2.1	**3.3**	2.1	2.1	1.6	L/M/Leu		19
	rc_AI178971_at	Similar to alpha globin/ Hemoglobin alpha	(HBA1)	AI178971	141.3	2.4	**0.3**	0.4	0.5	0.5	E		20
	D86297_at	Aminolevulinic acid synthase 2	Alas2	D86297	131.4	2.7	**0.5**	**0.5**	0.8	0.9	E		Entrez gene
	Y07704_g_at	Best5 protein	Best5	Y07704	33.8	2.9	**0.3**	0.5	0.5	0.5	"No information"		
	U50412_at	Phosphatidylinositol 3-kinase, r egulatory subunit, polypeptide 1	Pik3r1	U50412	43.8	1.3	***0.0***	***0.0***	0.5	0.8	M		4
	AB015191_g_at	Rhesus blood group	Rh	AB015191	*22.2*	1.4	***0.3***	***0.3***	*0.5*	0.6	E		Entrez gene
	M94918mRNA_f_at	Hemoglobin beta chain complex/ beta-globin	Hbb	M94918	3283.0	1.4	**0.5**	**0.5**	0.7	0.6	E		OMIM
	J04793_at	Solute carrier family 4 (anion exchanger), member 1	Slc4a1	J04793	58.0	1.2	0.7	**0.6**	1.0	0.7	E		Entrez gene
	U77697_at	Platelet-endothelial cell adhesion molecule/CD31	Pecam	U77697	45.5	0.7	**0.6**	0.9	0.8	0.8	M/L/E		OMIM
2	rc_AI009658_at	Chemokine, CC motif, ligand 5/secreted; RANTES	Ccl5	AI009658	*33.2*	*0.1*	1.8	**3.1**	1.4	*0.3*	L	T cell	OMIM
	rc_AA957923_at	Mast cell protease 2	Mcpt2	AA957923	*10.4*	*1.1*	3.3	**12.3**	8.7	5.8	M	mast cell	26
	U67914_at	Carboxypeptidase A3	Cpa3	U67914	19.3	*0.8*	1.0	**3.5**	2.7	1.6	M	mast cell	27

Group	Probe ID	Annotation	Symbol	GenBank	Whole liver						Cell type	Predicted classification	Reference
					D 0	4	7	14	21	28			
	U67911_s_at	Mast cell protease 9 or mast cell protease 8/mast cell protease 8 precursor (RMCP-8)	"Mcpt9/ Mcpt8"	U67911	*28.9*	*0.6*	2.2	**4.7**	3.0	2.1	M	mast cell	Entrez gene
	U67908_at	Chymase 1, mast cell	Cma1	U67908	*35.2*	*1.0*	*1.2*	**1.8**	1.5	*1.2*	M	mast cell	Entrez gene
	rc_AA957003_at	S100 calcium binding protein A8/ calgranulin A	S100a8	AA957003	6.5	7.8	3.2	**4.2**	1.1	1.4	M/Leu		29, 31
	L18948_at	S100 calcium binding protein A9/ calgranulin B	S100a9	L18948	16.9	5.6	*0.9*	**2.6**	*1.0*	*1.1*	M/Leu		31
	U31598_s_at	Major histocompatibility complex, class Ⅱ, DM alpha (RT1.DMa)	Hla-dma	U31598	98.1	1.9	2.5	**2.6**	2.6	2.2	M/L	Kupffer cell, mast cell	Entrez gene
	U31599_at	Major histocompatibility complex, class Ⅱ, DM beta (RT1. DMb)	Hla-dmb	U31599	20.7	2.3	4.3	3.5	**4.4**	2.8	M/L	Kupffer cell, mast cell	Entrez gene
	L06040_s_at	Arachidonate 12-lipoxygenase	Alox12	L06040	99.8	1.7	*0.2*	***0.1***	*0.3*	0.5	Leu/P		OMIM, 35
3	X06916_at	S100 calcium-binding protein A4 Mts1	S100a4	X06916	18.2	7.1	2.7	1.8	2.2	**3.1**	M/L		36
	M28671_at	Similar to Ig gamma-2B chain C region (rearranged IgG-2b)	---	M28671	*14.7*	*0.6*	*0.8*	*1.0*	**3.3**	*0.7*	L	B cell	
	rc_AI234828_g_at	Immunoglobulin heavy chain, alpha polypeptide	Igha	AI234828	28.8	0.6	1.0	1.7	2.0	**2.4**	L	B cell	
	X53517_at	CD37 antigen	Cd37	X53517	*32.5*	2.7	1.9	2.1	**2.3**	**2.3**	L	B cell	OMIM
	X58294_at	Carbonic anhydrase 2	Ca2	X58294	63.8	2.0	0.8	1.0	1.6	**2.1**	M/L/E		37, 38
	AF072411_g_at	cd36 antigen	Cd36	AF072411	67.0	1.3	1.2	0.9	1.2	**1.8**	M/E		OMIM
	U75689_s_at	Deoxyribonuclease I-like 3/ DNase gamma	Dnase1l3	U75689	106.6	0.1	1.1	1.1	0.5	**0.2**	M		40
	X73371_at	Fc receptor, IgG, low affinity IIb/ Fc gamma receptor	Fcgr2b	X73371	190.7	0.5	0.8	1.1	0.7	**0.5**	M/L/Leu	B cell, mast cell	OMIM
	L04672_s_at	Adrenomedullin receptor	Admr	L04672	147.0	0.5	0.8	0.9	0.7	**0.4**	L	T cell possibly	17, 4
4	AF041083_at	Solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1	Slc11a1	AF041083	*15.1*	2.7	*0.7*	*0.9*	*1.0*	*0.9*	M		Entrez gene
	D14015_g_at	Cyclin E	CCNE1	D14015	27.1	1.5	1.3	0.9	0.9	1.0	M		44
	U31367_at	Myelin and lymphocyte protein /myelin protein MVP17	Mal	U31367	*19.9*	2.4	*1.0*	1.1	1.2	*0.7*	L	T cell	43

Expression profiles of the whole liver and the isolated Kupffer cell fraction during fibrogenesis were obtained using a rat genome U34A array (Affymetrix). Marker genes for hematopoietic cells in the Kupffer cell fraction are listed. Expression intensities are given for d 0, and expression intensity data for d 4, 7, 14, 21 and 28 are shown as ratios to the d 0 expression data for analysis of hematopoietic cells in whole liver. The classification of genes into groups 1-4 and corresponds to the maximum change of expression at the different time points on d 7, 14, 21, 28 and 4, respectively. Italicized values indicate an "absent" call by the Affymetrix software. The bold values indicate the highest or lowest ratio in the chronic phase (d 7, 14, 21 and 28). □ between 0.667 and 1.5; ■ ⩾ 1.5; ■ ⩽ 0.667. Cell types in the inflammatory cell fraction are as follows: M: Monocytes and their progenitors; L: Lymphocytes and their progenitors; E: Erythrocytes and their progenitors; P: Platelets and their progenitors; Leu: Other kinds of leukocytes and their progenitors.

fibrosis. Regarding other genes, Hao2 and Hpcl2 had a role in fatty acid oxidation in peroxisome, while Amacr was associated with beta-oxidation of pristanoyl-CoA and C27-bile acyl-CoAs. The down-regulation of these genes and other metabolic enzymes related to fatty acid oxidation (Figure 6) suggested abnormalities in the fatty acid oxidation process in fibrosis. The expression of Amacr might be related to that of the nuclear receptor subfamily 0, group B, member 2 (Nr0b2), and changes in NrOb2 expression may affect one of the key molecules in cholesterol biosynthesis. Up-regulation of Gk and down-regulation of Pepck1, PC and Slc37a4 suggested abnormalities in gluconeogenesis, glycogen storage and glycolysis, while down-regulation of Ste and Hsd17b2 suggested an abnormality of estrogen metabolism in the liver. Decreased expression of many Cyp drug metabolism enzymes was also found in progression of fibrosis, and abnormalities in hormonal signaling were suggested by the down-regulation of Inhbe, Ghr and Dio1. These results showed that identification of hepatocyte-specific marker genes could allow analysis of functional changes in fibrosis, and all the identified abnormalities might have major effects on hepatic function.

DISCUSSION

Marker genes for HSCs

Markers of HSCs such as Acta 2, Cryab, Spp1, Prnp, and Pai-1 were strongly up-regulated on d 4, and then quickly decreased in expression following a gradual up-regulation. On the other hand, other HSC markers such as Gpc3, Lox, and Mgp did not show marked up-regulation on d 4, but their expression level increased linearly during fibrogenesis. Therefore, HSCs may be associated with events in two

Table 4 Marker genes in hematopoietic cells in the Kupffer cell fraction

Group	Probe ID	Annotation	Symbol	GenBank	Whole liver						Isolated Kupffer cells						Cell type	Predicted classification	Ref.
					D 0	4	7	14	21	28	d 0	4	7	14	21	28			
1	M34097_at	Granzyme B/natural killer (NK) cell protease 1 (RNKP-1)	Gzmb	M34097	21.4	1.2	**2.1**	1.1	0.9	0.8	42.1	1.9	7.5	7.6	3.2	1.3	M/L	Kupffer cell, T1 cell	
	rc_AA892775_at	Lysozyme	Lyz	AA892775	220.8	9.1	**6.4**	3.4	5.3	**6.4**	1164.3	5.5	2.9	4.1	2.9	3.9	M	Kupffer cell	2.3
	Y12009_at	Chemokine, cc motif, receptor 5	Ccr5	Y12009	11.7	1.8	**2.6**	1.4	1.4	1.2	29.4	11.9	8.7	6.8	4.5	5.4	M/L	T cell	4
	E13732cds_at	Macrophage inflammatory protein-1 alpha receptor/chemokine, CCmotif, receptor 1/RANTES receptor	Ccr1	E13732	9.4	3.8	**2.3**	1.5	2.2	**2.3**	40.6	11.8	1.7	2.3	1.8	4.0	M/L	T cell	5
	X13044_g_at	CD74 antigen/ invariant polpypeptide of major histocompatibility class II antigen-associated	Cd74	X13044	131.7	1.9	**13.9**	7.8	7.7	5.6	1672.0	1.9	2.4	3.1	2.3	2.6	L	B cell	2,6,7
	X04139_s_at	Protein kinase C, beta 1	Prkcb1	X04139	14.5	1.5	**3.7**	2.4	2.2	1.7	33.5	2.2	3.7	4.5	2.5	4.9	M		8
	X03369_s_at	Similar to tubulin, beta	TUBB	X03369	18.6	0.9	**2.0**	1.8	1.1	1.4	28.6	0.9	2.5	10.5	4.2	5.3	M		9
	M98820_at	Interleukin 1 beta	Il1b	M98820	*19.4*	1.6	**3.6**	1.6	1.1	1.1	884.8	6.8	2.7	2.6	2.2	2.8	M	Kupffer cell	10,11,13
	U87627_at	Solute carrier family16 (monocarboxylate transporter), member 3	Slc16a3	U87627	24.8	1.9	**1.5**	0.8	1.2	0.9	52.0	10.4	2.8	9.3	5.4	3.2	M/L		14,15,16
	D00403_g_at	Interleukin 1 alpha	Il1a	D00403	27.6	0.6	**1.7**	0.8	0.5	0.5	1395.6	1.9	1.1	0.4	0.4	0.6	M		17,18
	rc_AI639534_at	Properdin factor, complement/Factor P PROPERDIN P FACTOR, COMPLEMENT; PFC	Pfc	AI639534	68.5	1.2	**2.1**	1.6	1.1	0.8	49.8	11.9	5.1	5.8	8.7	7.3	M		19
	X03015_at	CD8 antigen, alpha chain	Cd8a	X03015	3.3	8.5	**15.7**	5.0	9.1	3.8	31.3	2.3	4.4	4.5	2.2	0.9	L	T cell	OMIM
	M18854_at	Similar to T-cell receptor beta-chain/ T-cell receptor active beta-chain C-region	---	M18854	*22.7*	*2.0*	**3.7**	2.6	2.6	2.5	37.9	1.0	7.1	15.5	9.3	4.3	L	T cell	
	rc_AA892506_at	Coronin 1A	Coro1a	AA892506	*30.5*	3.8	**4.1**	3.7	3.2	3.6	216.3	1.4	3.5	5.2	3.8	3.0	M/L		OMIM
	M55050_at	Interleukin 2 receptor, beta chain	Il2rb	M55050	48.5	0.9	**1.5**	0.9	0.9	0.8	64.7	2.0	4.1	4.1	2.9	1.7	L	T cell	OMIM
	M30691_at	Ly6-C antigen gene /CD56	Ly6c	M30691	30.2	3.6	**5.0**	1.6	1.7	1.1	173.8	3.7	2.1	2.6	1.8	1.4	L	T cell, NK cell	20,22
	rc_AA891302_at	Similar to Ser/Thr kinase (BL44)	---	AA891302	*6.9*	3.2	***5.4***	4.5	*3.9*	4.1	57.3	1.1	2.6	6.9	4.6	5.8	L	B cell	23
	M10072mRNA_s_at	Protein tyrosine phosphatase, receptor type,C/CD45	Ptprc	M10072	8.2	5.9	**4.3**	3.1	3.3	2.7	70.6	6.9	4.6	4.7	1.6	4.3	M		24
	S74141_s_at	Hemopoietic cell kinase hck tyrosine kinase	Hck	S74141	*42.8*	2.3	**3.5**	2.3	2.5	2.1	114.0	10.6	4.1	4.5	4.2	4.7	M		OMIM
	X52196cds_at	Arachidonate 5-lipoxygenase activating protein	Alox5ap	X52196	*34.5*	2.3	***2.1***	1.7	1.7	1.8	82.4	9.1	3.2	8.4	5.3	6.5	M	Kupffer cell	25,26
	U93306_at	kinase insert domain protein receptor	Kdr	U93306	31.3	0.5	**1.6**	1.2	0.8	0.6	1516.6	0.1	0.2	0.1	0.1	0.1	M		27,28,29
	U55192_at	Inositol polyphosphate-5-phosphatase D	Inpp5d	U55192	*11.6*	2.1	**3.3**	2.1	2.1	1.6	56.8	3.3	2.6	4.4	3.4	3.4	L/M/Leu		OMIM
	rc_AI178971_at	Similar to alpha globin/Hemoglobin alpha	(HBA1)	AI178971	141.3	2.4	**0.3**	0.4	0.5	0.5	18.2	*0.8*	*0.6*	*0.5*	15.6	*1.2*	E		OMIM
	D86297_at	Aminolevulinic acid synthase 2	Alas2	D86297	131.4	2.7	**0.5**	**0.5**	0.8	0.9	29.9	*0.2*	4.1	5.2	40.9	4.9	E		Entrez gene
	Y07704_g_at	Best5 protein	Best5	Y07704	33.8	2.9	**0.3**	0.5	0.5	0.5	668.1	*0.0*	0.2	0.1	0.4	0.2	No information		
	U50412_at	Phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1	Pik3r1	U50412	43.8	1.3	***0.0***	***0.0***	0.5	0.8	*5.0*	14.0	*2.6*	*0.5*	*2.1*	3.4	M		30,31,32
	AB015191_g_at	Rhesus blood group	Rh	AB015191	*22.2*	1.4	***0.3***	***0.3***	*0.5*	0.6	*14.1*	*0.5*	4.6	4.8	40.7	4.8	E		Entrez gene

Group	Probe ID	Annotation	Symbol	GenBank	Whole liver						Isolated Kupffer cells						Cell type	Predicted classification	Ref.
					D 0	4	7	14	21	28	d 0	4	7	14	21	28			
	M94918mRNA_f_at	Hemoglobin beta chain complex/ beta-globin	Hbb	M94918	3283.0	1.4	**0.5**	**0.5**	0.7	0.6	1212.2	0.3	2.0	2.7	4.8	2.9	E		OMIM
	J04793_at	Solute carrier family 4(anion exchanger), member 1	Slc4a1	J04793	58.0	1.2	0.7	**0.6**	1.0	0.7	29.0	1.4	3.7	3.1	18.6	2.8	E		Entrez gene
	U77697_at	Platelet-endothelial cell adhesion molecule/CD31	Pecam	U77697	45.5	0.7	**0.6**	0.9	0.8	0.8	821.2	0.3	0.3	0.2	0.3	0.3	M/L /E		OMIM
2	rc_AI009658_at	Chemokine, CC motif, ligand 5/secreted; RANTES	Ccl5	AI009658	*33.2*	*0.1*	1.8	**3.1**	1.4	*0.3*	456.2	0.5	0.4	1.6	1.1	0.3	L	T cell	OMIM
	rc_AA957923_at	Mast cell protease 2	Mcpt2	AA957923	*10.4*	*1.1*	3.3	**12.3**	8.7	5.8	23.8	2.9	3.8	89.6	45.0	43.7	M	mast cell	33,34
	U67914_at	Carboxypeptidase A3	Cpa3	U67914	19.3	*0.8*	1.0	**3.5**	2.7	1.6	*24.6*	1.4	2.0	31.4	13.7	19.6	M	mast cell	35,36,37
	U67911_s_at	Mast cell protease 9 or mast cell protease 8/ mast cell protease 8 precursor (RMCP-8)	"Mcpt9/ Mcpt8"	U67911	*28.9*	*0.6*	2.2	**4.7**	3.0	2.1	81.2	1.2	2.9	24.9	13.4	8.4	M	mast cell	Entrez gene
	U67908_at	Chymase 1, mast cell	Cma1	U67908	*35.2*	*1.0*	*1.2*	**1.8**	1.5	*1.2*	*32.4*	*1.2*	*1.4*	16.1	11.3	6.7	M	mast cell	Entrez gene
	rc_AA957003_at	S100 calcium binding protein A8/ calgranulin A	S100a8	AA957003	6.5	7.8	3.2	**4.2**	1.1	1.4	31.6	6.2	2.6	8.9	7.4	1.0	M/ Leu		38,39
	L18948_at	S100 calcium binding protein A9/ calgranulin B	S100a9	L18948	16.9	5.6	*0.9*	**2.6**	*1.0*	*1.1*	48.9	6.7	4.4	8.9	10.7	1.3	M/ Leu		40
	U31598_s_at	Major histocompatibility complex, class II, DM alpha (RT1.DMa)	Hla-dma	U31598	98.1	1.9	2.5	**2.6**	**2.6**	2.2	172.1	4.5	3.3	4.9	4.3	6.6	M/L	Kupffer cell, mast cell	Entrez gene
	U31599_at	Major histocompatibility complex, class II, DM beta (RT1.DMb)	Hla-dmb	U31599	20.7	2.3	4.3	3.5	**4.4**	2.8	33.5	8.3	8.7	8.8	14.4	15.5	M/L	Kupffer cell, mast cell	Entrez gene
	L06040_s_at	Arachidonate 12-lipoxygenase	Alox12	L06040	99.8	1.7	*0.2*	***0.1***	*0.3*	0.5	*9.2*	*3.3*	*3.0*	7.6	35.1	*2.5*	Leu/ P		41,42,43
3	X06916_at	S100 calcium-binding protein A4 Mts1	S100a4	X06916	18.2	7.1	2.7	1.8	2.2	**3.1**	132.0	9.5	1.5	4.0	2.8	2.3	M/L		45
	M28671_at	Similar to Ig gamma-2B chain C region (rearranged IgG-2b)	---	M28671	*14.7*	*0.6*	*0.8*	*1.0*	**3.3**	*0.7*	61.7	3.3	5.8	13.9	3.9	3.8	L	B cell	
	rc_AI234828_g_at	Immunoglobulin heavy chain, alpha polypeptide	Igha	AI234828	28.8	0.6	1.0	1.7	2.0	**2.4**	905.3	0.5	0.9	0.9	0.4	0.7	L	B cell	
	X53517_at	CD37 antigen	Cd37	X53517	*32.5*	2.7	1.9	2.1	**2.3**	**2.3**	105.9	3.2	1.8	3.6	2.7	2.1	L	B cell	OMIM
	X58294_at	Carbonic anhydrase 2	Ca2	X58294	63.8	2.0	0.8	1.0	1.6	**2.1**	*36.7*	0.4	7.2	5.8	35.8	6.3	M/ L/E		46,47
	AF072411_g_at	CD36 antigen	Cd36	AF072411	67.0	1.3	1.2	0.9	1.2	**1.8**	77.9	9.4	4.0	2.8	3.5	4.6	M/E		OMIM
	U75689_s_at	Deoxyribonuclease I-like 3/DNase gamma	Dnase1l3	U75689	106.6	0.1	1.1	1.1	0.5	**0.2**	2583.7	0.1	0.2	0.2	0.2	0.1	M		48
	X73371_at	Fc receptor, IgG, low affinity IIb/Fc gamma receptor	Fcgr2b	X73371	190.7	0.5	0.8	1.1	0.7	**0.5**	2913.7	0.5	0.4	0.4	0.4	0.2	M/ L/ Leu	B cell, mast cell	OMIM
	L04672_s_at	Adrenomedullin receptor	Admr	L04672	147.0	0.5	0.8	0.9	0.7	**0.4**	2004.8	0.2	0.3	0.2	0.4	0.1	L	T cell possibly	49,50
4	AF041083_at	Solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1	Slc11a1	AF041083	*15.1*	2.7	*0.7*	*0.9*	*1.0*	*0.9*	77.2	1.8	1.6	4.6	2.0	2.1	M		Entrez gene
	D14015_g_at	Cyclin E	CCNE1	D14015	27.1	1.5	1.3	0.9	0.9	1.0	37.6	1.4	2.0	1.5	7.6	1.3	M		
	U31367_at	Myelin and lymphocyte protein/ myelin protein MVP17	Mal	U31367	*19.9*	2.4	*1.0*	1.1	1.2	*0.7*	32.2	14.2	2.4	3.9	2.2	1.8	L	T cell	51

Expression profiles of the whole liver and the isolated Kupffer cell fraction during fibrogenesis were obtained using a rat Genome U34A Array (Affymetrix). Marker genes of hematopoietic cells in the Kupffer cell fraction are listed. Expression intensities are shown for d 0, and the expression intensity data for d 4, 7, 14, 21 and 28 are displayed as ratios to the d 0 expression levels. Italics indicate an "absent" call by the Affymetrix software. Bold values indicate the highest or lowest ratio in the chronic phase (d 7, 14, 21 and 28). □ between 0.667 and 1.5; ■ ⩾ 1.5; ■ ⩽ 0.667. The cell types in the inflammatory cell fraction are as follows: M: Monocytes and their progenitors; L: Lymphocytes and their progenitors; E: Erythrocytes and their progenitors; P: Platelets and their progenitors; Leu: Other kinds of leukocytes and their progenitors.

Table 5 References used in the definition of types of hematopoietic cells in the Kupffer cell fraction

No.	Reference
1	**Tordjmann T**, Soulie A, Guettier C, Schmidt M, Berthou C, Beaugrand M, Sasportes M. Perforin and granzyme B lytic protein expression during chronic viral and autoimmune hepatitis. *Liver* 1998; **18**: 391-397
2	**Lautenschlager I**. Characteristics of the strongly Ia-positive cells in rat liver. *Scand J Immunol* 1984; **20**: 333-338
3	**Boisclair J**, Dore M, Beauchamp G, Chouinard L, Girard C. Characterization of the inflammatory infiltrate in canine chronic hepatitis. *Vet Pathol* 2001; **38**: 628-635
4	**Ahlenstiel G**, Woitas RP, Rockstroh J, Spengler U. CC-chemokine receptor 5 (CCR5) in hepatitis C--at the crossroads of the antiviral immune response? *J Antimicrob Chemother* 2004; **53**: 895-898
5	**Eis V**, Luckow B, Vielhauer V, Siveke JT, Linde Y, Segerer S, De Lema GP, Cohen CD, Kretzler M, Mack M, Horuk R, Murphy PM, Gao JL, Hudkins KL, Alpers CE, Grone HJ, Schlondorff D, Anders HJ. Chemokine receptor CCR1 but not CCR5 mediates leukocyte recruitment and subsequent renal fibrosis after unilateral ureteral obstruction. *J Am Soc Nephrol* 2004; **15**: 337-347
6	**McCabe MJ Jr**, Dias JA, Lawrence DA. Lead influences translational or posttranslational regulation of Ia expression and increases invariant chain expression in mouse B cells. *J Biochem Toxicol* 1991; **6**: 269-276
7	**Wilson KM**, Labeta MO, Pawelec G, Fernandez N. Cell-surface expression of human histocompatibility leucocyte antigen (HLA) class II-associated invariant chain (CD74) does not always correlate with cell-surface expression of HLA class II molecules. *Immunology* 1993; **79**: 331-335
8	**Kiley SC**, Parker PJ. Differential localization of protein kinase C isozymes in U937 cells: evidence for distinct isozyme functions during monocyte differentiation. *J Cell Sci* 1995; **108** (Pt 3): 1003-1016
9	**Allen JN**, Liao Z, Moore SA, Wewers MD. Changes in mononuclear phagocyte microtubules after endotoxin stimulation. II. Changes in microtubule composition. *Am J Respir Cell Mol Biol* 1997; **16**: 127-132
10	**Devergne O**, Peuchmaur M, Humbert M, Navratil E, Leger-Ravet MB, Crevon MC, Petit MA, Galanaud P, Emilie D. In vivo expression of IL-1 beta and IL-6 genes during viral infections in human. *Eur Cytokine Netw* 1991; **2**: 183-194
11	**Thornton AJ**, Ham J, Kunkel SL. Kupffer cell-derived cytokines induce the synthesis of a leukocyte chemotactic peptide, interleukin-8, in human hepatoma and primary hepatocyte cultures. *Hepatology* 1991; **14**: 1112-1122
12	**Manthey CL**, Kossmann T, Allen JB, Corcoran ML, Brandes ME, Wahl SM. Role of Kupffer cells in developing streptococcal cell wall granulomas. Streptococcal cell wall induction of inflammatory cytokines and mediators. *Am J Pathol* 1992; **140**: 1205-1214
13	**Zhu XL**, Zellweger R, Zhu XH, Ayala A, Chaudry IH. Cytokine gene expression in splenic macrophages and Kupffer cells following haemorrhage. *Cytokine* 1995; **7**: 8-14
14	**Loike JD**, Kaback E, Silverstein SC, Steinberg TH. Lactate transport in macrophages. *J Immunol* 1993; **150**: 1951-1958
15	**Daberkow RL**, White BR, Cederberg RA, Griffin JB, Zempleni J. Monocarboxylate transporter 1 mediates biotin uptake in human peripheral blood mononuclear cells. *J Nutr* 2003; **133**: 2703-2706
16	**Merezhinskaya N**, Ogunwuyi SA, Mullick FG, Fishbein WN. Presence and localization of three lactic acid transporters (MCT1, -2, and -4) in separated human granulocytes, lymphocytes, and monocytes. *J Histochem Cytochem* 2004; **52**: 1483-1493
17	**Marra F**, Valente AJ, Pinzani M, Abboud HE. Cultured human liver fat-storing cells produce monocyte chemotactic protein-1. Regulation by proinflammatory cytokines. *J Clin Invest* 1993; **92**: 1674-1680
18	**Guc D**, Gulati P, Lemercier C, Lappin D, Birnie GD, Whaley K. Expression of the components and regulatory proteins of the alternative complement pathway and the membrane attack complex in normal and diseased synovium. *Rheumatol Int* 1993; **13**: 139-146
19	**Schwaeble W**, Huemer HP, Most J, Dierich MP, Strobel M, Claus C, Reid KB, Ziegler-Heitbrock HW. Expression of properdin in human monocytes. *Eur J Biochem* 1994; **219**: 759-764
20	**Yamanouchi S**, Kuwahara K, Sakata A, Ezaki T, Matsuoka S, Miyazaki J, Hirose S, Tamura T, Nariuchi H, Sakaguchi N. A T cell activation antigen, Ly6C, induced on CD4+ Th1 cells mediates an inhibitory signal for secretion of IL-2 and proliferation in peripheral immune responses. *Eur J Immunol* 1998; **28**: 696-707
21	**Manoussaka MS**, Smith RJ, Conlin V, Toomey JA, Brooks CG. Fetal mouse NK cell clones are deficient in Ly49 expression, share a common broad lytic specificity, and undergo continuous and extensive diversification in vitro. *J Immunol* 1998; **160**: 2197-2206
22	**Wrammert J**, Kallberg E, Agace WW, Leanderson T. Ly6C expression differentiates plasma cells from other B cell subsets in mice. *Eur J Immunol* 2002; **32**: 97-103
23	**Katz P**, Whalen G, Kehrl JH. Differential expression of a novel protein kinase in human B lymphocytes. Preferential localization in the germinal center. *J Biol Chem* 1994; **269**: 16802-16809
24	**Valent P**, Ashman LK, Hinterberger W, Eckersberger F, Majdic O, Lechner K, Bettelheim P. Mast cell typing: demonstration of a distinct hematopoietic cell type and evidence for immunophenotypic relationship to mononuclear phagocytes. *Blood* 1989; **73**: 1778-1785
25	**Titos E**, Claria J, Planaguma A, Lopez-Parra M, Villamor N, Parrizas M, Carrio A, Miquel R, Jimenez W, Arroyo V, Rivera F, Rodes J. Inhibition of 5-lipoxygenase induces cell growth arrest and apoptosis in rat Kupffer cells: implications for liver fibrosis. *FASEB J* 2003; **17**: 1745-1747
26	**Titos E**, Claria J, Planaguma A, Lopez-Parra M, Gonzalez-Periz A, Gaya J, Miquel R, Arroyo V, Rodes J. Inhibition of 5-lipoxygenase-activating protein abrogates experimental liver injury: role of Kupffer cells. *J Leukoc Biol* 2005; **78**: 871-878
27	**Sawano A**, Iwai S, Sakurai Y, Ito M, Shitara K, Nakahata T, Shibuya M. Flt-1, vascular endothelial growth factor receptor 1, is a novel cell surface marker for the lineage of monocyte-macrophages in humans. *Blood* 2001; **97**: 785-791
28	**Coppola S**, Narciso L, Feccia T, Bonci D, Calabro L, Morsilli O, Gabbianelli M, De Maria R, Testa U, Peschle C. Enforced expression of KDR receptor promotes proliferation, survival and megakaryocytic differentiation of TF1 progenitor cell line. *Cell Death Differ* 2006; **13**: 61-74
29	**Fernandez Pujol B**, Lucibello FC, Zuzarte M, Lutjens P, Muller R, Havemann K. Dendritic cells derived from peripheral monocytes express endothelial markers and in the presence of angiogenic growth factors differentiate into endothelial-like cells. *Eur J Cell Biol* 2001; **80**: 99-110
30	**Bowling WM**, Flye MW, Qiu YY, Callery MP. Inhibition of phosphatidylinositol-3'-kinase prevents induction of endotoxin tolerance in vitro. *J Surg Res* 1996; **63**: 287-292
31	**Capodici C**, Hanft S, Feoktistov M, Pillinger MH. Phosphatidylinositol 3-kinase mediates chemoattractant-stimulated, CD11b/CD18-dependent cell-cell adhesion of human neutrophils: evidence for an ERK-independent pathway. *J Immunol* 1998; **160**: 1901-1909
32	**Bracke M**, Nijhuis E, Lammers JW, Coffer PJ, Koenderman L. A critical role for PI 3-kinase in cytokine-induced Fcalpha-receptor activation. *Blood* 2000; **95**: 2037-2043
33	**Zweifel M**, Breu K, Matozan K, Renner E, Welle M, Schaffner T, Clavien PA. Restoration of hepatic mast cells and expression of a different mast cell protease phenotype in regenerating rat liver after 70%-hepatectomy. *Immunol Cell Biol* 2005; **83**: 587-595
34	**Pemberton AD**, Brown JK, Wright SH, Knight PA, Miller HR. The proteome of mouse mucosal mast cell homologues: the role of transforming growth factor beta1. *Proteomics* 2006; **6**: 623-631

No.	Reference
35	**Schwartz LB**. Analysis of MC(T) and MC(TC) mast cells in tissue. *Methods Mol Biol* 2006; **315**: 53-62
36	**Henningsson F**, Yamamoto K, Saftig P, Reinheckel T, Peters C, Knight SD, Pejler G. A role for cathepsin E in the processing of mast-cell carboxypeptidase A. *J Cell Sci* 2005; **118**: 2035-2042
37	**Chen ZQ**, He SH. Cloning and expression of human colon mast cell carboxypeptidase. *World J Gastroenterol* 2004; **10**: 342-347
38	**Lagasse E**, Clerc RG. Cloning and expression of two human genes encoding calcium-binding proteins that are regulated during myeloid differentiation. *Mol Cell Biol* 1988; **8**: 2402-2410
39	**Bhardwaj RS**, Zotz C, Zwadlo-Klarwasser G, Roth J, Goebeler M, Mahnke K, Falk M, Meinardus-Hager G, Sorg C. The calcium-binding proteins MRP8 and MRP14 form a membrane-associated heterodimer in a subset of monocytes/macrophages present in acute but absent in chronic inflammatory lesions. *Eur J Immunol* 1992; **22**: 1891-1897
40	**Ryckman C**, Vandal K, Rouleau P, Talbot M, Tessier PA. Proinflammatory activities of S100: proteins S100A8, S100A9, and S100A8/A9 induce neutrophil chemotaxis and adhesion. *J Immunol* 2003; **170**: 3233-3242
41	**Yoshimoto T**, Suzuki H, Yamamoto S, Takai T, Yokoyama C, Tanabe T. Cloning and expression of arachidonate 12-lipoxygenase cDNA from porcine leukocytes. *Adv Prostaglandin Thromboxane Leukot Res* 1991; **21**A: 29-32
42	**Gu JL**, Natarajan R, Ben-Ezra J, Valente G, Scott S, Yoshimoto T, Yamamoto S, Rossi JJ, Nadler JL. Evidence that a leukocyte type of 12-lipoxygenase is expressed and regulated by angiotensin II in human adrenal glomerulosa cells. *Endocrinology* 1994; **134**: 70-77
43	**Nakamura M**, Ueda N, Kishimoto K, Yoshimoto T, Yamamoto S, Ishimura K. Immunocytochemical localization of platelet-type arachidonate 12-lipoxygenase in mouse blood cells. *J Histochem Cytochem* 1995; **43**: 237-244
44	**Kaminski WE**, Jendraschak E, Baumann K, Kiefl R, Fischer S, Marcus AJ, Broekman MJ, von Schacky C. Human mononuclear cells express 12-LX: coordinated mRNA regulation with 5-LX and FLAP genes. *Blood* 1996; **87**: 331-340
45	**Takenaga K**, Nakamura Y, Sakiyama S. Expression of a calcium binding protein pEL98 (mts1) during differentiation of human promyelocytic leukemia HL-60 cells. *Biochem Biophys Res Commun* 1994; **202**: 94-101
46	**Yancopoulos GD**, Oltz EM, Rathbun G, Berman JE, Smith RK, Lansford RD, Rothman P, Okada A, Lee G, Morrow M. Isolation of coordinately regulated genes that are expressed in discrete stages of B-cell development. *Proc Natl Acad Sci USA* 1990; **87**: 5759-5763
47	**Quelo I**, Jurdic P. Differential regulation of the carbonic anhydrase II gene expression by hormonal nuclear receptors in monocytic cells: identification of the retinoic acid response element. *Biochem Biophys Res Commun* 2000; **271**: 481-491
48	**Baron WF**, Pan CQ, Spencer SA, Ryan AM, Lazarus RA, Baker KP. Cloning and characterization of an actin-resistant DNase I-like endonuclease secreted by macrophages. *Gene* 1998; **215**: 291-301
49	**Kitabatake Y**, Kawamura S, Yamashita M, Okuyama K, Takayanagi M, Ohno I. The expression of mRNA for calcitonin gene-related peptide receptors in a mucosal type mast cell line, RBL-2H3. *Biol Pharm Bull* 2004; **27**: 896-898
50	**Makino Y**, Nakamura H, Ikeda E, Ohnuma K, Yamauchi K, Yabe Y, Poellinger L, Okada Y, Morimoto C, Tanaka H. Hypoxia-inducible factor regulates survival of antigen receptor-driven T cells. *J Immunol* 2003; **171**: 6534-6540
51	**Frank M**. MAL, a proteolipid in glycosphingolipid enriched domains: functional implications in myelin and beyond. *Prog Neurobiol* 2000; **60**: 531-544

The reference numbers correspond to the numbers in Table 4 in the supplementary data.

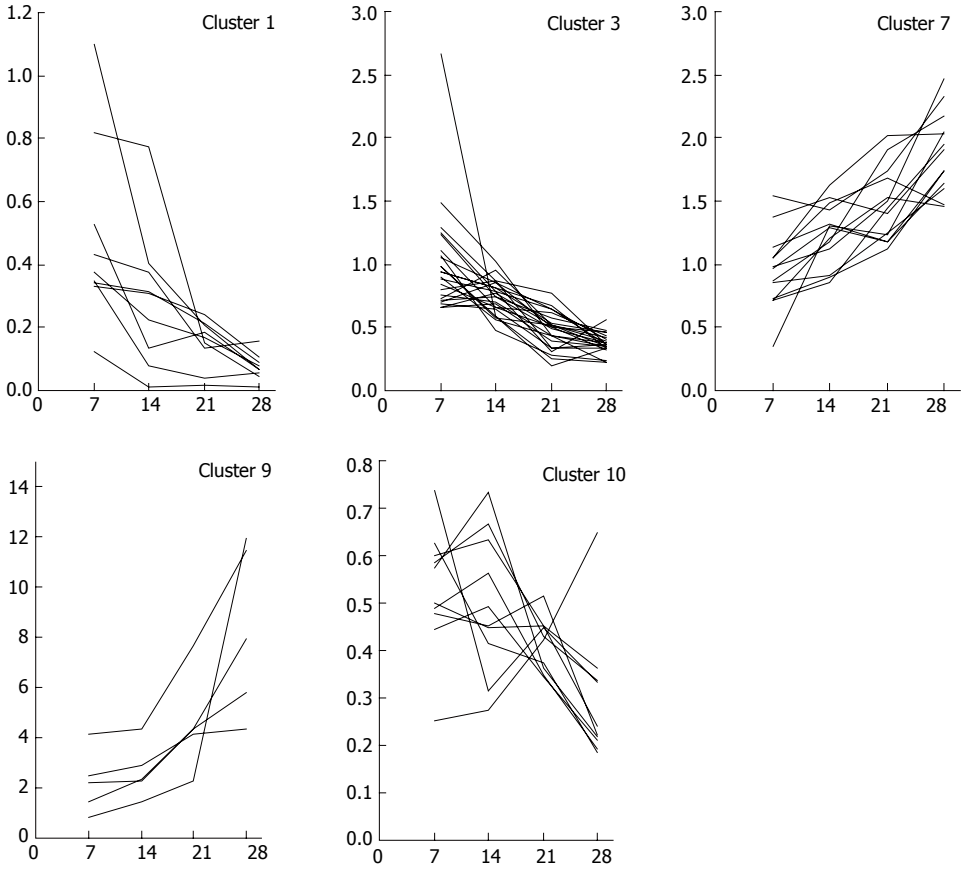

Figure 2 Cluster of genes that increased or decreased in expression with progression of fibrogenesis. Gene expression profiles in the chronic phase (d 7, 14, 21 and 28) were clustered into 10 patterns using K-means analysis. Clustered genes with a tendency to temporally decrease (clusters 1, 3 and 10) or increase (clusters 7 and 9) were selected as gene markers that had a strong relationship with fibrogenesis.

Table 6 Marker genes for hepatocytes

Probe ID	Functional category	Annotation	Symbol	GenBank	UniGene	Whole Liver						Isolated Hepatocytes					
						d 0	4	7	14	21	28	d 0	4	7	14	21	28
U68168_at	Amino acid metabolism	Kynureninase (L-kynurenine hydrolase)	Kynu	U68168	Rn.10575	487.4	0.4	1.0	1.2	0.9	1.2	981.6	0.5	0.8	0.5	0.5	0.4
Z50144_g_at		Aminoadipate aminotransferase	Aadat	Z50144	Rn.11133	336.1	0.4	0.7	0.9	0.9	1.0	360.3	0.6	0.9	0.7	0.6	0.8
AF056031_at		Kynurenine 3-monooxygenase (kynurenine 3-hydroxylase)	Kmo	AF056031	Rn.35029	292.1	0.4	0.9	1.0	0.9	1.1	483.1	0.6	0.5	0.7	0.5	0.5
D44494_at		3-hydroxyan-thranilate 3,4-dioxygenase	Haao	D44494	Rn.48675	1071.6	0.5	0.7	0.9	0.7	0.8	948.6	0.6	1.0	0.8	0.8	0.8
M84648mRNA_s_at		Dopa decarboxylase	Ddc	M84648		219.5	0.4	0.5	0.8	0.6	0.6	241.3	0.4	1.1	0.6	0.7	0.3
J02827_at		Branched chain ketoacid dehydrogenase E1, alpha polypeptide	Bckdha	J02827	Rn.3489	194.2	0.5	0.4	0.7	0.7	0.7	164.1	0.7	0.9	0.6	1.0	1.3
rc_AI168942_at		Branched chain keto acid dehydrogenase E1, beta polypeptide	Bckdhb	AI168942	Rn.15623	335.2	0.3	0.6	0.7	0.7	0.7	248.2	0.4	1.0	1.2	1.6	1.9
rc_AI102838_s_at		Isovaleryl coenzyme A dehydrogenase	Ivd	AI102838	Rn.147	345.5	0.4	0.6	0.5	0.7	0.8	439.6	0.6	0.7	0.6	0.7	0.7
M93401_at		Aldehyde dehydrogenase family 6, subfamily A1	Aldh6a1	M93401	Rn.2098	564.1	0.7	0.6	1.2	1.0	1.2	796.3	0.5	0.5	0.8	0.6	0.8
J05499_at		Glutaminase 2 (liver, mitochondrial)	Gls2	J05499	Rn.10202	254.0	0.6	0.9	0.9	0.8	1.3	410.8	0.6	0.4	0.6	0.4	2.1
D10354_s_at		Glutamic pyruvic transaminase 1, soluble	Gpt1	D10354	Rn.6318	229.0	0.6	0.9	0.9	1.2	1.6	201.7	0.9	0.9	1.2	1.0	4.1
J04171_at		Glutamate oxaloacetate transaminase 1	Got1	J04171	Rn.5819	262.9	1.0	0.7	0.9	1.1	1.7	342.5	0.7	0.8	0.8	0.6	4.9
M58308_at		Histidine ammonia lyase	Hal	M58308	Rn.10037	244.9	1.1	1.5	1.4	1.2	2.3	413.3	1.3	0.7	0.6	0.9	2.7
X13119cds_s_at		Serine dehydratase	Sds	X13119		425.9	0.7	0.7	1.1	0.7	1.2	116.7	0.6	0.2	1.2	0.3	23.0
rc_AA892112_g_at		Proline dehydrogenase (oxidase) 2 (predicted)	(Prodh2)	AA892112	Rn.4247	423.3	0.5	0.7	1.0	0.9	0.9	597.2	0.5	0.9	0.7	1.1	1.0
rc_AA892345_at		Dimethylglycine dehydrogenase precursor	Dmgdh	AA892345	Rn.3646	219.5	0.4	0.7	0.8	1.0	0.8	276.6	0.5	0.8	0.7	0.8	0.8
AF067650_at		Sarcosine dehydrogenase	Sardh	AF067650	Rn.37484	133.1	0.7	0.5	0.8	0.6	0.6	112.7	0.5	0.5	0.7	0.6	1.3
J03588_at		Guanidinoacetate methyltransferase	Gamt	J03588	Rn.33890	2017.0	0.3	0.7	0.8	0.6	0.7	1684.6	0.4	1.0	0.8	1.0	0.7
X06150cds_at		Glycine N-methyltransferase	Gnmt	X06150	Rn.11142	605.4	0.2	0.7	0.6	0.5	0.6	801.9	0.3	0.5	0.5	0.3	0.8
X15734_at		Methionine adenosyl-transferase I, alpha	Mat1a	X15734	Rn.10418	1290.3	0.5	0.5	0.6	0.6	0.5	438.8	0.6	0.6	0.6	0.8	2.9
AF038870_at		Betaine-homocysteine methyltransferase	Bhmt	AF038870	Rn.11406	2239.0	0.3	0.7	0.8	0.8	0.8	3346.2	0.3	0.7	0.8	0.7	0.8
M59861_at		Formyltetra-hydrofolate dehydrogenase	Fthfd	M59861	Rn.2328	554.2	0.5	0.9	1.3	1.4	1.0	924.3	0.5	1.0	0.9	1.0	1.0
rc_AA942685_at		Cytosolic cysteine dioxygenase 1	Cdo1	AA942685	Rn.2589	1815.9	0.4	0.9	0.8	0.5	0.5	1960.7	0.4	0.6	0.6	0.6	0.5
M64755_at		Cysteine sulfinic acid decarboxylase	Csad	M64755	Rn.43232	394.3	0.1	0.4	0.5	0.3	0.2	403.3	0.2	0.7	0.3	0.6	0.1
rc_AI012802_at		Hydroxyacyl glutathione hydrolase	Hagh	AI012802	Rn.11048	537.6	0.6	0.6	0.9	0.7	0.9	657.2	0.5	0.8	0.7	0.7	0.8

Probe ID	Functional category	Annotation	Symbol	GenBank	UniGene	Whole Liver						Isolated Hepatocytes					
						d 0	4	7	14	21	28	d 0	4	7	14	21	28
E01415cds_s_at		Glutathione S-transferase, mu type 3	Gstm3	E01415		314.8	0.2	0.7	0.5	0.5	0.6	456.3	0.2	0.4	0.5	0.4	0.5
rc_AI235747_at		Glutathione transferase YA subunit	Gsta5	AI235747	Rn.10460	77.2	0.6	2.1	1.5	1.2	0.8	94.0	0.7	0.8	1.3	0.8	0.3
rc_AA945082_at		Glutathione-S-transferase, alpha type2	Gsta2	AA945082	Rn.40574	10.3	2.0	2.2	2.3	4.4	7.9	24.1	1.4	2.5	3.1	2.2	2.6
K03041mRNA_s_at		Ornithine transcarbamylase	Otc	K03041		219.7	0.4	0.7	1.0	0.8	0.9	259.2	0.4	0.5	0.8	0.4	0.5
X12459_at		Arginosuccinate synthetase	Ass	X12459	Rn.5078	2507.3	0.6	0.7	0.7	0.7	0.8	2828.7	0.3	0.6	0.6	0.5	1.4
J03959_g_at		Urate oxidase	Uox	J03959	Rn.11330	74.9	0.4	0.4	0.7	0.7	0.6	67.0	0.4	0.9	0.7	1.0	1.0
M33648_at	Cholesterol synthesis & bile acid synthesis	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2	Hmgcs2	M33648	Rn.29594	3672.7	0.4	0.5	0.7	0.5	0.5	3097.5	0.6	0.8	0.6	0.9	0.8
rc_AI180442_at		Farensyl diphosphate synthase	Fdps	AI180442	Rn.2622	193.8	0.2	0.6	0.6	0.5	0.5	212.0	0.4	1.2	0.8	1.6	0.2
M95591_at		Farnesyl diphosphate farnesyl transferase 1	Fdft1	M95591	Rn.3252	594.7	0.2	0.3	0.6	0.4	0.5	480.9	0.4	0.8	0.7	0.9	0.2
AB016800_at		7-dehydro-cholesterol reductase	Dhcr7	AB016800	Rn.228	198.3	0.3	0.7	1.1	0.8	1.1	312.6	0.6	0.9	0.8	1.0	0.4
D86745exon_s_at		Nuclear receptor subfamily 0, group B, member 2	Nr0b2	D86745		201.3	0.2	0.6	0.4	0.4	0.2	147.5	0.4	0.3	0.4	0.6	0.5
D14989_f_at		Similar to Alcohol sulfotransferase (Hydroxysteroid sulfotransferase) (ST) (ST-60)	LOC361510	D14989	Rn.40365	334.8	0.3	1.0	0.9	0.4	1.4	372.1	1.0	0.7	1.5	0.8	0.5
D43964_at		Bile acid-Coenzyme A: amino acid N-acyltransferase	Baat	D43964	Rn.11129	743.8	0.5	1.5	1.2	0.9	0.7	1038.5	0.8	0.8	0.6	0.8	0.6
E12625cds_at		Sterol-C4-methyl oxidase-like	Sc4mol	E12625		579.8	0.1	0.2	0.5	0.2	0.3	452.9	0.1	0.4	0.4	0.8	0.1
L16995_at		Sterol regulatory element binding factor 1	Srebf1	L16995		116.7	0.7	1.0	1.5	1.2	1.6	227.6	0.2	0.6	1.3	0.7	0.3
rc_AA866264_s_at	Steroid hormone synthesis & metabolism	Similar to 20-alpha -hydroxysteroid dehydrogenase	---	AA866264	Rn.14713	37.8	0.5	1.0	1.1	1.5	2.5	100.8	0.2	0.7	0.8	0.6	1.2
S76489_s_at		Sulfotransferase, estrogen preferring estrogen sulfotransferase	"Ste, ste2"	S76489		1787.7	0.3	0.7	0.9	0.5	0.3	1919.8	0.3	0.8	0.4	0.3	0.1
J05035_at		Steroid 5 alpha-reductase 1	Srd5a1	J05035	Rn.4620	483.3	0.4	1.1	1.0	0.7	1.1	1175.7	0.6	0.3	0.6	0.8	0.2
U72497_at		Fatty acid amide hydrolase	Faah	U72497	Rn.10619	493.0	0.5	0.7	0.9	0.7	0.6	439.6	0.5	1.0	0.7	1.1	0.7
D17310_s_at		3-alpha-hydroxysteroid dehydrogenase	LOC191574	D17310	Rn.10021	440.6	0.5	0.8	0.8	0.6	0.8	576.3	0.8	0.8	0.7	0.7	0.5
rc_AI105448_at		Hydroxysteroid 11-beta dehydrogenase 1	Hsd11b1	AI105448	Rn.888	2057.5	0.5	0.8	0.7	0.7	0.6	2047.0	0.3	0.9	0.6	0.8	0.7
X91234_at		Hydroxysteroid (17-beta) dehydrogenase 2	Hsd17b2	X91234	Rn.10515	1060.3	0.2	0.8	0.8	0.2	0.0	158.0	0.4	0.8	0.3	1.7	0.5
rc_AI101743_s_at		Hydroxysteroid (17-beta) dehydrogenase 4	Hsd17b4	AI101743	Rn.2082	151.5	0.9	0.5	1.1	0.7	0.9	267.3	0.5	0.4	0.8	0.5	1.1
U89280_at		Hydroxysteroid (17-beta) dehydrogenase 9	Hsd17b9	U89280	Rn.10857	901.9	0.6	0.7	0.9	1.4	1.9	734.2	1.4	0.7	2.0	1.6	3.3
rc_AA893495_at		Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 6	Serpina6	AA893495	Rn.2374	1880.6	0.2	0.9	0.8	0.6	0.6	2046.0	0.4	0.7	0.6	0.9	0.3

Probe ID	Functional category	Annotation	Symbol	GenBank	UniGene	Whole Liver						Isolated Hepatocytes					
						d 0	4	7	14	21	28	d 0	4	7	14	21	28
S80431_s_at		Aldo-keto reductase family 1, member D1	Akr1d1	S80431		284.2	0.7	0.5	1.4	0.8	1.0	786.7	0.3	0.2	0.8	0.3	0.9
rc_AI172293_at		Sterol-C4-methyl oxidase-like	Sc4mol	AI172293	Rn.7167	677.2	0.2	0.3	0.6	0.3	0.4	694.9	0.2	0.6	0.4	0.9	0.1
U89905_at	Lipid biosynthesis, fatty acid metabolism & lipid transport	Alpha-methylacyl-CoA racemase	Amacr	U89905	Rn.2590	590.0	0.1	0.6	0.7	0.4	0.2	303.5	0.9	0.9	0.5	0.9	0.6
J02749_at		Acetyl-Coenzyme A acyltransferase 1 (peroxisomal 3-oxoacyl-Coenzyme A thiolase)	Acaa1	J02749	Rn.8913	130.3	0.4	0.8	1.4	0.9	1.7	79.4	2.1	2.3	1.9	2.4	3.8
X95189_at		Acyl-Coenzyme A oxidase 2, branched chain	Acox2	X95189	Rn.10622	510.4	0.2	0.7	1.2	0.5	0.3	797.7	0.2	0.3	0.5	0.6	0.4
X95188_at		Acyl-Coenzyme A oxidase 3, pristanoyl	Acox3	X95188	Rn.10546	73.8	0.5	*0.4*	0.7	*0.6*	*0.4*	56.7	0.4	0.4	0.7	0.9	1.0
K03249_at		Enoyl-Coenzyme A, hydratase/ 3-hydroxyacyl Coenzyme A dehydrogenase	Ehhadh	K03249	Rn.3671	198.2	0.2	0.9	1.0	0.8	0.8	372.1	0.4	0.7	0.9	0.9	0.3
U64451_at		Acyl-Coenzyme A dehydrogenase, short/branched chain	Acadsb	U64451	Rn.44423	91.6	0.6	0.6	0.7	0.5	0.6	82.9	0.5	0.1	0.6	0.2	0.9
AF044574_g_at		2-4-dienoyl-Coenzyme A reductase 2, peroxisomal	Decr2	AF044574	Rn.7879	212.3	0.5	1.0	1.4	0.9	1.5	399.5	0.9	1.0	0.7	0.8	0.6
rc_AA893239_at		2-hydroxy-phytanoyl -Coenzyme A lyase	Hpcl2	AA893239	Rn.21425	480.5	0.1	0.4	0.4	0.1	0.2	236.1	0.5	0.3	0.3	0.4	0.6
rc_AI175764_s_at		Stearoyl-Coenzyme A desaturase 1	Scd1	AI175764	Rn.1023	52.7	0.2	0.7	1.5	1.7	0.8	47.2	0.1	2.6	5.3	2.3	0.1
rc_AA893242_g_at		Acyl-CoA synthetase long-chain family member 1	Acsl1	AA893242	Rn.6215	415.1	0.3	0.5	1.0	0.7	0.8	822.4	0.2	0.3	0.6	0.4	0.4
rc_AI171506_at		Malic enzyme 1	Me1	AI171506	Rn.3519	39.6	0.6	1.1	1.4	0.9	1.8	52.7	0.8	1.7	1.1	1.8	0.3
X70223_at		Peroxisomal membrane protein 2	Pxmp2	X70223	Rn.10292	1059.1	0.3	0.8	0.7	0.6	0.6	907.9	0.7	0.9	0.8	1.0	0.5
rc_AI232087_at		Hydroxyacid oxidase 2 (long chain)	Hao2	AI232087	Rn.10417	731.5	0.4	1.2	0.7	0.6	0.5	693.5	0.2	0.9	0.4	0.3	0.4
U10697_s_at		Carboxylesterase 1	Ces1	U10697	Rn.82692	574.2	0.4	1.2	0.8	0.7	0.6	691.0	0.9	0.9	0.6	1.1	0.4
M20629_s_at		Esterase 2	Es2	M20629	Rn.2549	2513.3	0.2	0.6	0.6	0.5	0.2	2429.2	0.5	0.7	0.6	1.0	0.0
AB010635_s_at		Carboxylesterase 2 (intestine, liver)	Ces2	AB010635	Rn.14535	42.1	9.7	4.2	4.4	7.7	11.5	45.3	15.2	4.9	7.0	10.1	15.6
L46791_at		Carboxylesterase 3	Ces3	L46791	Rn.34885	223.0	0.1	1.0	0.6	0.5	0.4	463.6	0.1	0.8	0.4	0.6	0.0
M16235_at		Lipase, hepatic	Lipc	M16235	Rn.1195	760.7	0.4	0.7	0.9	0.7	0.6	826.2	0.6	0.7	0.9	1.1	0.5
X03468_at		Apolipoprotein A-Ⅱ	Apoa2	X03468	Rn.10309	2543.4	0.3	0.8	0.6	0.8	0.7	3332.3	0.5	0.6	0.4	0.7	0.2
M00002_at		Apolipoprotein A-Ⅳ	Apoa4	M00002	Rn.15739	298.8	0.4	0.6	0.6	0.5	0.6	411.7	0.6	0.7	0.2	0.8	0.5
U53873cds_at		Apolipoprotein B	Apob	U53873		731.2	0.8	0.2	0.4	0.3	0.2	246.2	0.1	0.1	0.7	0.0	1.2
rc_AA945171_at		Apolipoprotein C-Ⅳ	Apoc4	AA945171	Rn.33157	720.3	0.7	0.6	0.9	0.7	0.8	1066.0	0.6	0.8	0.5	0.5	0.7
rc_AA893213_at		Apolipoprotein M	Apom	AA893213	Rn.262	1525.1	0.6	0.7	0.8	0.7	0.8	1912.6	0.8	0.9	0.6	0.8	0.6
U02096_at		Fatty acid binding Protein 7, brain	Fabp7	U02096	Rn.10014	190.4	0.3	1.1	0.5	0.3	0.2	129.4	0.7	0.8	0.3	0.3	0.1
U26033_at		Carnitine O-octanoyl-transferase	Crot	U26033	Rn.4896	100.7	0.9	1.7	2.3	1.9	2.0	142.9	1.9	2.2	2.8	1.1	1.9
K03045cds_r_at	Retiniod synthesis & metabolism	Retinol binding protein 4, plasma	Rbp4	K03045		3156.1	0.6	0.6	0.6	0.6	0.4	2863.7	0.5	0.6	0.6	0.9	0.6
U33500_g_at		Retinol dehydrogenase type Ⅱ (RODHⅡ)	RoDHII	U33500	Rn.37873	255.1	0.7	1.3	0.9	0.8	0.4	95.0	0.8	2.1	0.4	0.7	3.2
U18762_at		Retinol dehydrogenase type Ⅲ	Rdh3	U18762	Rn.31786	72.9	0.2	0.3	0.3	0.4	0.6	110.2	0.5	0.3	0.8	0.9	0.3

Probe ID	Functional category	Annotation	Symbol	GenBank	UniGene	Whole Liver						Isolated Hepatocytes					
						d 0	4	7	14	21	28	d 0	4	7	14	21	28
X53588_at	Glucolysis & Gluconeogenesis	Glucokinase	Gck	X53588	Rn.10447	56.7	0.5	0.4	1.3	1.2	2.0	218.3	0.1	0.3	0.3	0.1	0.1
M86235_at		Ketohexokinase	Khk	M86235		813.7	0.4	0.8	1.0	0.8	0.9	701.4	0.5	1.4	1.0	1.3	0.9
rc_AA945442_at		Glucokinase regulatory protein	Gckr	AA945442	Rn.7863	248.0	0.3	0.8	1.2	1.0	1.0	449.8	0.4	1.0	0.6	0.8	0.4
rc_AA892395_s_at		Aldolase B	Aldob	AA892395	Rn.10592	2821.0	0.5	0.8	0.9	0.7	0.8	3179.1	0.6	0.8	0.7	0.7	0.9
AB002558_at		Glycerol-3 -phosphate dehydrogenase 1 (soluble)	Gpd1	AB002558	Rn.44452	164.6	0.9	0.7	1.1	0.7	0.7	102.5	0.6	0.9	1.5	0.9	1.0
X05684_at		Pyruvate kinase, liver and RBC	Pklr	X05684		111.0	0.2	0.8	0.8	0.8	0.4	107.4	0.3	1.1	0.7	0.9	0.2
U32314_at		Pyruvate carboxylase	Pc	U32314	Rn.11094	398.3	0.3	0.7	0.8	0.5	0.4	456.4	0.5	0.8	0.5	0.8	0.5
X15580complete_seq_s_at		6-phosphofructo-2-kinase/ fructose-2,6-biphosphatase 1	Pfkfb1	X15580	Rn.10115	262.8	0.1	0.3	0.4	0.5	0.4	89.2	0.4	1.0	0.5	0.8	0.7
rc_AA892799_i_at		Glyoxylate reductase/ hydroxypyruvate reductase (predicted)	(Grhpr)	AA892799	Rn.7815	669.4	0.4	0.4	1.0	0.7	0.7	561.6	0.4	0.8	0.7	0.8	0.5
L37333_s_at		Glucose-6-phosphatase, catalytic	G6pc	L37333	Rn.10992	864.9	0.2	0.8	1.2	0.3	0.4	1915.9	0.4	0.5	0.6	1.0	0.6
X04069_at		Liver glycogen phosphorylase	Pygl	X04069	Rn.21399	129.7	0.5	1.0	1.4	1.1	0.8	248.9	0.5	0.8	0.7	0.7	0.3
K03243mRNA_s_at		Phosphoenol-pyruvate carboxykinase 1	Pck1	K03243		2955.1	0.5	0.7	0.6	0.3	0.3	2764.6	0.9	0.3	0.5	0.9	1.0
AF080468_at		Solute carrier family 37 (glycerol-6-phosphate transporter), member 4	Slc37a4	AF080468	Rn.1592	681.2	0.2	0.7	0.7	0.5	0.4	609.2	0.4	0.7	0.6	1.0	0.4
rc_AI030175_s_at		Sorbitol dehydrogenase	Sord	AI030175	Rn.11334	762.4	0.7	0.7	1.1	0.9	0.8	1108.8	0.5	0.6	0.8	0.7	0.6
D63704_g_at	Nucleotide-related enzymes	Dihydropy-rimidinase	Dpys	D63704	Rn.10586	388.7	0.4	0.7	0.7	0.5	0.5	466.5	0.6	0.7	0.7	0.8	0.3
M97662_at		Ureidopropionase, beta	Upb1	M97662	Rn.11110	1487.6	0.4	0.5	0.7	0.9	0.7	1151.1	0.4	1.0	0.8	1.1	0.8
D85035_at		Dihydropy-rimidine dehydrogenase	Dpyd	D85035	Rn.17564	230.4	0.3	0.8	0.7	0.8	1.0	238.1	0.5	0.9	0.9	0.5	1.7
AF041066_at		Ribonuclease, RNase A family 4	Rnase4	AF041066	Rn.22804	1617.5	0.3	1.0	0.9	0.6	0.4	1395.3	0.5	0.8	0.8	1.1	0.6
M57507_at		Guanylate cyclase 1, soluble, beta 2	Gucy1b2	M57507	Rn.10933	85.8	0.3	0.5	0.8	0.5	0.6	95.5	0.3	0.9	0.4	0.5	0.3
E01184cds_s_at	Drug-metabolism	Cytochrome P450, family 1, subfamily a, polypeptide 2	Cyp1a2	E01184		1518.5	0.1	0.3	0.3	0.2	0.1	1898.4	0.4	0.4	0.3	0.4	0.0
J04187_at		Cytochrome P450, subfamily 2A, polypeptide 1	Cyp2a2	J04187	Rn.9867	798.2	0.5	0.8	0.8	0.7	0.6	1022.0	0.4	0.8	0.6	0.6	0.5
K01721mRNA_s_at		Cytochrome P450, family 2, subfamily b, polypeptide 15	Cyp2b15	K01721	Rn.2287	462.6	0.2	0.7	0.4	0.3	0.7	1031.8	0.4	0.3	0.5	0.2	0.1
X79081mRNA_f_at		Cytochrome P450, subfamily IIC (mephenytoin 4-hydroxylase)	Cyp2c	X79081		591.2	0.0	0.4	0.2	0.2	0.1	411.3	0.1	0.2	0.1	0.1	0.1
J02861mRNA_s_at		Cytochrome P450 2c13	Cyp2c13	J02861	Rn.32070	1659.9	0.5	1.6	1.0	0.8	0.6	1914.7	0.5	0.9	0.7	0.6	0.7
AB008424_s_at		Cytochrome P450, family 2, subfamily d, polypeptide 13	Cyp2d13	AB008424	Rn.32106	1316.3	0.4	0.9	1.0	0.7	0.7	1998.3	0.5	0.8	0.5	0.8	0.5
AB008423_s_at		Cytochrome P450, family 2, subfamily d, polypeptide 26	Cyp2d26	AB008423	Rn.40137	3830.8	0.7	1.0	0.7	0.7	0.6	4520.5	0.7	0.7	0.6	0.8	0.7
S48325_s_at		Cytochrome P450, family 2, subfamily e, polypeptide 1	Cyp2e1	S48325		5249.5	0.4	0.6	0.5	0.5	0.5	6219.9	0.4	0.5	0.5	0.6	0.6

Probe ID	Functional category	Annotation	Symbol	GenBank	UniGene	Whole Liver						Isolated Hepatocytes					
						d 0	4	7	14	21	28	d 0	4	7	14	21	28
AF017393_at		Cytochrome P450, family 2, subfamily f, polypeptide 2	Cyp2f2	AF017393	Rn.10817	180.5	0.5	0.7	1.0	0.9	1.0	234.2	0.8	0.9	0.7	1.0	0.5
U40004_s_at		Cytochrome P450, family 2, subfamily j, polypeptide 9	Cyp2j9	U40004	Rn.37480	336.9	0.3	0.7	0.5	0.5	0.4	440.5	0.5	0.7	0.6	0.9	0.3
X62086mRNA_s_at		"Cytochrome P450, subfamily 3A, polypeptide 3 cytochrome P450, family 3, subfamily a, polypeptide 11 cytochrome P-450PCN (PNCN inducible)"	Cyp3a3, Cyp3a11, RGD:628626	X62086		3742.3	0.3	0.5	0.5	0.4	0.5	3344.0	0.5	0.5	0.6	0.4	0.6
M13646_s_at		Cytochrome P450, family 3, subfamily a, polypeptide 11	Cyp3a11	M13646	Rn.37424	2198.3	0.4	1.1	0.7	0.5	0.5	2356.8	0.8	0.7	0.6	0.5	0.1
U46118_at		Cytochrome P450, family 3, subfamily a, polypeptide 13	Cyp3a13	U46118	Rn.10489	37.7	*0.7*	1.5	1.4	1.7	2.3	28.7	2.8	2.2	1.7	2.2	7.3
D38381_s_at		Cytochrome P450, 3a18	RGD:628709	D38381	Rn.32085	444.8	0.6	1.9	1.0	0.9	1.3	682.8	0.8	0.9	0.7	0.4	1.1
U39206_at		Cytochrome P450 4F4	RGD:708363	U39206	Rn.10170	357.7	0.3	0.8	0.7	0.5	0.7	311.9	0.4	0.6	0.8	0.7	0.9
M94548_at		Cytochrome P450, family 4, subfamily F, polypeptide 2	Cyp4f2	M94548	Rn.5722	1637.4	0.6	1.0	0.9	0.8	0.8	2192.8	0.7	0.7	0.6	0.9	0.4
J05460_s_at		Cytochrome P450, family 7, subfamily a, polypeptide 1	Cyp7a1	J05460	Rn.10737	305.0	0.1	1.3	1.5	0.4	0.6	224.3	*0.1*	0.6	1.3	0.5	*0.1*
M21208mRNA_s_at		Cytochrome P450, family 17, subfamily a, polypeptide 1	Cyp17a1	M21208	Rn.10172	36.6	0.3	2.7	0.6	0.3	*0.2*	67.4	0.9	0.5	0.4	0.5	1.7
U17697_s_at		Cytochrome P450, subfamily 51	Cyp51	U17697	Rn.6150	355.0	0.2	0.5	0.7	0.5	0.5	660.8	0.4	0.9	0.6	1.2	0.1
M13506_at		Liver UDP-glucuronosy-ltransferase, phenobarbital-inducible form	Udpgtr2	M13506	Rn.9969	198.2	1.7	1.4	0.7	0.9	0.8	424.1	2.7	0.5	0.8	1.1	0.6
D38069exon_s_at		UDP glycosyl-transferase 1 family, polypeptide A6	Ugt1a6	D38069		63.8	0.6	0.5	1.4	0.9	0.8	83.9	0.6	0.6	1.7	1.0	0.5
rc_AA818122_f_at		Sulfotransferase family, cytosolic, 2A, dehydroepian-drosterone (DHEA)-preferring, member 1	Sth2	AA818122	Rn.2151?	1199.3	0.3	1.3	0.8	0.3	0.6	1674.5	0.9	0.3	0.7	0.5	0.3
L22339_g_at		Sulfotransferase family 1A, member 2	Sult1a2	L22339	Rn.9937	1303.6	0.4	1.1	0.9	0.7	0.8	1587.5	0.4	0.9	0.6	0.4	0.3
rc_AA926193_at		Sulfotransferase family, cytosolic, 1C, member 2	RGD:621064	AA926193	Rn.22471	119.3	0.2	0.7	0.5	0.6	0.5	99.8	0.2	1.0	0.5	0.4	0.2
X56228_g_at		Thiosulfate sulfurtransferase	Tst	X56228	Rn.6360	1476.9	0.4	0.5	0.8	0.7	0.7	1565.7	0.4	0.8	0.6	0.8	0.5
rc_AA892821_at		Aldo-keto reductase family 7, member A2 (aflatoxin aldehyde reductase)	RGD:620311	AA892821	Rn.8548	184.3	0.4	0.7	1.0	0.8	0.8	223.4	0.6	1.0	0.6	0.6	0.8
AF045464_s_at		Aldo-keto reductase family 7, member A3 (aflatoxin aldehyde reductase)	Akr7a3	AF045464	Rn.6043	337.0	1.5	2.0	1.6	1.4	1.7	240.8	2.9	2.1	1.6	2.0	2.6

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						d 0	4	7	14	21	28	d 0	4	7	14	21	28
X65083cds_at		Epoxide hydrolase 2, cytoplasmic	Ephx2	X65083	Rn.54495	44.5	0.8	0.8	1.6	0.6	0.2	22.3	2.6	2.5	1.3	0.2	4.3
AF001898_at		Aldehyde dehydrogenase family 1, member A1	Aldh1a1	AF001898	Rn.6132	304.5	4.1	2.5	2.9	4.1	4.4	752.5	2.2	1.4	1.2	1.5	2.9
M23995_g_at		Aldehyde dehydrogenase family 1, subfamily A4	Aldh1a4	M23995	Rn.74044	167.4	2.1	0.8	0.1	1.2	3.1	148.8	2.1	1.3	5.0	1.6	2.1
rc_AI172017_at		Aldehyde dehydrogenase 2	Aldh2	AI172017	Rn.2300	1125.0	0.5	0.7	0.7	0.6	0.6	1227.2	0.6	1.0	0.6	1.1	0.5
X90710_at		Alcohol dehydrogenase 4 (class II), pi polypeptide	Adh4	X90710	Rn.10302	201.0	0.6	1.5	1.0	0.4	0.4	182.8	0.9	0.5	0.5	0.6	0.5
rc_AA817846_at		3-hydroxy-butyrate dehydrogenase (heart, mitochondrial)	Bdh	AA817846	Rn.36635	574.7	0.3	0.9	0.8	0.7	0.3	723.4	0.3	0.4	0.2	0.7	0.3
rc_AA892382_at		Camello-like 1	Cml1	AA892382	Rn.3643	59.8	0.3	0.7	0.7	0.4	0.3	44.2	0.5	0.7	0.5	0.6	0.4
M26125_at		Epoxide hydrolase 1	Ephx1	M26125	Rn.3603	1661.4	1.7	1.8	1.6	1.5	1.7	2083.7	2.3	1.6	1.8	2.2	1.7
M84719_at		Flavin containing monooxygenase 1	Fmo1	M84719	Rn.867	320.7	0.0	0.5	0.1	0.2	0.1	466.1	0.0	0.5	0.1	0.1	*0.0*
rc_AA817964_s_at		Paraoxonase 1	Pon1	AA817964	Rn.20732	4497.8	0.4	0.8	0.7	0.7	0.6	4715.3	0.5	0.7	0.7	0.8	0.5
M31363mRNA_f_at		Sulfotransferase family, cytosolic, 2A, dehydroepian-drosterone (DHEA) -preferring, member 1	Sth2	M31363	Rn.2151	2071.8	0.3	1.0	0.6	0.2	0.3	2228.8	0.7	0.3	0.4	0.4	0.3
M11670_at	Anti-oxidant enzymes	Catalase	Cat	M11670	Rn.3001	697.4	0.4	1.4	0.9	0.7	0.7	723.0	0.6	0.8	0.9	0.8	0.5
U94856_at		Paraoxonase 1	Pon1	U94856	Rn.20732	3127.6	0.4	0.7	0.7	0.6	0.6	3370.6	0.5	0.7	0.7	0.8	0.5
M15481_at	Growth factors & their receptors	Insulin-like Growth factor 1	Igf1	M15481	Rn.6282	534.3	0.4	0.3	0.6	0.4	0.4	367.7	0.6	0.7	0.6	0.7	0.6
M58634_at		Insulin-like Growth factor binding protein 1	Igfbp1	M58634	Rn.34026	521.1	1.8	0.7	0.3	0.4	0.3	914.9	1.8	0.3	0.3	0.9	0.9
J04486_at		Insulin-like growth factor binding protein 2	Igfbp2	J04486	Rn.6813	17.4	2.4	1.5	2.3	4.3	5.8	13.1	6.0	3.3	4.9	5.2	14.6
rc_AA924289_s_at		Insulin-like growth factor binding protein, acid labile subunit	Igfals	AA924289	Rn.7327	217.0	0.3	0.6	0.8	0.5	0.6	234.0	0.3	0.5	0.8	0.8	0.4
AF089825_at		Inhibin beta E	Inhbe	AF089825	Rn.30020	156.2	0.3	0.8	0.9	0.3	0.4	147.0	0.6	1.2	0.8	1.1	0.2
S49003_s_at		Growth hormone receptor	Ghr	S49003		1081.0	0.2	0.5	0.4	0.5	0.4	1080.8	0.2	0.7	0.7	0.9	0.4
AF076619_at		Growth factor receptor bound protein 14	Grb14	AF076619	Rn.30028	210.0	0.7	0.6	0.7	0.4	0.6	221.9	0.8	0.8	0.8	0.5	0.6
M32167_g_at		Vascular endothelial growth factor A	Vegfa	M32167	Rn.1923	42.1	1.0	0.6	0.7	0.4	0.5	36.3	0.8	0.4	0.8	0.7	0.7
M37394_at		Epidermal growth factor receptor	Egfr	M37394	Rn.37227	134.6	0.4	0.6	0.7	0.5	0.4	116.6	0.7	0.8	0.6	0.9	0.5
L48060_s_at		Prolactin receptor	Prlr	L48060	Rn.9757	64.0	0.3	0.6	0.8	1.0	1.5	133.3	0.5	0.5	0.8	1.3	1.1
rc_AA892251_at		Arginine vasopressin receptor 1A	Avpr1a	AA892251	Rn.32282	204.4	0.1	0.4	1.0	1.0	0.6	455.0	0.3	0.7	0.6	1.1	*0.1*
L32132_at		Lipopoly-saccharide binding protein	Lbp	L32132	Rn.48863	54.1	3.1	0.9	1.2	1.9	2.2	39.4	8.1	1.5	1.9	3.1	5.7
L13025UTR#1_f_at		Polymeric immunoglobulin receptor	Pigr	L13025		421.3	0.5	0.5	0.5	0.5	0.7	1974.1	0.6	0.6	0.8	0.8	0.7
D14869_s_at		Prostaglandin E receptor 3 (subtype EP3)	Ptger3	D14869	Rn.10361	72.3	0.7	0.5	0.5	0.3	0.4	46.6	0.4	0.4	0.5	0.5	0.5
K01934mRNA#2_at		Thyroid hormone responsive protein	Thrsp	K01934		1556.7	0.2	0.8	1.0	0.8	0.7	1201.3	0.1	1.1	1.0	1.4	0.1
X57999cds_at		Deiodinase, iodothyronine, type I	Dio1	X57999	Rn.42914	139.8	0.4	0.6	0.7	0.4	*0.3*	168.8	0.4	0.5	0.2	0.6	*0.2*

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						d 0	4	7	14	21	28	d 0	4	7	14	21	28
X76456cds_at	Hepatic secretory proteins	Afamin	Afm	X76456		3150.5	0.3	0.8	0.8	0.7	0.5	3565.8	0.5	0.8	0.9	0.9	0.3
X02361_at		Alpha-fetoprotein	Afp	X02361	Rn.9174	*46.5*	*0.4*	*0.7*	1.2	1.5	1.5	35.4	0.5	1.8	1.2	1.3	0.6
rc_AA817854_s_at		Ceruloplasmin	Cp	AA817854	Rn.32777	418.5	0.9	0.5	1.1	0.8	0.8	362.8	1.1	0.9	1.6	0.5	1.1
X86178mRNA_g_at		Alpha-2-glycoprotein 1, zinc	Azgp1	X86178		871.9	0.5	0.7	0.7	0.7	0.8	1252.1	0.5	0.6	0.9	0.8	0.9
M27434_s_at		Alpha-2u globulin PGCL1	LOC259246	M27434		6719.1	0.2	1.1	0.4	0.2	0.1	4046.4	0.6	0.8	0.1	0.5	0.0
J00738_s_at		Alpha-2u globulin PGCL4	RGD:708508	J00738	Rn.81155	527.0	0.0	0.1	0.0	0.0	*0.0*	53.4	0.1	0.2	0.1	0.1	0.2
X51615_at		Pregnancy-zone protein	Pzp	X51615		55.0	0.7	0.9	0.8	0.6	0.8	63.3	0.6	0.6	2.0	0.9	0.4
rc_AA945608_at		Serum amyloid P-component	Sap	AA945608	Rn.1902	1278.3	0.6	0.8	0.7	0.7	0.7	1286.9	0.9	0.9	0.6	0.9	0.6
rc_AI102562_at		Metallothionein	Mt1a	AI102562	Rn.54397	8150.5	0.6	0.5	0.5	0.5	0.2	2209.3	2.2	0.6	0.6	1.2	1.2
X86561cds#2_at	Blood-function	Fibrinogen, alpha Polypeptide	Fga	X86561		1512.9	0.8	0.5	0.6	0.6	0.5	1223.6	1.8	1.1	1.0	0.9	0.5
D21215cds_s_at		Coagulation factor X	F10	D21215		641.3	0.4	0.6	0.9	0.7	0.8	850.3	0.6	0.7	0.9	1.0	0.6
U20194_g_at		Complement component 8, beta polypeptide	C8b	U20194	Rn.10152	1279.6	0.5	0.5	0.6	0.5	0.4	954.2	0.7	0.7	0.5	1.0	0.6
M62832_at		Plasminogen	Plg	M62832		1783.6	0.5	0.5	0.7	0.7	0.6	1618.8	0.5	0.8	0.9	1.2	0.5
M12112mRNA#3_s_at		Angiotensinogen	Agt	M12112		780.9	0.5	0.6	0.9	0.8	0.7	1279.3	0.6	1.0	0.7	1.1	0.9
L00117_at	Protease & protease inhibitors	Elastase 1, pancreatic	Ela1	L00117		132.6	0.0	0.3	0.1	0.0	0.1	74.5	*0.2*	0.3	*0.1*	*0.0*	*0.0*
rc_AI230712_at		Subtilisin-like endoprotease	Pace4	AI230712	Rn.950	63.8	1.0	0.5	0.9	1.2	1.1	78.2	1.2	0.9	0.5	1.0	1.0
AF097723_s_at		Plasma glutamate carboxypeptidase	Pgcp	AF097723	Rn.17112	519.4	0.3	0.6	0.6	0.4	0.4	464.5	0.4	0.8	0.6	0.7	0.4
X70900_at		Hepsin	Hpn	X70900	Rn.11139	799.9	0.5	0.7	0.8	0.7	0.7	792.6	0.6	0.8	0.7	1.0	0.7
rc_AA946503_at		Lipocalin 2	Lcn2	AA946503	Rn.11303	26.4	4.0	*0.8*	1.4	2.3	12.0	7.1	126.5	5.2	35.9	4.3	157.1
X69834_at		Serine (or cysteine) proteinase inhibitor, clade A, member 3M	Serpina3m	X69834	Rn.10424	875.6	0.5	0.8	0.8	1.0	1.0	752.4	1.2	0.5	1.6	2.6	0.5
D00752_at		Serine protease inhibitor	Spin2a	D00752	Rn.34396	5647.3	0.3	0.8	0.7	0.6	0.4	6388.0	0.4	0.7	0.5	0.8	0.1
M35299_s_at		Serine protease inhibitor, Kazal type 1	Spink1	M35299	Rn.9767	55.7	0.6	0.8	0.7	0.4	0.5	39.6	0.5	0.3	0.7	0.1	1.1
X16273cds_at		Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	Serpina1	X16273		6888.3	0.6	1.0	0.7	0.6	0.6	6788.4	1.0	0.8	0.6	0.7	0.9
rc_AA893552_at		Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 4	Serpina4	AA893552	Rn.11152	813.0	0.4	0.7	0.6	0.7	0.6	809.8	0.5	1.0	0.6	1.0	0.4
M63991_at		Serine (or cysteine) peptidase inhibitor, clade A (alpha-1 antipeptidase, antitrypsin), member 7	Serpina7	M63991	Rn.9948	25.6	1.8	2.2	1.5	3.8	1.6	10.7	7.1	7.9	4.6	1.8	1.6
M22993cds_s_at		"Murinoglobulin 1 homolog (mouse) alpha-1-inhibitor III similar to Murinoglobulin 1 homolog murinoglobulin 2"	"Mug1, LOC297568, LOC297572, RGD:1302962"	M22993		979.0	0.5	0.3	0.8	0.5	0.5	1427.7	0.1	0.1	0.6	0.2	0.6
V01216_at	Cell surface proteins & structural proteins	Orosomucoid 1	Orm1	V01216	Rn.10295	1695.5	1.5	1.3	1.0	1.2	1.3	1097.2	3.1	1.9	2.3	2.2	4.6
X05023_at		Mannose-binding protein C (liver)	Mbl2	X05023	Rn.9667	107.8	0.5	0.8	1.0	0.8	0.7	179.0	0.6	0.9	0.8	0.9	0.7
AF080507_at		Mannose-binding protein mRNA	---	AF080507		462.7	0.6	1.2	1.3	1.2	1.1	739.8	0.7	1.0	1.2	1.1	0.6
K02817cds_s_at		Asialoglycoprotein receptor 1	Asgr1	K02817		544.5	0.8	0.6	0.7	0.6	0.6	661.3	1.0	0.8	0.6	0.9	0.7

Probe ID	Functional category	Annotation	Symbol	GenBank	UniGene	Whole Liver						Isolated Hepatocytes					
						d 0	4	7	14	21	28	d 0	4	7	14	21	28
U82612cds_g_at		Fibronectin 1	Fn1	U82612		626.7	1.0	0.4	0.8	0.6	0.5	607.1	0.4	0.4	0.8	0.8	0.6
M81687_at		Syndecan 2	Sdc2	M81687	Rn.11127	438.7	0.4	0.7	0.9	0.7	0.6	542.6	0.6	0.7	0.8	0.7	0.3
AF090134_at		Lin-7 homolog a (C. elegans)	Lin7a	AF090134	Rn.31766	86.2	0.8	0.6	0.9	0.5	0.7	69.9	1.2	1.4	1.0	0.8	0.5
X04070_at		Gap junction membrane channel protein beta 1	Gjb1	X04070	Rn.10444	1214.5	0.5	0.5	0.6	0.6	0.5	1377.4	0.4	0.7	0.6	1.0	0.6
rc_AA799879_at		Synaptogyrin 1	Syngr1	AA799879	Rn.11188	75.1	0.6	0.6	0.7	0.6	0.5	60.0	1.0	0.7	0.6	0.8	1.1
S76054_s_at		Keratin complex 2, basic, gene 8	Krt2-8	S76054		305.6	2.3	1.0	1.3	1.2	1.7	345.3	2.8	1.6	1.6	1.4	2.0
rc_AI072634_at		Keratin complex 1, acidic, gene 18	Krt1-18	AI072634	Rn.3603	417.8	1.9	1.1	1.3	1.2	1.6	384.1	3.7	2.2	1.8	1.8	3.3
X59864mRNA_at		H19 fetal liver mRNA	H19	X59864		3.3	17.7	7.5	7.8	48.5	21.2	1.3	1.0	52.8	86.2	1.2	1.1
D31662exon#4_s_at	Signal transduction & transcription factors	Regucalcin	Rgn	D31662		865.7	0.2	0.7	1.0	0.5	0.4	863.6	0.2	1.2	0.7	0.6	0.1
rc_AA893485_at		RAB10, member RAS oncogene family	Rab10	AA893485	Rn.65864	90.1	0.5	0.5	1.3	0.7	0.7	68.0	0.3	1.3	0.6	1.0	0.4
U68544_at		Peptidylprolyl isomerase F (cyclophilin F)	Ppif	U68544	Rn.2923	276.1	0.4	0.7	0.9	0.7	1.1	399.5	0.4	0.5	0.5	0.4	1.1
rc_AA799560_at		N-myc downstream regulated gene 2	Ndrg2	AA799560	Rn.3407	799.7	0.4	1.0	1.2	0.8	0.7	1329.5	0.5	0.6	0.6	0.6	0.4
rc_AA891194_s_at		Arg/Abl-interacting protein ArgBP2	Argbp2	AA89119	Rn.24612	35.9	1.3	2.0	1.4	1.5	2.0	38.5	4.3	2.1	1.9	2.3	1.5
X57133mRNA_at		Rat mRNA for hepatocyte nuclear factor 4	HNF4	X57133		214.9	0.9	0.4	0.5	0.5	0.3	210.3	0.4	0.4	0.6	0.6	0.8
Y14933mRNA_s_at		One cut domain, family member 1	Onecut1	Y14933	Rn.48812	70.3	0.4	0.2	0.6	0.1	0.2	212.2	0.5	0.4	0.4	0.0	0.3
D86745cds_s_at		Nuclear receptor subfamily 0, group B, member 2	Nr0b2	D86745		486.2	0.2	0.9	0.6	0.4	0.2	304.0	0.5	0.4	0.5	0.7	0.6
X12752_at		CCAAT/enhancer binding protein (C/EBP), alpha	Cebpa	X12752		224.3	0.5	0.3	0.5	0.4	0.3	206.1	0.3	0.4	0.5	0.6	0.4
M81855_at	Transporters	ATP-binding cassette, sub-family B (MDR/TAP), member 1	Abcb1	M81855	Rn.82691	3.2	124.3	24.8	40.6	90.6	108.0	9.1	85.3	20.9	34.8	23.8	63.9
AB010466_s_at		ATP-binding cassette, sub-family C (CFTR/MRP), member 6	Abcc6	AB010466	Rn.29976	452.7	0.4	0.8	0.6	0.5	0.6	415.3	0.5	0.7	0.6	0.9	0.5
U53927_at		Solute carrier family 7 (cationic amino acid transporter, y+ system), member 2	Slc7a2	U53927		29.7	1.6	0.7	0.8	0.7	1.0	27.7	1.1	0.6	0.9	0.5	5.2
M77479_at		Solute carrier family 10 (sodium/bile acid cotransporter family), member 1	Slc10a1	M77479	Rn.9913	1054.3	0.2	0.8	0.7	0.4	0.4	1314.8	0.3	0.5	0.5	0.8	0.3
L23413_at		Solute carrier family 26 (sulfate transporter), member 1	Slc26a1	L23413	Rn.10016	456.1	0.5	0.7	0.8	0.5	0.6	426.3	0.6	1.1	0.6	1.2	0.7
U76379_s_at		Solute carrier family 22 (organic cation transporter), member 1	Slc22a1	U76379	Rn.11186	518.4	0.6	0.6	0.6	0.5	0.5	565.6	0.6	0.8	0.5	0.6	0.4
L27651_g_at		Solute carrier family 22 (organic anion transporter), member 7	Slc22a7	L27651	Rn.10009	423.3	0.6	0.7	0.9	0.5	0.7	477.6	0.9	1.3	0.9	1.3	0.6
U88036_at		Solute carrier organic anion transporter family, member 1a4	Slco1a4	U88036	Rn.5641	560.4	0.4	1.1	1.1	0.9	1.0	588.3	0.6	0.9	0.9	0.5	0.6

Probe ID	Functional category	Annotation	Symbol	GenBank	UniGene	Whole Liver						Isolated Hepatocytes					
						d 0	4	7	14	21	28	d 0	4	7	14	21	28
M95762_at		Solute carrier family 6 (neurotransmitter transporter, GABA), member 13	Slc6a13	M95762	Rn.10527	205.2	0.4	0.7	0.7	0.5	0.5	262.9	0.7	0.6	0.4	0.8	0.5
U28504_g_at		Solute carrier family 17 (sodium phosphate), member 1	Slc17a1	U28504	Rn.11150	36.0	0.6	1.1	1.6	2.0	2.0	78.7	0.9	1.2	1.1	1.3	0.5
M64862_at		Solute carrier organic anion transporter family, member 2a1	Slco2a1	M64862	Rn.9671	241.6	0.6	0.6	0.5	0.6	0.5	166.2	0.3	0.4	0.7	0.9	0.5
AB013112_s_at		Aquaporin 9	Aqp9	AB013112		693.4	0.6	0.7	0.8	0.7	0.7	730.2	0.9	1.2	1.2	1.6	0.5
AB005547_at		Aquaporin 8	Aqp8	AB005547	Rn.6315	158.0	1.1	0.9	0.9	0.9	1.6	103.4	1.7	2.5	2.9	3.5	2.1
AB000507_at		Aquaporin 7	Aqp7	AB000507	Rn.11111	*35.9*	1.8	1.4	1.5	1.4	1.9	13.7	4.0	4.9	5.9	5.1	4.2
rc_AA799645_g_at		FXYD domain-containing ion transport regulator 1	Fxyd1	AA799645	Rn.3828	169.6	0.4	0.6	0.5	0.5	0.4	200.9	0.5	0.3	0.3	0.5	0.5
AF080568_at	Enzymes related phospholipids	Phosphate cytidyly-ltransferase 2, ethanolamine	Pcyt2	AF080568	Rn.7291	617.9	0.4	0.4	0.8	0.6	0.7	569.7	0.6	0.8	0.6	0.8	0.6
D28560_at		Ectonucleotide pyrophosphatase/ phospho-diesterase 2	Enpp2	D28560	Rn.20403	410.9	0.1	0.9	0.8	0.6	0.4	353.6	0.4	0.7	0.7	0.8	0.5
L14441_at		Phosphatidy-lethanolamine N-methyl-transferase	Pemt	L14441	Rn.9875	758.5	0.4	0.8	0.9	0.7	0.6	809.4	0.6	0.9	0.8	1.1	0.5
rc_AA875050_at		(Ethanolamine kinase)	---	AA875050	Rn.65516	533.0	0.4	1.0	0.9	0.7	0.6	421.1	0.6	0.7	0.6	0.9	1.0
D16339_at	Protein related vitamins	Tocopherol (alpha) transfer protein	Ttpa	D16339		597.7	0.4	1.0	1.0	0.8	0.8	498.8	0.8	0.9	0.9	0.4	0.5
D14564cds_s_at		L-gulonolactone oxidase	Gulo	D14564		1483.9	0.2	0.5	0.6	0.3	0.2	1205.8	0.2	0.8	0.5	0.8	0.2
U19485_g_at	Others	Secreted phosphoprotein 2	Spp2	U19485	Rn.84	2059.5	0.6	0.8	0.8	0.7	0.6	2454.4	0.8	0.8	0.7	0.9	0.6
AF022774_g_at		Rabphilin 3A-like (without C2 domains)	Rph3al	AF022774	Rn.10986	39.9	*1.4*	*1.0*	1.5	1.7	1.5	28.5	2.4	1.9	1.2	1.7	2.9
rc_AA945050_f_at		Rat senescence marker protein 2A gene, exons 1 and 2	Smp2a	AA945050	Rn.40124	385.7	0.6	0.9	1.2	0.7	1.4	547.7	1.4	0.5	1.8	0.9	1.0
AF062389_at		Kidney-specific protein (KS)	RGD:708383	AF062389	Rn.14875	44.9	1.1	3.0	2.0	1.4	2.0	50.7	0.5	2.2	4.6	3.8	3.3
AF037072_at		Carbonic anhydrase 3	Ca3	AF037072	Rn.1647	1189.5	0.1	0.3	0.3	0.2	0.1	3196.8	0.0	0.4	0.1	0.1	*0.0*

Expression profiles of whole liver or isolated hepatocytes during fibrogenesis were obtained using a rat Genome U34A Array (Affymetrix). Marker genes for hepatocytes, which are the main contributors to the expression profile of the whole liver, are listed. Expression intensities are given for d 0, and expression intensity data for d 4, 7, 14, 21 and 28 are displayed as ratios to the d 0 expression data. Italics indicate an "absent" call by the Affymetrix software. Genes with the highest or lowest ratio in the chronic phase are shown in bold text. □ between 0.667 and 1.5; ■ ⩾ 1.5; ■ ⩽ 0.667.

different phases, such as repair in the acute phase response and fibrosis in the chronic phase response. These two groups of HSC marker genes may also be differentially regulated. Marker genes in one group may be expressed both in undifferentiated and in differentiated HSCs, while marker genes in the other group may be expressed mainly in differentiated HSCs. The functional changes associated with differentiation of HSCs during fibrosis are not clear. Schnabel *et al*[15] have temporally divided the activation process of HSCs into an initiation phase and a perpetuation phase, and HSCs in the initiation phase may play a role in wound healing[16,17] and are then eliminated by apoptosis[18,19], although some HSCs in the initiation phase differentiate into cells in the perpetuation phase. The two kinds of marker genes found in our work may be associated with the two phases.

In the present study, gene expression in HSCs *in vivo* was mostly similar to that found *in vitro*, suggesting that HSCs can be activated and produce ECM with few factors contributed by other cell types. Since HSCs also produce auto-stimulating factors such as TGF-beta, chemokines, PDGF, and IGF-1, our results strongly suggest that HSCs have self-supporting properties and few exogenous factors are required for their activation and differentiation. Even if

Table 7 Marker genes for hepatocytes that showed a strong relationship with fibrogenesis

Functional category	Cluster No.	Direction of change	Annotation	Symbol	Common
Amino acid metabolism	7	Increase	Glutamate oxaloacetate transaminase 1	Got1	J04171
	7	Increase	Glutamic-pyruvate transaminase (alanine aminotransferase)	Gpt	D10354
	9	Increase	Glutathione-S-transferase, alpha type2	Gsta2	AA945082
	3	Decrease	Cytosolic cysteine dioxygenase 1	Cdo1	AA942685
	10	Decrease	Cysteine-sulfinate decarboxylase	Csad	M64755
Cholesterol synthesis	3	Decrease	Nuclear receptor subfamily 0, group B, member 2	Nr0b2	D86745
Steroid hormone synthesis	7	Increase	Hydroxysteroid (17-beta) dehydrogenase 9	Hsd17b9	U89280
	7	Increase	(20-alpha-hydroxysteroid dehydrogenase)	---	AA866264
	1	Decrease	Hydroxysteroid (17-beta) dehydrogenase 2	Hsd17b2	X91234
	3	Decrease	Sulfotransferase, estrogen preferring	Ste	S76489
Lipid biosynthesis, metabolism, fatty acid & lipid transport	9	Increase	Carboxylesterase 2 (intestine, liver) (drug metabolism)	Ces2	AB010635
	1	Decease	2-hydroxyphytanoyl-CoA lyase(peroxisomal) alpha-oxidation	Hpcl2	AA893239
	3	Decease	Carboxylesterase 3	Ces3	L46791
	3	Decease	Hydroxyacid oxidase 2 (long chain)(peroxisomal)(alpha-oxidation)	Hao2	AI232087
	3	Decease	Fatty acid binding protein 7, brain (cytosolic)	Fabp7	U02096
	10	Decease	Alpha-methylacyl-CoA racemase Peroxisomal)	Amacr	U89905
Retinoid synthesis & metabolism	3	Decease	Retinol dehydrogenase type Ⅱ (RODH Ⅱ)	RoDHII	U33500
	10	Decease	Retinol dehydrogenase type Ⅲ	Rdh3	U18762
Dlucolysis & gluconeogenesis	7	Increase	Glucokinase	Gck	X53588
	3	Decrease	Phosphoenolpyruvate carboxykinase 1(PEPCK1) (cytosolic)	Pck1	K03243
	3	Decrease	Pyruvate carboxylase	Pc	U32314
	3	Decrease	Solute carrier family 37 (glycerol-6-phosphate transporter), member 4	Slc37a4	AF080468
	3	Decrease	Ribonuclease, RNase A family 4	Rnase4	AF041066
	3	Decrease	Ectonucleotide pyrophosphatase/phosphodiesterase 2(lysophospholipaseD)	Enpp2	D28560
Drug-metabolism	7	Increase	Cytochrome P450, family 3, subfamily a, polypeptide 13	Cyp3a13	U46118
	9	Increase	Aldehyde dehydrogenase family 1, member A1	Aldh1a1	AF001898
	1	Decrease	Cytochrome P450, family 1, subfamily a, polypeptide 2	Cyp1a2	E01184
	1	Decrease	Cytochrome P450, subfamily ⅡC (mephenytoin 4-hydroxylase)	Cyp2c	X79081
	1	Decrease	Flavin containing monooxygenase 1	Fmo1	M84719
	3	Decrease	Cytochrome P450, family 17, subfamily a, polypeptide 1	Cyp17a1	M21208
	3	Decrease	Cytochrome P450, family 3, subfamily a, polypeptide 11	Cyp3a11	M13646
	3	Decrease	Alcohol dehydrogenase 4 (class Ⅱ), pi polypeptide	Adh4	X90710
	3	Decrease	3-hydroxybutyrate dehydrogenase (heart, mitochondrial)	Bdh	AA817846
	3	Decrease	Camello-like 1 N-acetyltransferase 8, NAT8)	Cml1	AA892382
	3	Decrease	(hydroxysteroid sulfotransferase)	---	M31363
	3	Decrease	(hydroxysteroid sulfotransferase subunit)	---	AA818122
Growth factors & their receptors	7	Increase	Lipopolysaccharide binding protein	Lbp	L32132
	9	Decrease	Insulin-like growth factor binding protein 2	Igfbp2	J04486
	3	Decrease	Activin beta E	Inhbe	AF089825
	10	Decrease	Growth hormone receptor	Ghr	S49003
	10	Decrease	Insulin-like growth factor binding protein 1	Igfbp1	M58634
	10	Decrease	Deiodinase, iodothyronine, type I	Dio1	X57999
Hepatic secretory proteins	7	Increase	Alpha-fetoprotein	Afp	X02361
	1	Decrease	Alpha-2u globulin PGCL4 /// alpha-2u globulin PGCL2 /// alpha-2u-globulin (L type)/// alpha-2u globulin PGCL1 /// alpha-2u globulin PGCL3	Obp3 /// LOC298109 /// LOC298116 /// LOC259246 /// LOC259244	M27434
	1	Decrease	Alpha-2u globulin PGCL4 (Ppp2r2a protein phosphatase 2 (formerly 2A), regulatory subunit B (PR 52), alpha isoform)	Obp3	J00738
	10	Decrease	Metallothionein	Mt1a	AI102562
Protease & protease inhibitor	9	Increase	Lipocalin 2	Lcn2	AA946503
	1	Decrease	Elastase 1, pancreatic	Ela1	L00117
	10	Decrease	Esterase 2 (liver carboxylesterase)	Es2	M20629
	3	Decrease	Serine protease inhibitor	Spin2a	D00752
Cell surface proteins & structural proteins	7	Increase	Keratin complex 2, basic, gene 8 (cytokeratin-8)	Krt2-8	S76054
	7	Increase	Similar to cytokeratin(keratin complex 1, acidic, gene 18)	(Krt1-18)	AI072634
Signal transduction	3	Decrease	Regucalcin	Rgn	D31662
Transporters	7	Increase	solute carrier family 17 (sodium phosphate), member 1	Slc17a1	U28504
	7	Increase	Aquaporin 7	Aqp7	AB000507
	9	Increase	ATP-binding cassette, sub-family B (MDR/TAP), member 1	Abcb1	M81855
	3	Decrease	Solute carrier family 10 (sodium/bile acid cotransporter family), member 1	Slc10a1	M77479

Functional category	Cluster No.	Direction of change	Annotation	Symbol	Common
Protein related vitamins	10	Decrease	L-gulono-gamma-lactone oxidase	Gulo	D14564
Others	7	Increase	Rabphilin 3A-like (without C2 domains)	Rph3al	AF022774
	1	Decrease	Carbonic anhydrase 3	Ca3	AF037072

Gene expression profiles in the chronic phase (d 7, 14, 21 and 28) were clustered into 10 patterns using K-means analysis. Clustered genes that showed a tendency to temporally decrease (clusters 1, 3 and 10) or increase (clusters 7 and 9) were selected, as shown in Table 5 in the supplemental data. Among these genes, those showing a strong relation with fibrogenesis were further selected based on a *t*-test statistical analysis of the rate of change in expression intensity and the number of days of fibrosis. Hepatocyte-specific gene markers showing a strong relation with fibrogenesis are listed.

inflammation induced by virus activation stimulates long-term or weak hepatitis, HSCs may be able to autonomously activate and promote fibrosis, and this property of HSCs may be central to promotion of fibrogenesis. Sancho-Bru *et al*[20] have recently reported that a culture model of HSCs could not exactly match the activated phenotype found in DNA microarray analysis of isolated HSCs from cirrhotic human livers, because in culture marker genes for HSCs in the perpetuation phase are predominantly expressed, relative to those in the initiation phase. However, isolation of HSCs may also alter the expression of some genes, especially the expression of genes associated with inflammation, as shown in Figures 2 and 4. Therefore, compared to studies of isolated HSCs, our approach reveals the actual behavior of HSCs *in vivo* during fibrosis.

Gpc3 has been recently proposed as a serum and histochemical marker for hepatocellular carcinoma[21,22], since it is only weakly expressed in hepatocytes in normal and cirrhotic livers. However, in our study, Gpc3 was expressed in isolated HSCs during fibrogenesis, but weakly in cultured HSCs, suggesting that HSCs require extracellular factors for expression of Gpc3 during fibrogenesis, or that Gpc3-expressing cells with abnormal characteristics may contaminate the HSC fraction. The cell type showing expression of Gpc3 requires further study.

Marker genes for inflammatory cells

In the present study, gene expression in the inflammatory cell-fraction indicated the presence of several kinds of hematopoietic cells in this fraction, leading to some uncertainty in the data from the rat fibrosis model. However, the behavior of mast cells is of note, since temporary up-regulation of mast cell markers such as chemokines and mast cell proteases indicated invasion and/or activation of mast cells around d 14. Invasion of a marked number of mast cells could not be detected with HE staining, and therefore the number of invading mast cells must be small. Mast cells not only cause acute inflammation, but also have a role in induction of chronic inflammation[12,14], and involvement of mast cells in hepatic fibrosis has been reported[12,23-25]. RANTES, a chemokine that is produced by T cells and stimulates mast cells[26,27], showed its peak expression on d 14, as shown in Figure 4. RANTES has been suggested to be a mediator of progression from acute to chronic inflammation in colitis[28], and further studies are of importance to determine whether RANTES activation of mast cells is essential for liver fibrosis.

Marker genes for hepatocytes

DNA microarray analysis of whole liver in experimental animal models of hepatic fibrosis has been reported[1,2], and characteristic behavior of hepatocyte-specific marker genes over the time course of fibrogenesis was found in this study. DNA microarray data for the whole liver are similar to those for hepatocytes, since 70% of hepatic cells are hepatocytes[8,9], and data on d 28 are generally similar to those in previous reports. However, our temporal data indicate progressive abnormal gene expression in fibrogenesis, including in the early phase of fibrogenesis. Furthermore, since our data did not contain genes expressed in other hepatic cells, the hepatocyte-specific gene set allowed examination of the molecular network in hepatocytes.

Clustered abnormalities were found in genes associated with metabolism of sulfur-containing amino acids in this study. Similar abnormalities in metabolism of sulfur-containing amino acids have been reported[29,30], and an increase in methionine concentration in blood has been found in cirrhosis[31,32], which may be related to changes in gene expression in sulfur-containing amino acid metabolism. Furthermore, S-adenosylmethionine has an important role in methylation, including the methylation of DNA. Since abnormalities likely induce tumorigenesis due to DNA instability, it is of note that long-term suppression of S-adenosylmethionine synthetase increases the risk of tumorigenesis[33]. Glycine methyltransferase also has been implicated in DNA instability[34,35], and long-term suppression of this enzyme also has an associated risk of tumorigenesis during liver fibrosis. A metabolite of methionine, homocysteine, is also suspected as a risk factor for cardiovascular disease[36,37], and an increase in the concentration of homocysteine has been found in cirrhosis[38]. Abnormal homocysteine metabolism may also have an important role in the pathogenesis of liver failure, including fatty liver, activation of HSCs (i.e., enhancement of fibrosis), cardiovascular disease, and HCC. Finally, biosynthesis of taurine from cysteine may be suppressed, possibly leading to painful muscle cramps, which are a complication of cirrhosis caused by taurine deficiency[39]. Therefore, in summary, it is apparent that abnormalities in sulfur-containing amino acid metabolism can result in development of serious diseases.

Down-regulation of enzymes related to beta-oxidation in TAA-induced experimental fibrosis has been reported[29]. Studies of the Aox knockout mouse[40] suggest that metabolites of fatty acids in beta oxidation accumulate in

the liver and stimulate PPAR-alpha, resulting in peroxisome proliferation, and tumorigenesis is a potential risk in long-term administration of PPAR-alpha agonists and in the Aox-1 knockout mouse[40,41]. Radical accumulation by blocking metabolic enzymes associated with beta-oxidation has been suggested as one explanation of tumorigenesis in Aox knockout mice, and furthermore, microarray analysis of Aox-deficient mice can show up-regulation of Lcn2, a marker of carcinogenesis[42]. Interestingly markers such as Lcn2 and Cd36 were up-regulated in hepatocytes during fibrogenesis and in the inflammatory cell fraction in the late phase in our model. Although tumorigenesis due to PPAR alpha agonist is thought not to occur in humans[41,43], abnormalities in lipid metabolism during liver fibrosis may have a role in steatofibrosis and/or enhancement of fibrosis and HCC.

Up-regulation of Gck and down-regulation of Pck1 in hepatocytes suggest a decrease in gluconeogenesis, and suppression of gluconeogenesis has been reported in cirrhosis[44]. A deficiency of Scl37a4 enzyme activity in humans causes glycogen storage disease type 1 (GSD-1) genetic disorders[45] and suppression of this molecule is associated with hepatic steatosis[46]. Abnormalities in metabolism and synthesis of sex hormone have also been found in fibrotic liver, and an increased ratio of estrogen to testosterone in serum induces feminization, which is a complication in cirrhotic males[47]. Abnormality of sex-hormone metabolism is also related to liver malignancies[48,49].

Down-regulation of Ghr is also found in hepatocytes, and long-term suppression of growth signals may greatly influence fundamental hepatic vitality and produce abnormal hepatic regeneration. Insensitivity to growth hormones in cirrhosis has been reported[50,51], and administration of growth hormone protects against experimental liver fibrosis[52]. Rgn, a regulator of calcium signaling, may have an important role in regulation of proliferation and apoptosis of hepatocytes, as well as in formation of HCC[53,54], and down-regulation of regucalcin in our model suggested a risk of HCC development. An association of Afp, Ste, Mt1a, Lcn2, Abcb1 Cml1 (Nat8) and Ca3 with hepatocellular carcinoma (HCC) formation has also been reported. Accumulation of estrogen in the liver is suspected to promote HCC[55,56], and NAT8 polymorphism may be related to HCC[57,58]. Down-regulation of metallothionein[59-61] and carbonic anhydrase[62,63], up-regulation of Mdr/Tap[64] and Lcn[42,65] occur in HCC, and proliferation of hepatoma cells is suppressed by over-expression of Rgn[53,66]. The abnormal expression of all these genes in our model is similar to that in HCC, suggesting the importance of understanding whether such changes in gene expression reflect a tumorigenic environment or even promote tumorigenesis.

Differential regulations of genes involved in key events of liver fibrosis

In addition to the expression profiles of cell type specific marker genes discussed above, we here describe how genes involved in key events of liver fibrogenesis are differentially regulated. Gene expression profiles of the whole liver for different functional categories in liver fibrogenesis, such as ECM synthesis/degradation, inflammation and oxidative stress, are shown in Figure 8. Since both synthesis and degradation of ECM occur simultaneously, both genes are put together. Figure 8 shows that most genes in each category have a common and mutually correlated expression pattern, showing different regulations for different categories. Most genes of ECM synthesis/degradation as shown in Figure 8A have a peak of up-regulation on d 4 and a following gradual up-regulation along with the progression of fibrosis, and interestingly these genes are classified into group 1 of the HSC -specific genes as shown in Figure 2. Another type of genes of ECM synthesis/degradation as shown in Figure 8B have no peak on d 4 and only a gradual up-regulation along with the progression of fibrosis, and are classified into group 2 of the HSC-specific genes as shown in Figure 2. Many genes are also involved in the inflammatory category, and here only some of them are plotted as representative in Figure 8C. Most genes of inflammation have a peak on d 4 or 7 commonly, and are classified in group 1 of the Kupffer cell fraction specfic genes as shown in Figure 4. Most genes in the catregory of oxidative stress in Figure 8D have a minimum expression on d 4, in contrast to the genes of inflammation having a peak on d 4 or 7, and are involved in hepatocyte specific genes as shown in Figure 6.

Gene expression profiles viewed from both cell types and functional categories have made more clear image on how the temporal expression pattern are closely associated in terms of both the cell specificity and functions in liver fibrosis, and are regulated differently in different categories but in a mutually correlated manner within the same category.

Pathological overview of the behavior of HSCs, inflammatory cells, and hepatocytes in fibrogenesis

Our results from gene-expression profiling using hepatic cell-specific marker genes support the hypothesis shown in Figure 9. Comparisons of gene expression in HSCs *in vivo* and *in vitro* strongly suggest that HSCs have self-supporting properties and that few exogenous molecules are required to activate and differentiate HSCs. Hepatocytes have been shown to suffer from serious stress during fibrogenesis, and signals from suppressed hepatocytes, such as radicals, prophlogistic substances and toxic metabolites due to abnormal metabolism, are able to stimulate Kupffer cells and HSCs, leading to subsequent production of HSC-stimulating-factors such as TNF-α and IL-1 by Kupffer cells. Therefore, HSCs, Kupffer cells and other inflammatory cells produce factors such as TGF-beta that suppress hepatocyte vitality and result in hepatocyte injury. Sequential activation of inflammatory cells such as lymphocytes and mast cells may be essential in this process, and even if this stimulatory circuit is small in scale during early remission of fibrogenesis, it can be maintained with appropriate stimulation even at long intervals, with small-scale inflammation induced by C-type hepatitis virus propagation. The self-supporting characteristics of HSCs may have a central role in maintenance of this circuit.

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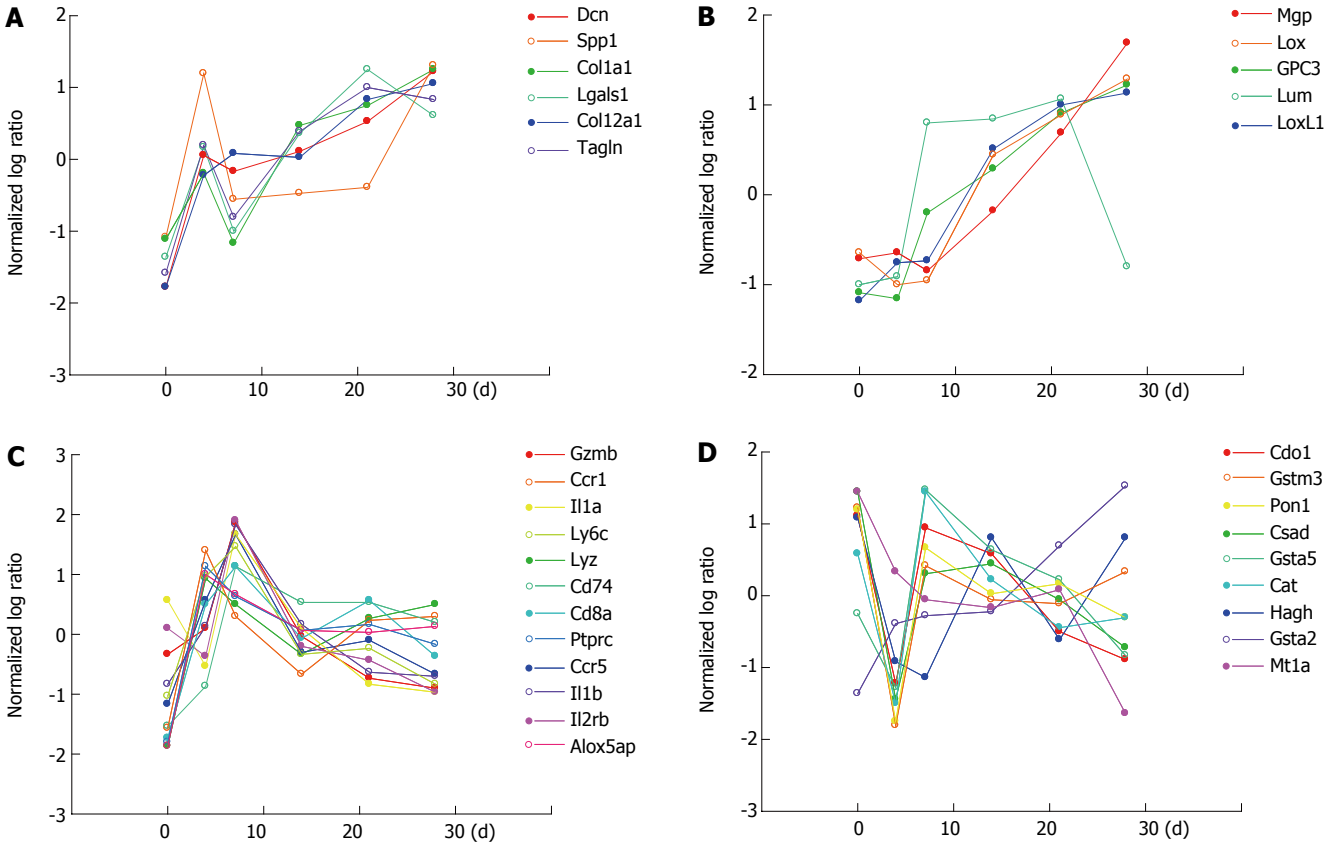

Figure 3 Differential regulations of genes involved in key events of liver fibrosis. Gene expression profiles of different events in liver fibrogenesis such as ECM synthesis/degradation, inflammation and oxidative stress, are shown. The x-axis showing the days of fibrogenesis (d 0, 4, 7 14, 21, 28) and the y-axis the normalized log ratio(scaled in terms of mean and SD, and the log base 2) of the whole liver gene expression. **A** and **B**: genes of ECM synthesis/degradation classified into group 1 (2) of HSCs as shown in Table 1; **C**: genes of inflammation involved in the Kupffer cell fraction as shown in Table 3; **D**: genes of oxidative stress involved in the hepatocytes as shown in Table 6.

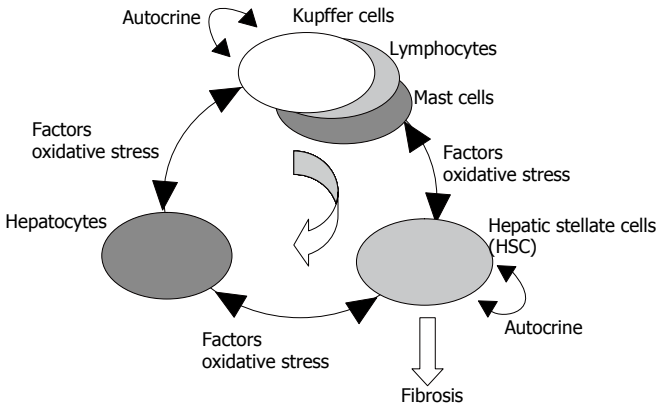

Figure 4 Circuit model of hepatic cells in fibrogenesis. Gene expression profiles show that HSCs have self-activating properties, and that widespread damage to hepatocytes occurred in development of fibrosis, suggesting a self-activating circuit model of fibrogenesis. After an initial stimulatory trigger caused by events such as virus infection, hepatic cells are able to stimulate each other, and the self-activating properties of HSCs maintain this cycle over the long term. Details are given in the text.

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REFERENCES

1 **Utsunomiya T**, Okamoto M, Hashimoto M, Yoshinaga K, Shiraishi T, Tanaka F, Mimori K, Inoue H, Watanabe G, Barnard GF, Mori M. A gene-expression signature can quantify the degree of hepatic fibrosis in the rat. *J Hepatol* 2004; **41**: 399-406

2 **Jiang Y**, Liu J, Waalkes M, Kang YJ. Changes in the gene expression associated with carbon tetrachloride-induced liver fibrosis persist after cessation of dosing in mice. *Toxicol Sci* 2004; **79**: 404-410

3 **Geerts A**. History, heterogeneity, developmental biology, and functions of quiescent hepatic stellate cells. *Semin Liver Dis* 2001; **21**: 311-335

4 **Kmiec Z**. Cooperation of liver cells in health and disease. *Adv Anat Embryol Cell Biol* 2001; **161**: III-XIII, 1-151

5 **Horie T**, Sakaida I, Yokoya F, Nakajo M, Sonaka I, Okita K. L-cysteine administration prevents liver fibrosis by suppressing hepatic stellate cell proliferation and activation. *Biochem Biophys Res Commun* 2003; **305**: 94-100

6 **Kawada N**, Seki S, Inoue M, Kuroki T. Effect of antioxidants, resveratrol, quercetin, and N-acetylcysteine, on the functions of cultured rat hepatic stellate cells and Kupffer cells. *Hepatology* 1998; **27**: 1265-1274

7 **Ishizaki-Koizumi S**, Sonaka I, Fujitani S, Nishiguchi S. Mechanisms of the protective effect of L-alanine to D-galactosamine-induced hepatocellular injury: comparative studies of L-alanine and pyruvate. *Biochem Biophys Res Commun* 2002; **291**:738-743

8 **Friedman SL**, Rockey DC, McGuire RF, Maher JJ, Boyles JK, Yamasaki G. Isolated hepatic lipocytes and Kupffer cells

from normal human liver: morphological and functional characteristics in primary culture. *Hepatology* 1992; **15**: 234-243
9 **ten Hagen TL**, van Vianen W, Bakker-Woudenberg IA. Isolation and characterization of murine Kupffer cells and splenic macrophages. *J Immunol Methods* 1996; **193**: 81-91
10 **Margolin AA**, Greshock J, Naylor TL, Mosse Y, Maris JM, Bignell G, Saeed AI, Quackenbush J, Weber BL. CGHAnalyzer: a stand-alone software package for cancer genome analysis using array-based DNA copy number data. *Bioinformatics* 2005; **21**: 3308-3311
11 **Kozlowska J**, Loch T, Jablonska J, Cianciara J. Biochemical markers of fibrosis in chronic hepatitis and liver cirrhosis of viral origin. *Przegl Epidemiol* 2001; **55**: 451-458
12 **Armbrust T**, Batusic D, Ringe B, Ramadori G. Mast cells distribution in human liver disease and experimental rat liver fibrosis. Indications for mast cell participation in development of liver fibrosis. *J Hepatol* 1997; **26**: 1042-1054
13 **Shimizu S**, Satomura K, Aramaki T, Katsuta Y, Takano T, Omoto Y. Hepatic chymase level in chronic hepatitis: co-localization of chymase with fibrosis. *Hepatol Res* 2003; **27**: 62-66
14 **Stoyanova II**. Relevance of mast cells and hepatic lobule innervation to liver injury. *Rom J Gastroenterol* 2004; **13**: 203-209
15 **Schnabl B**, Purbeck CA, Choi YH, Hagedorn CH, Brenner D. Replicative senescence of activated human hepatic stellate cells is accompanied by a pronounced inflammatory but less fibrogenic phenotype. *Hepatology* 2003; **37**: 653-664
16 **Diegelmann RF**, Evans MC. Wound healing: an overview of acute, fibrotic and delayed healing. *Front Biosci* 2004; **9**: 283-289
17 **Lalazar A**, Wong L, Yamasaki G, Friedman SL. Early genes induced in hepatic stellate cells during wound healing. *Gene* 1997; **195**: 235-243
18 **Elsharkawy AM**, Oakley F, Mann DA. The role and regulation of hepatic stellate cell apoptosis in reversal of liver fibrosis. *Apoptosis* 2005; **10**: 927-939
19 **Janoschek N**, van de Leur E, Gressner AM, Weiskirchen R. Induction of cell death in activated hepatic stellate cells by targeted gene expression of the thymidine kinase/ganciclovir system. *Biochem Biophys Res Commun* 2004; **316**: 1107-1115
20 **Sancho-Bru P**, Bataller R, Gasull X, Colmenero J, Khurdayan V, Gual A, Nicolas JM, Arroyo V, Gines P. Genomic and functional characterization of stellate cells isolated from human cirrhotic livers. *J Hepatol* 2005; **43**: 272-282
21 **Capurro M**, Wanless IR, Sherman M, Deboer G, Shi W, Miyoshi E, Filmus J. Glypican-3: a novel serum and histochemical marker for hepatocellular carcinoma. *Gastroenterology* 2003; **125**: 89-97
22 **Moriguchi H**, Sato C. The values and limitations of glypican-3 as a novel tumor marker for hepatocellular carcinoma from clinical and economic viewpoints. *Gastroenterology* 2004; **127**: 679-680
23 **Akiyoshi H**, Terada T. Mast cell, myofibroblast and nerve terminal complexes in carbon tetrachloride-induced cirrhotic rat livers. *J Hepatol* 1998; **29**: 112-119
24 **Matsunaga Y**, Kawasaki H, Terada T. Stromal mast cells and nerve fibers in various chronic liver diseases: relevance to hepatic fibrosis. *Am J Gastroenterol* 1999; **94**: 1923-1932
25 **Yamashiro M**, Kouda W, Kono N, Tsuneyama K, Matsui O, Nakanuma Y. Distribution of intrahepatic mast cells in various hepatobiliary disorders. An immunohistochemical study. *Virchows Arch* 1998; **433**: 471-479
26 **Conti P**, DiGioacchino M. MCP-1 and RANTES are mediators of acute and chronic inflammation. *Allergy Asthma Proc* 2001; **22**: 133-137
27 **Juremalm M**, Nilsson G. Chemokine receptor expression by mast cells. *Chem Immunol Allergy* 2005; **87**: 130-144
28 **Ajuebor MN**, Hogaboam CM, Kunkel SL, Proudfoot AE, Wallace JL. The chemokine RANTES is a crucial mediator of the progression from acute to chronic colitis in the rat. *J Immunol* 2001; **166**: 552-558
29 **Low TY**, Leow CK, Salto-Tellez M, Chung MC. A proteomic analysis of thioacetamide-induced hepatotoxicity and cirrhosis in rat livers. *Proteomics* 2004; **4**: 3960-3974
30 **Pisi E**, Marchesini G. Mechanisms and consequences of the impaired trans-sulphuration pathway in liver disease: Part II. Clinical consequences and potential for pharmacological intervention in cirrhosis. *Drugs* 1990; **40** Suppl 3: 65-72
31 **Cascino A**, Cangiano C, Calcaterra V, Rossi-Fanelli F, Capocaccia L. Plasma amino acids imbalance in patients with liver disease. *Am J Dig Dis* 1978; **23**: 591-598
32 **Almasio P**, Bianchi G, Marchesini G, Luca A, Bugianesi E, Le Grazie C, Pagliaro L. Sulphur amino acid pattern in chronic liver disease. *Ital J Gastroenterol* 1994; **26**: 21-25
33 **Matsui H**, Kawada N. Effect of S-adenosyl-L-methionine on the activation, proliferation and contraction of hepatic stellate cells. *Eur J Pharmacol* 2005; **509**: 31-36
34 **Singh V**, Miranda TB, Jiang W, Frankel A, Roemer ME, Robb VA, Gutmann DH, Herschman HR, Clarke S, Newsham IF. DAL-1/4.1B tumor suppressor interacts with protein arginine N-methyltransferase 3 (PRMT3) and inhibits its ability to methylate substrates in vitro and in vivo. *Oncogene* 2004; **23**: 7761-7771
35 **Tseng TL**, Shih YP, Huang YC, Wang CK, Chen PH, Chang JG, Yeh KT, Chen YM, Buetow KH. Genotypic and phenotypic characterization of a putative tumor susceptibility gene, GNMT, in liver cancer. *Cancer Res* 2003; **63**: 647-654
36 **Lalouschek W**, Aull S, Deecke L, Schnider P, Uhl F, Zeiler K. Hyperhomocyst(e)inemia--an independent risk factor of stroke. *Fortschr Neurol Psychiatr* 1996; **64**: 271-277
37 **Tsai MY**, Arnett DK, Eckfeldt JH, Williams RR, Ellison RC. Plasma homocysteine and its association with carotid intimal-medial wall thickness and prevalent coronary heart disease: NHLBI Family Heart Study. *Atherosclerosis* 2000; **151**: 519-524
38 **Look MP**, Riezler R, Reichel C, Brensing KA, Rockstroh JK, Stabler SP, Spengler U, Berthold HK, Sauerbruch T. Is the increase in serum cystathionine levels in patients with liver cirrhosis a consequence of impaired homocysteine transsulfuration at the level of gamma-cystathionase? *Scand J Gastroenterol* 2000; **35**: 866-872
39 **Yamamoto S**. Oral taurine therapy for painful muscle cramp in liver cirrhosis. *Am J Gastroenterol* 1994; **89**: 457-458
40 **Chu R**, Lim H, Brumfield L, Liu H, Herring C, Ulintz P, Reddy JK, Davison M. Protein profiling of mouse livers with peroxisome proliferator-activated receptor alpha activation. *Mol Cell Biol* 2004; **24**: 6288-6297
41 **Gonzalez FJ**. Recent update on the PPAR alpha-null mouse. *Biochimie* 1997; **79**: 139-144
42 **Meyer K**, Lee JS, Dyck PA, Cao WQ, Rao MS, Thorgeirsson SS, Reddy JK. Molecular profiling of hepatocellular carcinomas developing spontaneously in acyl-CoA oxidase deficient mice: comparison with liver tumors induced in wild-type mice by a peroxisome proliferator and a genotoxic carcinogen. *Carcinogenesis* 2003; **24**: 975-984
43 **Roglans N**, Bellido A, Rodriguez C, Cabrero A, Novell F, Ros E, Zambon D, Laguna JC. Fibrate treatment does not modify the expression of acyl coenzyme A oxidase in human liver. *Clin Pharmacol Ther* 2002; **72**: 692-701
44 **Changani KK**, Jalan R, Cox IJ, Ala-Korpela M, Bhakoo K, Taylor-Robinson SD, Bell JD. Evidence for altered hepatic gluconeogenesis in patients with cirrhosis using in vivo 31-phosphorus magnetic resonance spectroscopy. *Gut* 2001; **49**: 557-564
45 **Chou JY**. The molecular basis of type 1 glycogen storage diseases. *Curr Mol Med* 2001; **1**: 25-44
46 **Bandsma RH**, Wiegman CH, Herling AW, Burger HJ, ter Harmsel A, Meijer AJ, Romijn JA, Reijngoud DJ, Kuipers F. Acute inhibition of glucose-6-phosphate translocator activity leads to increased de novo lipogenesis and development of hepatic steatosis without affecting VLDL production in rats. *Diabetes* 2001; **50**: 2591-2597
47 **Maruyama Y**, Adachi Y, Aoki N, Suzuki Y, Shinohara H, Yamamoto T. Mechanism of feminization in male patients with non-alcoholic liver cirrhosis: role of sex hormone-binding globulin. *Gastroenterol Jpn* 1991; **26**: 435-439
48 **Rossi L**, Leveri M, Gritti C, De Silvestri A, Zavaglia C, Sonzogni L, Silvestri L, Civardi E, Mondelli MU, Silini EM.

Genetic polymorphisms of steroid hormone metabolizing enzymes and risk of liver cancer in hepatitis C-infected patients. *J Hepatol* 2003; **39**: 564-570

49 **Granata OM**, Carruba G, Montalto G, Miele M, Bellavia V, Modica G, Blomquist CH, Castagnetta LA. Altered androgen metabolism eventually leads hepatocellular carcinoma to an impaired hormone responsiveness. *Mol Cell Endocrinol* 2002; **193**: 51-58

50 **Bucuvalas JC**, Horn JA, Chernausek SD. Resistance to growth hormone in children with chronic liver disease. *Pediatr Transplant* 1997; **1**: 73-79

51 **Donaghy AJ**, Delhanty PJ, Ho KK, Williams R, Baxter RC. Regulation of the growth hormone receptor/binding protein, insulin-like growth factor ternary complex system in human cirrhosis. *J Hepatol* 2002; **36**: 751-758

52 **Chen S**, Wang HT, Yang B, Fu YR, Ou QJ. Protective effects of recombinant human growth hormone on cirrhotic rats. *World J Gastroenterol* 2004; **10**: 2894-2897

53 **Izumi T**, Yamaguchi M. Overexpression of regucalcin suppresses cell death and apoptosis in cloned rat hepatoma H4-II-E cells induced by lipopolysaccharide, PD 98059, dibucaine, or Bay K 8644. *J Cell Biochem* 2004; **93**: 598-608

54 **Tsurusaki Y**, Yamaguchi M. Role of regucalcin in liver nuclear function: binding of regucalcin to nuclear protein or DNA and modulation of tumor-related gene expression. *Int J Mol Med* 2004; **14**: 277-281

55 **Farrell GC**, Koltai A, Murray M. Source of raised serum estrogens in male rats with portal bypass. *J Clin Invest* 1988; **81**: 221-228

56 **Lampropoulou-Karatzas C**, Goritsas P, Makri MG. Low serum testosterone: a special feature of hepatocellular carcinoma. *Eur J Med* 1993; **2**: 23-27

57 **Agundez JA**, Olivera M, Ladero JM, Rodriguez-Lescure A, Ledesma MC, Diaz-Rubio M, Meyer UA, Benitez J. Increased risk for hepatocellular carcinoma in NAT2-slow acetylators and CYP2D6-rapid metabolizers. *Pharmacogenetics* 1996; **6**: 501-512

58 **Yu MW**, Pai CI, Yang SY, Hsiao TJ, Chang HC, Lin SM, Liaw YF, Chen PJ, Chen CJ. Role of N-acetyltransferase polymorphisms in hepatitis B related hepatocellular carcinoma: impact of smoking on risk. *Gut* 2000; **47**: 703-709

59 **Endo T**, Yoshikawa M, Ebara M, Kato K, Sunaga M, Fukuda H, Hayasaka A, Kondo F, Sugiura N, Saisho H. Immunohistochemical metallothionein expression in hepatocellular carcinoma: relation to tumor progression and chemoresistance to platinum agents. *J Gastroenterol* 2004; **39**: 1196-1201

60 **Huang GW**, Yang LY. Metallothionein expression in hepatocellular carcinoma. *World J Gastroenterol* 2002; **8**: 650-653

61 **Waalkes MP**, Diwan BA, Rehm S, Ward JM, Moussa M, Cherian MG, Goyer RA. Down-regulation of metallothionein expression in human and murine hepatocellular tumors: association with the tumor-necrotizing and antineoplastic effects of cadmium in mice. *J Pharmacol Exp Ther* 1996; **277**: 1026-1033

62 **Kuo WH**, Chiang WL, Yang SF, Yeh KT, Yeh CM, Hsieh YS, Chu SC. The differential expression of cytosolic carbonic anhydrase in human hepatocellular carcinoma. *Life Sci* 2003; **73**: 2211-2223

63 **Saarnio J**, Parkkila S, Parkkila AK, Pastorekova S, Haukipuro K, Pastorek J, Juvonen T, Karttunen TJ. Transmembrane carbonic anhydrase, MN/CA IX, is a potential biomarker for biliary tumours. *J Hepatol* 2001; **35**: 643-649

64 **Nagasue N**, Dhar DK, Makino Y, Yoshimura H, Nakamura T. Overexpression of P-glycoprotein in adenomatous hyperplasia of human liver with cirrhosis. *J Hepatol* 1995; **22**: 197-201

65 **Hanai J**, Mammoto T, Seth P, Mori K, Karumanchi SA, Barasch J, Sukhatme VP. Lipocalin 2 diminishes invasiveness and metastasis of Ras-transformed cells. *J Biol Chem* 2005; **280**: 13641-13647

66 **Misawa H**, Inagaki S, Yamaguchi M. Suppression of cell proliferation and deoxyribonucleic acid synthesis in the cloned rat hepatoma H4-II-E cells overexpressing regucalcin. *J Cell Biochem* 2001; **84**: 143-149

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BASIC RESEARCH

Establishment and characterization of a cholangiocarcinoma cell line (RMCCA-1) from a Thai patient

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Abstract

AIM: To establish and characterize a new cell line derived from peripheral cholangiocarcinoma of a Thai patient.

METHODS: The peripheral cholangiocarcinoma specimen surgically obtained from the patient was aseptically processed by washing and mincing before culturing in Ham's F12 medium containing 10% fetal bovine serum. After 3 mo, when the cell line has become homogeneous and stabilized, several features were investigated, including growth characteristics, immunofluorescence staining for cytokeratins, expression of tumor markers, chromosomal analysis by G-banding and multicolour fluorescence *in situ* hybridization (mFISH), *in vitro* migration and invasion characteristics.

RESULTS: The RMCCA-1 cell line has been established. These cells proliferated as a monolayer with a population doubling time of 48 h. Immunofluorescence staining showed positive staining for human cytokeratin 7 and 19 verifying the biliary epithelial origin. RMCCA-1 secreted carbohydrate antigen 19-9 (CA19-9), but insignificant levels of carcinoembryonic antigen (CEA) and α-fetoprotein (AFP). Chromosome analysis identified aneuploidy karyotypes with a modal chromosome number of 59. RMCCA-1 exhibited a low level of *in vitro* invasiveness, but a high degree of motility. The cell line exhibited a significant number of chromosomal aberrations as shown by mFISH and G-banding methods.

CONCLUSION: A new cell line derived from peripheral cholangiocarcinoma of a Thai patient has been established. This cell line shows a low level of *in vitro* invasiveness, but a high degree of motility. It will serve as a valuable tool for further studies on tumor biology, molecular pathogenesis, metastatic mechanism and response to therapeutic drugs of cholangiocarcinoma.

Key words: Cholangiocarcinoma; Cell line; Establishment; mFISH; Invasion; Migration

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INTRODUCTION

Cholangiocarcinoma is a highly malignant epithelial neoplasm that arises within the intrahepatic and extrahepatic biliary tract[1]. The pathogenesis of this disease has been strongly associated with chronic inflammation and cellular injury within bile ducts, as well as partial obstruction of bile flow, manifested by various high risk conditions such as PSC (primary sclerosing chloangitis), hepatolithiasis and infestation by liver fluke (*Ophisthorchis viverrini* or *Clonorchis sinensis*)[2,3]. Although considered as a rare disease, cholangiocarcinoma occurs at a particularly high rate in Northeastern Thailand, with 84.6:100 000 males and 36.8:100 000 females affected by the disease. This is the area where the incidence rate of cholangiocarcinoma is the highest in the world, largely accounted by the habit of consuming uncooked cyprinoid fish, which are infected with the liver fluke[4].

Cholangiocarcinoma has become a serious threat to public health due to increasing worldwide incidence and mortality rates associated with lack of early detection and limited therapeutic options. At diagnosis, most patients are presented with advanced disease, possibly with undetected metastasis, resulting in less than 12 mo survival. Even those with operable tumor, the recurrence rate was extremely high, with a 5-year survival rate of less

than 40%[2,5]. Various routes of tumor spreading have been reported in cholangiocarcinoma, including direct invasion, infiltration along the biliary tree, vascular and lymphatic permeation and perineural or intraneural invasion[6].

Progress in understanding the molecular mechanisms governing cholangiocarcinoma invasion and metastasis has been limited by the lack of suitable cell lines and experimental models. Here, we described an establishment and preliminary characterization of a human cell line originated from a Thai patient presented with peripheral cholangiocarcinoma. This new cell line, which we named RMCCA-1, exhibits various characteristics typical of the biliary epithelial cells, as well as invasiveness and motility as shown by the *in vitro* assay. Thus this cell line will be useful for the studies of not only the tumor biology, molecular pathogenesis and drug response, but also the molecular mechanisms governing the metastatic spread of cholangiocarcinoma.

MATERIALS AND METHODS

Clinical specimen

A 40-year-old male patient was admitted to Rajavithi Hospital, Bangkok, Thailand with a professional diagnosis of peripheral cholangiocarcinoma. The patient's serum was analyzed for alkaline phosphatase (ALP), total bilirubin and direct bilirubin using a COBAS Integra 800 instrument (Roche, USA), whereas CA19-9, carcinoembryonic antigen (CEA), and α-fetoprotein (AFP) were analyzed on a ELECHYS 2012 instrument (Roche, USA). Significant laboratory analysis at admission showed elevated serum levels of ALP (374 U/L, normal 39-117 U/L), total bilirubin (19.73 mg/dL, normal 0.0-1.5 mg/dL), direct bilirubin (15.45 mg/dL, normal 0-0.5 mg/dL), CA19-9 (85.05 U/mL, normal 0-39 U/mL) and CEA (7.56 ng/mL, normal 0.0-3.4 ng/mL), whereas the AFP (2.24 U/mL, normal 0.0-5.8 U/mL), and CA125 (19.71 U/mL, normal 0.0-35 U/mL) levels were normal.

The CT examination evaluated for this study was performed on a helical CT scanner (Somatom Plus 4, Siemens Medical Solutions) using the following parameters: 5-mm collimation, 5-mm reconstruction interval, and a 1:1 table pitch. Both unenhanced and contrast-enhanced CT scans were obtained. With intravenous injection of 120 mL of nonionic contrast material [iopromide (Ultravist 370, Schering)], both hepatic artery phase (HAP) and portal venous phase (PVP) images were obtained with a scanning delay of 30 and 65 s, respectively. The results showed an ill defined hypo-density mass occupying the whole left lobe of the liver. At laparotomy, no ascites was found, and the surface of liver was smooth. Intraoperative ultrasonography revealed a solitary mass at left lobe of liver with moderate dilatation of left intrahepatic duct. The patient was then subjected to left hepatectomy and lymph node dissection. The tumor specimen was removed and subjected to histopathological study and to tissue culture under the approval of the Ethics Committee of Rajavithi Hospital.

Tumor histopathology

The lesion was classified as a well-differentiated peripheral cholangiocarcinoma at stage T2N0M0 according to the UICC standardization (Figure 1).

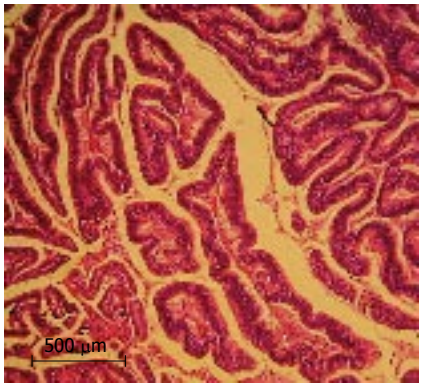

Figure 1 Hematoxylin and Eosin staining of the liver specimen (10 × magnification). The histopathological analysis of the specimen indicates a well-differentiated peripheral cholangiocarcinoma with no vascular invasion.

Primary culture

After the tumor tissue was surgically removed from the patient, it was immediately suspended in transfer medium [HAM's F12 (GIBCO, Grand Island, NY, USA) containing antibiotics (GIBCO, Grand Island, NY, USA)] at 4℃. The tissue was quickly washed in PBS pH 7.4 several times before being minced. Later the cell suspension was placed in a 100 mm × 20 mm plastic tissue culture dish (CORNING, New York, USA) containing 10 mL of growth medium (HAM's F12, 20% fetal bovine serum (FBS) (GIBCO, Grand Island, NY, USA), 1×10^{-5} g/L epidermal growth factor (EGF) (Pacific Science, Peprotech, New Jersey, USA), 0.1 U/L penicillin G sodium, 0.1 g/L streptomycin sulfate and 2.5×10^{4} g/L amphotericin B). The cell cultures were then incubated at 37℃ in a humidified 50 mL/L CO_2 atmosphere and observed daily. Tumor cells were separated from the contaminating fibroblast cells by manually dropping 2.5 mL/L trypsin containing 0.2 mL/L EDTA (GIBCO, Grand Island, NY, USA) in PBS onto an isolated tumor colony. Subsequently the detached tumor cells were transferred by pipettes to a new culture dish under a phase contrast microscope. After about 1 mo, a homogeneous layer of epithelial tumor cells with sustained growth pattern was established.

Growth kinetics

A suspension of 2×10^{3} cells was cultured in triplicates in 100 μL of Ham's F12 medium supplemented with 100 mL/L FBS in a 96-well plate (CORNING, New York, USA). At time intervals, 10 μL of 5 mg/mL MTT (USB, Cleveland, OH, USA) solution was added to the individual wells, followed by incubation for 4 h at 37℃ in a humidified atmosphere containing 50 mL/L CO_2. MTT converted to insoluble formazan dye in live cells was then dissolved by addition of 200 μL DMSO (Sigma, St. Louis, MO, USA) before the absorbency was read at 540 nm. The doubling time of the cell population was determined from the exponential phase of the growth curve.

Immunofluorescence staining

The monoclonal antibody mixture AE-1/AE-3 (DAKO, Denmark) recognized all known basic and most acidic keratin, thus it was used as a general marker of epithelial cells. Cytokeratin 7 (monoclonal mouse Anti-Human

Cytokeratin 7, DAKO, Denmark) and cytokeratin 19 (monoclonal mouse Anti-Human Cytokeratin 19, DAKO, Denmark) were used to specifically distinguish biliary epithelial cells from hepatocytes[7,8]. RMCCA-1 cells were grown on sterile coverslips until confluent before being fixed in methanol for 15 min, blocked in 10 mg/L bovine serum albumin (BSA), and incubated with primary antibody for 60-min. After that, a secondary antibody conjugated with fluorescein isothiocyanate (DAKO, Denmark) was added and the incubation was allowed to proceed for 60 min at 37℃. After washing, the coverslips were examined under a fluorescence microscope (Nikon Eclipse TE 2000-U, Kanagawa, Japan).

Chromosome preparation and G-banding analysis

The established tumor cells at 12th passage were subjected to chromosomal analysis. The cells were treated with 10 μmol/L Colchicine (Sigma, St. Louis, MO, USA) for 30 min and suspended in hypotonic solution, 0.075 mol/L KCl (Sigma, St. Louis, MO, USA), for 7 min, fixed in Carnoy's fixative and spreaded onto cold glass slides. The cells were stained with Giemsa and the representative chromosome sets were photographed for karyotype analysis. Interpretation of the karyotype was based on ISCN (1995). The modal chromosome number was determined from 20 cells.

Multicolour fluorescence in situ hybridization (mFISH) analysis

A duplicate slide, prepared from the same culture used for G-banding, was subjected to mFISH analysis according to the manufacturer's protocol (Metasystem, Germany). The 24 XCyte mFISH kit (Metasystem, Germany) with five fluorophors were used for hybridization, including FITC, Spectrum Orange, Texas Red, Cy5 and DEAC. Each chromosome (1-22, X and Y) was painted with different colors using various combinations of the fluorophores. After hybridization, the slides were evaluated under a fluorescence microscope (Axioimage, Zeiss, Germany), and the images were captured and analyzed using the Program Isis (Metasystem, Germany).

Tumor marker detection

A suspension of 10^5 tumor cells was cultured in serum-free HAM's F12 medium for 24 h before the conditioned medium was collected and centrifuged at 1800 r/min for 10 min. The supernatant was then collected for detection of CA 19-9, CEA and AFP by using chemiluminescence detection system on an ELECHYS 2012 instrument (Roche, USA).

In vitro invasion assay

Cancer cell invasiveness was determined using transwell chamber (Costar, Cambridge, MA, USA) coated with 0.3 g/L matrigel (Collaborative Research Inc., Bedford, MA, USA). Approximately, 1 × 10^5 cells (RMCCA-1, KKU-100, KKU-213 and HuCCA-1) in culture medium containing FBS were added into the upper compartment of the transwell, and incubated at 37℃ in a humidified atmosphere containing 50 mL/L CO_2 for 6 h. The lower compartment contained culture medium plus FBS. The filters were fixed with methanol and stained with 0.5% crystal violet in 25% methanol for 1 h, before rinsing with tap water several times. The cells on the upper surface of the filters were gently removed using cotton swabs and the cells that have invaded into the lower surface were counted under a microscope. The numbers of invaded cells in five random × 10 microscopic fields were counted and expressed as number of cells per well. The results shown represented mean ± SE of the number of invaded cells from three independent experiments, each carried out in triplicates.

In vitro motility assay

Motility assay was performed in a similar fashion to the invasion assay, except no matrigel coating was applied to the upper surface of the transwell filters. The numbers of migrating cells in five random × 10 microscopic fields were counted and expressed as number of cells per well. The results shown represented mean ± SE of the number of migrating cells from three independent experiments, each carried out in triplicates.

Gelatin zymography assay

Cells were starved by culturing in the medium without FBS for 24 h before collection of the conditioned medium. The conditioned medium was mixed with 5 × SDS-sample buffer before separation in a 120 g/L SDS-PAGE containing 1 mg/mL gelatin (Sigma, St. Louis, MO, USA). After electrophoresis at 200 V for 1 h, the gel was washed in a 25 mL/L Triton X-100 (Amersham, Piscataway, NJ, USA) solution twice. The gel was then incubated in buffer containing 50 mmol/L Tris-HCl (Amersham, Piscataway, NJ, USA), pH 7.5, 10 mmol/L $CaCl_2$ (Merck, Denmark), 1 mmol/L $ZnCl_2$ (Merck, Denmark), 10 mL/L Triton X-100 for 16-18 h, after which the gel was stained with 5 g/L Coomassie blue in 300 mL/L methanol and 100 mL/L acetic acid. After destaining, the clear band of gelatinolytic activity was documented for size determination using a Biorad GS700 gel scanner (Biorad, Hercules, CA, USA)[9].

RESULTS

Primary cell culture

The human CCA tissue fragments adhered to the dish after plating for 6 h. After three weeks, a layer of epithelial cells appeared, from which contaminating spindle-shaped fibroblasts could be readily distinguished and separated from the epithelial tumor cells by differential trypsinization. After successive 16 passages, a homogeneous immortalized culture of tumor cells was established and was named RMCCA-1. The cells from passages 16th and 30th were then used for morphological analysis. These cells exhibited circular to spindle shape with many processes and ornamental fringes. The nucleus and cytoplasm appeared granulated (Figure 2).

Growth kinetics

RMCCA-1 cells were in lag phase until d 5, after which they entered a logarithmic growth phase. The doubling time determined from the slope of the growth curve was 48 h (Figure 3).

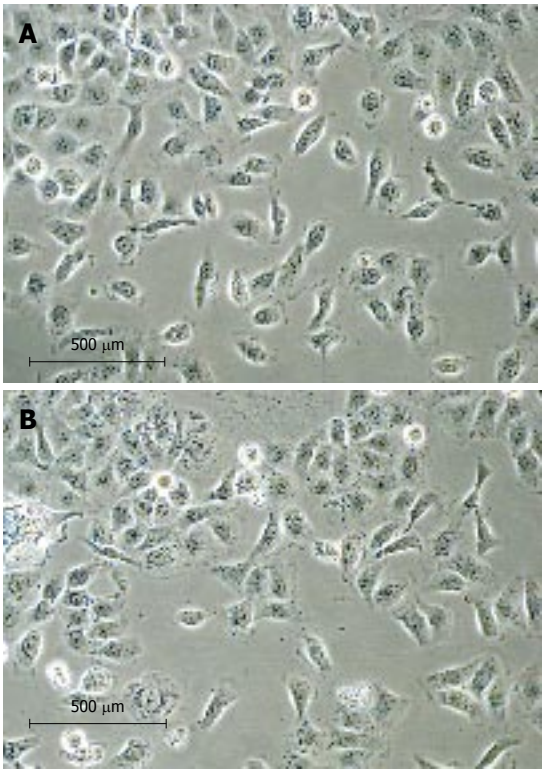

Figure 2 The RMCCA-1 culture under a phase contrast microscope at 20 × magnification. (**A**) at 16th passage; (**B**) at 30th passage. The RMCCA-1 cells exhibited circular to spindle shape with many processes and ornamental fringes. The nucleus and cytoplasm appeared granulated.

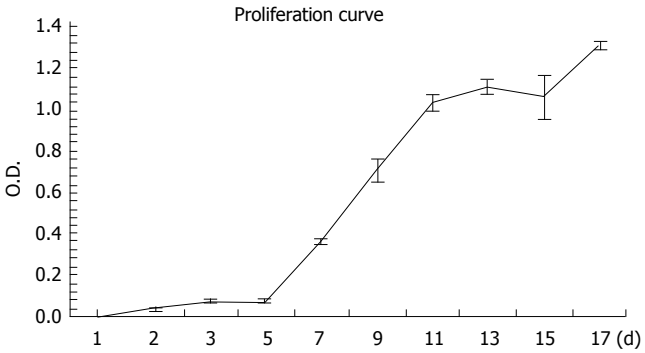

Figure 3 Growth kinetics of the RMCCA-1 cell line *in vitro* as analyzed by MTT assay. The tumor doubling time during the exponential phase of growth was 48 h. Each point represents mean ± SE from 3 independent experiments, each performed in triplicates.

Immunofluorescence staining

All RMCCA-1 cells showed positive staining with the AE-1/AE-3 monoclonal antibody mixture (Figure 4B), which recognizes the human epidermal cytokeratins, the signature of epithelial cells that distinguishes them from fibroblasts. Furthermore, specific markers for the adenocarcinoma and transitional cell carcinomas, cytokeratin 7 (Figure 4A) and 19 (data not shown), were also positive.

Chromosome analysis

The G-banding analysis demonstrated aneuploidy karyotype with marked structural abnormalities of the

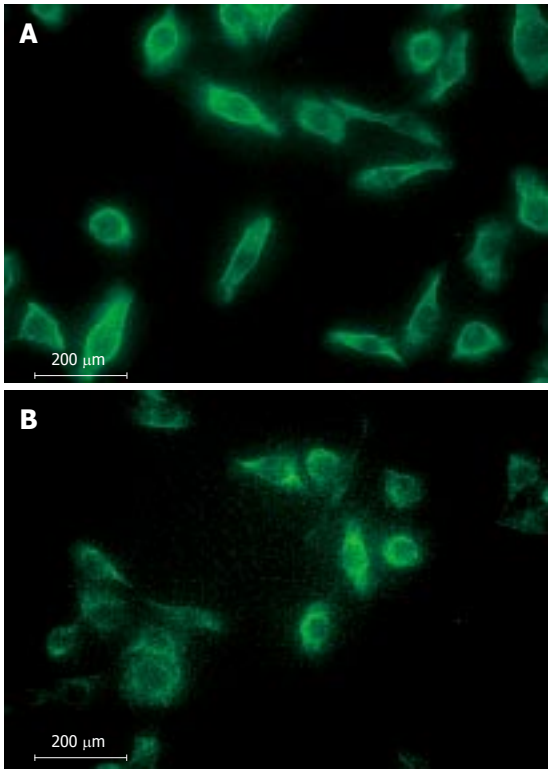

Figure 4 Immunofluoresence staining of RMCCA-1 cells with antibodies against (**A**) Cytokeratin 7; (**B**) AE-1/AE-3 at 40 × magnification.

chromosomes (Figure 5A and B). Although this cell line was established from a male patient, it lacks a Y chromosome and, instead, possesses two X chromosomes. The number of chromosomes ranges between 54 and 61, with a modal chromosome number of 59. The final karyotype was determined by consolidating the G-banding and mFISH results. Sixteen structural rearrangements were found, including 9 unbalanced translocations and 2 balanced translocations. In addition, the absence of Y chromosome in this cell line was also confirmed by both G-banding and mFISH techniques.

Tumor markers in spent media

The level of CA19-9 in the spent medium of RMCCA-1 cells (72.92 ± 4.35 U/mL) was similar to that of the patient's serum at admission (85.05 U/mL); both of which were about twice the maximum value of the normal range (39 U/mL, Table 1). The levels of CEA and AFP were normal (Table 1).

Invasiveness and motility

An important characteristic of metastatic cancer is the ability to migrate and invade the underlining basement membrane, the surrounding tissues and the blood vessels. We thus examine the invasiveness and motility of the RMCCA-1 cells, compared with those of three other established Cholangiocarcinoma cell lines from Thai patients (KKU-100, KKU-213 and HuCCA-1), using *in vitro* invasion and motility assay, respectively[10]. RMCCA-1 exhibited relatively higher migration rate (1,688 ± 207 cells/well) compared with the other 3 cell lines (1,514 ± 152 cells/well, 1,123 ± 163 cells/well, 40 ± 17 cells/well

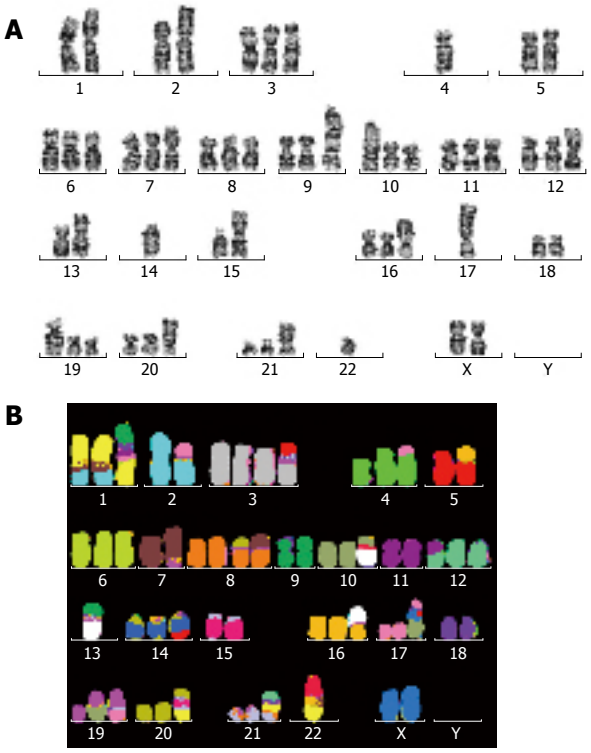

Figure 5 Representative karyotypes of RMCCA-1 cell line as assessed by (**A**) G-banding; (**B**) mFISH technique. The karyotype showed 54-61(3n)XX, -Y, der(1), t(1;2;7)(q31;q31;q22), t(2;17)(q33;q12), der(3)t(3;15)(q21;q?), t(4;17)(p14;q?), der(7)t(7;15)(p22;q?), der(8)t(8;7)(p12;?), der(10)t(10;13)(q21;q11), der(12)t(11;12)(q?,p11.2), der(13)t(13;9)(q11;q11), der(14)t(15;14)(q34;q32), der(16)t(13;16)(q11;p13.3), der(17)t(X;10;17)(p?,p?,p11.2), der(19)t(10;19)(q11.2;p13.3), der(19)t(17;19)(q?;q?), der(21)t(1;9;21)(?;?;q11), der(22)t(1;15;22)(?,?,?).

for KKU-213, KKU-100 and HuCCA-1, respectively). Surprisingly, the invasiveness of RMCCA-1 was significantly lower than those of KKU-213 and KKU-100 (181 ± 54 cells/well for RMCCA-1 *vs* 1021 ± 5 cells/well for KKU-213 and 765 ± 244 cells/well for KKU-100), while slightly higher than that of HuCCA-1 (38 ± 21 cells/well) (Figure 6).

Gelatin zymography

Most metastatic cells secrete proteinases to facilitate its invasion through tissue barriers. One of the most characterized families of tissue-degrading enzymes from cancer is the matrix-metalloproteinases (MMPs). Here we examined the ability to secrete the MMPs from RMCCA-1 cells by gelatin zymography. KKU-100 secretes a high level of *Mr* 72 000 band previously shown to correspond to MMP-2 (Figure 7)[11,12]. A *Mr* 72 000 band is also present in FBS. Presumably, this band corresponds to the endogenous gelatinase activity present in the FBS (our unpublished observation). In contrary, the conditioned medium of RMCCA-1 lacks the activity (Figure 7).

DISCUSSION

Cholangiocarcinoma (CCA) is a cancer arising from the bile duct epithelium. It is a lethal disease of which little is known about its biology, pathogenesis and behavior. Up to presence, there has been no effective early detection protocol or curative strategy for the disease; most patients

Table 1 Tumor marker levels in the spent media of RMCCA-1 and in the patient's serum at admission

	CEA (ng/mL)	AFP (U/mL)	CA 19-9 (U/mL)
[1]RMCCA-1	< 0.200	< 0.500	72.92 ± 4.35
Patient's serum	7.56	2.24	85.05
Normal serum	0.0-3.4	0.0-5.8	0.0-39

[1]The results shown represented mean ± SE from three independent experiments.

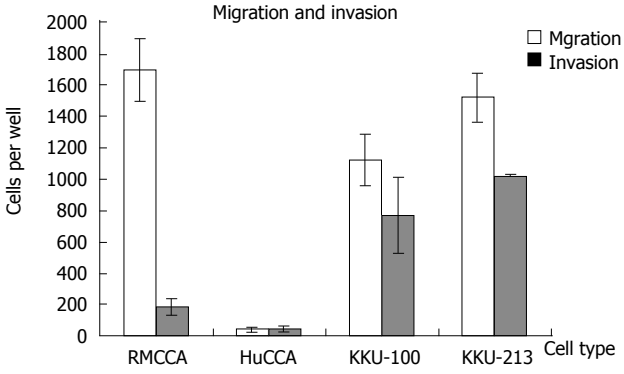

Figure 6 Invasion and migration rates of RMCCA-1 compared with KKU-213, KKU-100 and HuCCA-1 cell lines as determined by *in vitro* invasion and motility assay. Approximately 1 × 10^5 cells were seeded into the upper chamber of a transwell, and incubated for 6 h before the filter was fixed, stained, and the invading/migrating cells were counted under microscope.

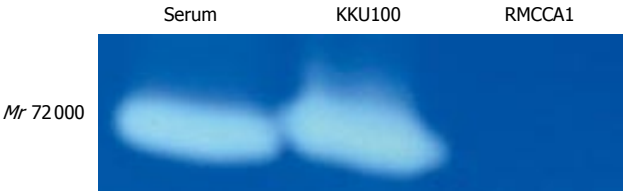

Figure 7 MMPs activity as determined by gelatin zymography. An intense band at *Mr* 72 000 was shown in the FBS (fetal bovine serum) and the conditioned medium of KKU-100, but not of the RMCCA-1 cells. The *Mr* 72 000 band in KKU-100 has previously been shown to correspond to MMP-2 activity in many cell lines[11,12].

seek curative treatment at advanced stage with poor prognosis. Therapeutic options for cholangiocarcinoma have been limited due to poor response to chemotherapy and radiation therapy. Surgery is perhaps the only effective cure although the 5-year survival after surgical treatment is less than 40%[2,5]. Therefore, it is urgently required that the molecular markers for early detection are mapped and molecular targets for effective treatment are deciphered. One way to understand the nature of such disease is by studying the behavior of cell lines derived from the tumor *in vitro*. Here we describe the establishment and preliminary characterization of a cell line derived from a peripheral cholangiocarcinoma of a Thai patient which we have named RMCCA-1.

The population doubling time of this cell line was about 48 h, where those of other cholangiocarcinoma cell lines originated from Thai patients were 55 h for HuCCA1[7] and 72 h for KKU100[8]. Although we have not yet deciphered the mechanism of growth regulation for

RMCCA-1, growth regulation mediated by COX-2, PGE_2, EGFR and Akt had been demonstrated in a number of cholangiocarcinoma cell lines, including CCLP1, HuCCT1, SG231[13].

Analysis of the spent medium from RMCCA-1 cells showed that these cells have retained some functional characteristics of the original tumor, including over expression of the tumor marker CA19-9. The levels of CA19-9 in the spent medium of RMCCA-1 cells (72.92 ± 4.35 U/mL) was similar to that of the patient's serum at admission (85.05 U/mL); both of which were approximately twice the maximum value of the normal range (39 U/mL, Table 1). CA19-9 was often detected in patients with malignant cholangiocarcinoma and pancreatic cancer[14]. Many kinds of cancer cell lines also secrete CA19-9, including those derived from cholangiocarcinoma (TK)[15], pancreatic carcinoma (SUIT-2)[16] and colon cancer (SW1116)[17].

Analysis by immunofluorescence staining using monoclonal antibodies to AE1 and AE3 showed positive staining for human cytokeratins, verifying the epithelial origin of the tumor. RMCCA-1 also stained positively with antibodies to cytokeratins 7 and 19, distinguishing the bile duct epithelial cells from the hepatocytes. Together, these data confirm that RMCCA-1 indeed derived from the epithelium of the bile duct.

Tumor metastasis involves a series of complex processes including dysregulation of cell adhesion, cell motility, and enzymatic proteolysis of basement membrane and extracellular matrix. The RMCCA-1 cells exhibit a high level of migration rate, contradicting the relatively low level of invasiveness and the absence of MMP activity (Figures 6 and 7). The low level of invasiveness in RMCCA-1 can, at least in part, be explained by the lack of MMP activity in this cell line. Unlike the RMCCA-1 cells, KKU-100 exhibits a high degree of invasiveness, correlating with a high level of MMPs activity (Figures 6 and 7). Karyotype analysis by G-banding revealed a complex pattern of chromosomal abnormalities. Most of the chromosomes were triplicates, suggesting that the cell line was originated from a tripoid cell. The lost of Y chromosome was not unusual as similar finding has been shown in human sarcomatous cholangiocarcinoma (SCK) cells[18]. Other aberrations involving Y chromosome have also been reported, including the translocation between Y chromosome and chromosome 1 as shown in a human cholangiocarcinoma cell line, PCI-SG231[19].

With recent development of mFISH technique, it has become possible to identify and characterize complex chromosomal aberrations, previously unrevealed by G-banding analysis. This technique has allowed us to characterize the karyotype of RMCCA-1 in much greater detail with a high level of accuracy.

We have detected aberrations of chromosomes 1, 5, 7, and 12 in the RMCCA-1 cells, which were consistent with those of the other human cholangiocarcinoma cell lines including SCK, JCK, Cho-CK, Choi-CK, CC-SW-I, CC-LP-I, PCI: SG231, and RPMI-7451, and a rat cholangiocarcinoma cell line, CC-62[18-21]. In contrast, we did not detect structural rearrangement of X chromosome and chromosome 6 in the RMCCA-1 cells, nor did we detect the lost of chromosome 18, as found in SCK, JCK, Cho-CK, and Choi-CK[18].

In conclusion, we successfully established a new cholangiocarcinoma cell line which we named RMCCA-1. We have also performed preliminary characterization of its growth characteristics, karyotype, secreted tumor markers and invasive properties. This cell line will be further used in our research towards understanding and combating against cholangiocarcinoma.

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REFERENCES

1 **Khan SA**, Davidson BR, Goldin R, Pereira SP, Rosenberg WM, Taylor-Robinson SD, Thillainayagam AV, Thomas HC, Thursz MR, Wasan H. Guidelines for the diagnosis and treatment of cholangiocarcinoma: consensus document. *Gut* 2005; **51**: vi1-vi9

2 **Sirica AE**. Cholangiocarcinoma: molecular targeting strategies for chemoprevention and therapy. *Hepatology* 2005; **41**: 5-15

3 **de Groen PC**, Gores GJ, LaRusso NF, Gunderson LL, Nagorney DM. Biliary tract cancers. *N Engl J Med* 1999; **341**: 1368-1378

4 **Vatanasapt V**, Sriamporn S, Vatanasapt P. Cancer control in Thailand. *Jpn J Clin Oncol* 2002; **32** Suppl: S82-S91

5 **Thongprasert S**. The role of chemotherapy in cholangiocarcinoma. *Ann Oncol* 2005; **16** Suppl 2: ii93-ii96

6 **Sripa B**, Pairojkul C. Pathology of Cholangiocarcinoma. In Liver Cancer in Thailand. 2000: 84-88

7 **Sirisinha S**, Tengchaisri T, Boonpucknavig S, Prempracha N, Ratanarapee S, Pausawasdi A. Establishment and characterization of a cholangiocarcinoma cell line from a Thai patient with intrahepatic bile duct cancer. *Asian Pac J Allergy Immunol* 1991; **9**: 153-157

8 **Sripa B**, Leungwattanawanit S, Nitta T, Wongkham C, Bhudhisawasdi V, Puapairoj A, Sripa C, Miwa M. Establishment and characterization of an opisthorchiasis-associated cholangiocarcinoma cell line (KKU-100). *World J Gastroenterol* 2005; **11**: 3392-3397

9 **MacDougall JR**, Bani MR, Lin Y, Rak J, Kerbel RS. The 92-kDa gelatinase B is expressed by advanced stage melanoma cells: suppression by somatic cell hybridization with early stage melanoma cells. *Cancer Res* 1995; **55**: 4174-4181

10 **Frandsen TL**. Assays for the study of human cancer cell invasion and metastasis. *Fibrinolysis* 1992; **6**: 71-76

11 **Quax PH**, de Bart AC, Schalken JA, Verheijen JH. Plasminogen activator and matrix metalloproteinase production and extracellular matrix degradation by rat prostate cancer cells in vitro: correlation with metastatic behavior in vivo. *Prostate* 1997; **32**: 196-204

12 **Muangman S**, Thippornwong M, Tohtong R. Anti-metastatic effects of curcusone B, a diterpene from Jatropha curcas. *In Vivo* 2005; **19**: 265-268

13 **Xu L**, Han C, Wu T. A novel positive feedback loop between PPARdelta and PGE2 signaling pathways for human cholangiocarcinoma cell growth. *J Biol Chem* 2006; **11**: 1-44

14 **Ozkan H**, Kaya M, Cengiz A. Comparison of tumor marker CA 242 with CA 19-9 and carcinoembryonic antigen (CEA) in pancreatic cancer. *Hepatogastroenterology* 2003; **50**: 1669-1674

15 **Watanabe M**, Chigusa M, Takahashi H, Nakamura J, Tanaka H, Ohno T. High level of CA19-9, CA50, and CEA-producible human cholangiocarcinoma cell line changes in the secretion ratios in vitro or in vivo. *In Vitro Cell Dev Biol Anim* 2000; **36**: 104-109

16 **Taniguchi S**, Iwamura T, Kitamura N, Yamanari H, Kojima A, Hidaka K, Seguchi K, Setoguchi T. Protein-free culture of the human pancreatic cancer cell line, SUIT-2. *Hum Cell* 1994; **7**: 207-214
17 **Adachi M**, Sekine T, Umemoto A, Tsukikawa S, Imai K, Yachi A. Mechanism of clearance of circulating CA19-9 in rats. *Tumour Biol* 1990; **11**: 51-58
18 **Kim DG**, Park SY, You KR, Lee GB, Kim H, Moon WS, Chun YH, Park SH. Establishment and characterization of chromosomal aberrations in human cholangiocarcinoma cell lines by cross-species color banding. *Genes Chromosomes Cancer* 2001; **30**: 48-56
19 **Storto PD**, Saidman SL, Demetris AJ, Letessier E, Whiteside TL, Gollin SM. Chromosomal breakpoints in cholangiocarcinoma cell lines. *Genes Chromosomes Cancer* 1990; **2**: 300-310
20 **Shimizu Y**, Demetris AJ, Gollin SM, Storto PD, Bedford HM, Altarac S, Iwatsuki S, Herberman RB, Whiteside TL. Two new human cholangiocarcinoma cell lines and their cytogenetics and responses to growth factors, hormones, cytokines or immunologic effector cells. *Int J Cancer* 1992; **52**: 252-260
21 **Gil-Benso R**, Martinez-Lorente A, Pellin-Perez A, Navarro-Fos S, Gregori-Romero MA, Carda C, Callaghan R, Peydro-Olaya A, Llombart-Bosch A. Characterization of a new rat cell line established from 2'AAF-induced combined hepatocellular cholangiocellular carcinoma. *In Vitro Cell Dev Biol Anim* 2001; **37**: 17-25

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CLINICAL RESEARCH

Enhanced expression of interleukin-18 in serum and pancreas of patients with chronic pancreatitis

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Abstract

AIM: To investigate interleukin-18 (IL-18) in patients with chronic panreatitis (CP).

METHODS: We studied 29 patients with CP and 30 healthy controls. Peripheral blood mononuclear cells (PBMC) were isolated and incubated with 50 mmol/L ethanol, lipopolysaccharide (LPS) (doses 25 g/L, 250 g/L, 2500 g/L) and both agents for 24 h. Levels of IL-18 in the supernatants, and levels of IL-18, IL-12, interferon (IFN)-γ and soluble CD14 in the serum were analysed by ELISA technique. Expression of IL-18 in PBMC was investigated by reverse-transcription (RT)-PCR. IL-18 protein levels in CP tissue and in normal pancreas were studied by ELISA technique. IL-18 levels in PBMC and pancreatic tissue were determined by Westernblot. Immunohistochemistry for pancreatic IL-18 expression was performed.

RESULTS: In patients, IL-18 serum levels were significantly enhanced by 76% (mean: 289.9 ± 167.7 ng/L) compared with controls (mean: 165.2 ± 43.6 ng/L; $P < 0.0005$). IL-12 levels were enhanced by 25% in patients (18.3 ± 7.3 ng/L) compared with controls (14.7 ± 6.8 ng/L, $P = 0.0576$) although not reaching the statistical significance. IFN-γ and soluble CD14 levels were not increased. *In vitro*, LPS stimulated significantly and dose-dependently IL-18 secretion from PBMC. Incubation with ethanol reduced LPS-stimulated IL-18 secretion by about 50%. The mRNA expression of IL-18 in PBMC and the response of PBMC to ethanol and LPS was similar in CP patients and controls. In PBMC, no significant differences in IL-18 protein levels were detected between patients and controls. IL-18 protein levels were increased in CP tissues compared to normal pancreatic tissues. IL-18 was expressed by pancreatic acinar cells and by infiltrating inflammatory cells within the pancreas.

CONCLUSION: IL-18 originates from the chronically inflammed pancreas and appears to be involved in the fibrotic destruction of the organ.

Key words: Chronic pancreatitis; Cytokines; Interleukin-18; Pancreatic fibrosis

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INTRODUCTION

Chronic pancreatitis represents an inflammatory disease characterized by repeated attacks of acute pancreatitis, severe abdominal pain, progressive destruction of the pancreatic tissue with fibrous replacement of the parenchyma leading to both exocrine and endocrine insufficiency[1]. In industrialized countries, excessive alcohol consumption is associated with the development of chronic pancreatitis in the majority of patients[2,3]. The early stages of the human disease remain almost inaccessible to investigation. Recent genetic findings suggest that premature digestive enzyme activation with subsequent pancreatic autodigestion represents a dominant factor in the initiation of acute pancreatitis[4,5]. These genetic studies also support a progressive link between repeated episodes

of acute pancreatitis and the development of chronic pancreatitis[5]. However, the exact immune mechanisms underlying the progression of chronic pancreatitis remain unclear.

The identification and characterization of pancreatic stellate cells (PSC) provided deep insights into the development of pancreatic fibrosis[6,7]. Activated PSC synthesize and secrete increased amounts of extracellular matrix proteins resulting in the fibrotic destruction of the pancreas[6,7]. Recent *in vitro* studies have demonstrated that alcohol and its metabolite acetaldehyde[8], oxidative stress[8], growth factors such as platelet derived growth factor[6], and the cytokine transforming growth factor (TGF)-β1[6] have the capacity to activate PSC during pancreatic injury. More recently, it was shown that PSC also responds to additional proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, and antiinflammatory cytokines such as IL-10[9].

During chronic pancreatitis, lymphocytes and mononuclear cells infiltrate the pancreas and contribute to the local progression of the disease through T-lymphocyte mediated cytotoxicity and production of cytokines[10-14]. However, data regarding the role of cytokines in chronic pancreatitis remain limited. IL-18 represents a proinflammatory cytokine that plays an important role in the Th-1 response due to its ability to induce interferon (IFN)-γ production in T-cells and natural killer cells[15,16]. IL-18 has been investigated in a variety of inflammatory and autoimmune human diseases[17]. In previous studies, our group and others have demonstrated an upregulation of serum IL-18 levels in patients with acute pancreatitis[18-20]. *In vitro* studies have shown that endotoxin induces IL-18 gene expression and secretion in human peripheral blood mononuclear cells (PBMC)[21]. In a previous investigation, the serum levels and gene expression of IL-18 in PBMC of patients with alcoholic liver cirrhosis were significantly enhanced compared to healthy controls, and IL-18 levels correlated with plasma endotoxin levels[22].

These data raise the possibility that IL-18 also participates in the immune mechanisms that result in the fibrotic destruction of the pancreas during chronic pancreatitis. Thus, the aim of the present study was to investigate this cytokine in patients with chronic pancreatitis. We determined the serum levels of IL-18, IFN, IL-12 and soluble CD14 in patients with chronic pancreatitis and healthy controls. We performed several *in vitro* studies with PBMC. We determined the protein expression of IL-18 in chronic pancreatitis tissue and in normal pancreas, and conducted immunohistochemical investigations in human pancreatic tissues.

MATERIALS AND METHODS

Patients

A total of 29 patients (22 males, 7 females; mean age 52 ± 11 years) with alcoholic and non-alcoholic chronic pancreatitis and 30 healthy controls with no history of alcohol abuse (10 males, 20 females; mean age 39 ± 11 year) were prospectively enrolled into the study at the University Hospital of Heidelberg at Mannheim, Mannheim, Germany. The study was approved by the Ethics Committee of the Faculty of Clinical Medicine Mannheim, University of Heidelberg, Germany. Written informed consent was obtained from each participant.

Data on the history of both alcohol consumption and the clinical course of pancreatic disease were assessed by patient self-report and review of the medical records. In each participant, a detailed history of alcohol intake was established using different screening methods, including the Lübeck alcohol dependence and abuse screening test (LAST)[23], the alcohol use disorders identification test (AUDIT)[24], the lifetime drinking history (LDH)[25], and a patient interview questioning ICD-10 criteria of chronic alcohol dependence[26]. Alcoholic disease etiology was established with the presence of at least one of the following criteria: (1) patient self-report of alcohol abuse as cause of the disease, (2) patient self-report with a history of excessive alcohol intake of at least 80 g per day in males and 60 g per day in females for some years, or (3) smaller amounts of daily alcohol intake in combination with answers gained from the above mentioned screening methods for alcohol consumption that allowed the diagnosis of chronic alcohol abuse.

The diagnosis of chronic pancreatitis required the typical clinical features of chronic pancreatitis with or without recurrent episodes of acute pancreatitis, and was based on the determination of pancreatic exocrine and endocrine function, pancreatic imaging or histological tissue examination. Pancreatic imaging was performed either by endoscopic retrograde pancreatography (ERP), computed tomography (CT), magnetic resonance imaging (MRI), or endosonography. In each patient, abdominal ultrasound was performed. According to the definitions of an international workshop on chronic pancreatitis[27], patients were further classified as suffering "definite" or "probable" chronic pancreatitis. Briefly, the classification of "definite" chronic pancreatitis required a typical clinical history of chronic pancreatitis and one or more of the following criteria: (1) calcifications in the pancreas, (2) moderate to marked ductal lesions, (3) marked exocrine insufficiency, and (4) typical histology of an adequate surgical specimen[27]. For the diagnosis of "probable" chronic pancreatitis, one or more of the following criteria were present in addition to the typical clinical features: (1) mild ductal alterations, (2) recurrent or persistent pseudocysts, (3) pathological secretin test, and (4) endocrine insufficiency[27].

In all patients, an episode of acute pancreatitis at the time of recruitment or within the last two months before inclusion into the study was excluded. Additional exclusion criteria were infections with fever or leukocytosis, liver cirrhosis and surgical or endoscopic interventions within 2 mo before examination.

Routine laboratory parameters

Routine laboratory parameters were determined in serum samples taken at the same time as samples for cytokine and endotoxin measurement. These parameters included serum concentrations of amylase, lipase, C-reactive protein (CRP), creatinine, and total white blood cell count. Endocrine pancreatic insufficiency was determined by the presence

of diabetes mellitus requiring antidiabetic treatment or records of an abnormal oral glucose tolerance test. Exocrine pancreatic insufficiency was defined by diarrhea, steatorrhea or maldigestion that was markedly reduced by enzyme supplementation. In some patients, pancreatic exocrine function was determined by using one or more of the following commercially available tests according to the manufacturer's recommendations: measurement of fecal chymotrypsin by a colorimetric method (Chymo, Boehringer, Germany), determination of fecal fat excretion by infrared reflection method (Esetek Analyser Fenir 8820, TSZ Stimotron AG, Wettenberg-Launsbach, Germany), measurement with the Pankreolauryl test N (Temmler Pharma GmbH, Marburg, Germany) or application of the pancreozymin-secretin-test (Sekretolin Diagnostikum, Hoechst AG, Germany or Takus, Pharmacia GmbH, Erlangen, Germany).

PBMC isolation and incubation with ethanol and endotoxin

Peripheral venous blood was collected from patients and healthy controls into sterile, pyrogen-free disposable syringes with endotoxin-free heparin (10 ku/L). As reported previously[22], PBMC was separated by standard density gradient centrifugation (Ficoll-Paque method) and adjusted to 3×10^9 cells/L in RPMI 1640 supplemented with 100 mL/L heat inactivated fetal bovine serum. PBMC were incubated with or without lipopolysaccharide (LPS) (doses 25 g/L, 250 g/L, 2500 g/L), in the absence or presence of 50 mol/L ethanol for 24 h. Cells were spun down, and PBMC supernatants were stored at -20℃ until measurement of cytokine levels. For RNA extraction, cells were stored at -80℃ in 4 mol/L GTC extraction buffer.

Immunoassay for IL-18, IFN, IL-12, soluble CD14

Concentrations of IL-18 in the serum, supernatants of PBMC and pancreatic tissues were determined by a specific sandwich enzyme linked immunoassay (ELISA; Fujisaki Institute, Hayashibara Biochemical Laboratories, Inc., Okayama, Japan) with minor modifications as described previously[22]. Serum concentrations of IFN were determined by a specific ELISA using two monoclonal antibodies as described previously[28]. Serum concentrations of IL-12 and soluble CD14 were also determined by ELISA technique as described previously[28].

RNA isolation and IL-18 RT-PCR analysis in PBMC

RNA extraction from PBMC was performed using acid phenol-chloroform extraction[22]. The RNA concentration was quantified spectrophotometrically. Complementary DNA of PBMC was obtained by reverse-transcription (RT) using 1 μg RNA and oligo d(T) primers. PCR for IL-18 and the housekeeping enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed as described previously[22]. Densitometric assessment of the PCR products was performed using EASY Plus 3.2 software (Herolab, Wiesloch, Germany). Semiquantitative PCR results were obtained by grading a ratio between the densitometry results of IL-18 and GAPDH.

Endotoxin assay

Plasma endotoxin levels were determined using an automated kinetic turbodimetric limulus amoebocyte lysate microtiter test as described previously[22].

Pancreatic tissue samples

Pancreatic tissue samples were obtained from 8 patients with chronic pancreatitis and from 2 individuals without pancreatic disease. Two of these patients with chronic pancreatitis were obtained from the present investigation and had later to be operated due to the development of a benign pancreatic mass and intractable pancreatic pain. The remaining 6 patients with chronic pancreatitis and the 2 individuals without pancreatic disease were obtained from clinical routine interventions. In 4 of these patients with chronic pancreatitis, surgery was necessary due to the development of pancreatic cancer.

Human pancreatic tissue and cell lysates of PBMC were homogenized in 5 mmol/L Hepes, pH 7.0, 280 mmol/L mannitol, 10 mmol/L KCl, 1 mmol/L $MgCl_2$, 1 mmol/L benzamidine, 20 mg/L trypsin inhibitor, 1 μmol/L leupeptin, and 0.2 mmol/L PMSF and boiled in electrophoresis sample buffer. Proteins were electrophoretically separated on SDS-125 g/L polyacrylamide gels. Electrotransfer to nitrocellulose membranes was done as described previously[29]. After staining with 2 g/L Ponceau S to check the efficiency of the transfer, free binding sites of the membrane were blocked with 10 g/L bovine serum albumin in Tris-buffered saline (10 mmol/L Tris-HCl, pH 8.0, and 150 mmol/L NaCl) for 60 min, followed by 75 min incubation with anti-IL-18 antibody (Natutec, Frankfurt, Germany) diluted in Tris-buffered saline plus 2 mL/L Tween 20. Bound antibodies were visualized with secondary antibodies conjugated to horse radish peroxidase using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Freiburg, Germany) and autoradiography film (Fujifilm Super HR-E 30, Fuji photo film, Düsseldorf, Germany).

Immunohistochemistry

Frozen tissue specimen were cut in 10 μm cryostat sections, transferred on glass slides and air-dried overnight. Immunohistochemistry was performed according to the streptavidin-biotin method. Sections were washed in tris-buffered saline (TBS) and incubated with 30 g/L bovine serum albumine (BSA) for 10 min at room temperature to block non-specific antibody reactions. The sections were incubated overnight at 4℃ with the primary antibody at a 1:5000 dilution (anti-human IL-18 polyclonal antibody, NatuTec, Frankfurt am Main, Germany). The slides were then rinsed repeatedly with TBS and were incubated with a biotin-streptavidin-conjugated secondary antibody (goat anti-rabbit immunoglobulin-specific antibody, Jackson Immuno Research, West Grove, USA) for 30 min at room temperature. The slides were washed again with TBS and were treated with a streptavidin-alkaline-phosphatase complex. Liquid diaminobenzidine was added as chromogen, and counterstaining was performed with hematoxylin. Controls were performed by using mouse serum (Sigma, Saint Louis, USA) as primary antibody and by administration of recombinant human IL-18 (MoBiTec, Göttingen, Germany) in excess to the primary antibody

before incubation. Light microscopical investigations were performed using a Zeiss Axioskop microscope.

Statistical analysis

We used the *t* test, if necessary with the Welch correction for unequal variances, for analysis of IL-18, IFN, IL-12 and soluble CD14 levels between patients and controls. These data are expressed as mean ± SD. $P < 0.05$ was considered significant. We used paired *t* tests for the comparison of IL-18 secretion from PBMC. The Bonferroni-Holm correction was applied to adjust for calculating three tests at a time to compare the IL-18 secretion from PBMC after different stimulations.

RESULTS

Clinical characteristics

Alcoholic chronic pancreatitis was diagnosed in 23 patients, and non-alcoholic chronic pancreatitis was found in 6 patients (Table 1). According to the recommendations of a workshop on chronic pancreatitis, 21 patients were classified with "definite" chronic pancreatitis, and 8 patients were classified with "probable" chronic pancreatitis (Table 1). An episode of acute pancreatitis was excluded in all patients by physical examination, abdominal ultrasound and determination of routine laboratory parameters (Table 2).

Table 1 Clinical characteristics of patients with chronic pancreatitis and healthy controls

Clinical characteristics	Patients	Controls
n	29	30
Age (yr)	52 ± 11	39 ± 11
Sex (Male/Female)	22/7	10/20
Alcoholic chronic pancreatitis	23/29	NA[1]
Non-alcoholic chronic pancreatitis	6/29	NA[1]
Definite chronic pancreatitis	21/29	NA[1]
Probable chronic pancreatitis	8/29	NA[1]
Exocrine insufficiency	16/29	NA[1]
Endocrine insufficiency	11/29	NA[1]

[1]Not applicable.

Table 2 Routine laboratory parameters in patients with chronic pancreatitis and healthy controls (mean ± SD)

Parameter	Normal range	Patients	Controls
White cell blood count	3.6-11.0 × 10^9/L	7.0 ± 1.9	6.0 ± 1.2
C-reactive protein	< 5 mg/L	6.4 ± 7.7	3.2 ± 0.9
Creatinine	6-11 mg/L	0.9 ± 0.3	0.9 ± 0.2
Amylase	8.4-31.7 U/L	33.0 ± 22.6	33.0 ± 17.4
Lipase	< 190 U/L	182.0 ± 98.0	161.0 ± 64.0

Serum levels of IL-18, IL-12 and IFN in CP patients

The fasted serum IL-18 levels were significantly enhanced by 76% in 29 patients with chronic pancreatitis (mean ± SD: 289.9 ± 167.7 ng/L) compared to healthy controls ($n = 30$; 165.2 ± 43.6 ng/L; Welch's T test, $P < 0.0005$). The IL-12 levels were enhanced by 25% in patients ($n = 27$ due to lack of sample in 2 patients; mean ± SD: 18.3 ± 7.3 ng/L) compared to controls ($n = 30$, 14.7 ± 6.8 ng/L). We observed a trend towards a statistically significant difference between patients and control subjects (T test, $P = 0.0576$). Serum IFN levels were not increased in patients ($n = 25$ due to lack of sample in 4 patients; 28 ± 15.4 ng/L) compared to controls ($n = 24$ due to lack of sample in 6 controls; 34.4 ± 18.0 ng/L; T test, $P = 0.18$).

Serum soluble CD14 and plasma endotoxin levels in CP patients

Serum levels of soluble CD14 were similar in patients ($n = 16$, mean ± SD: 2723.3 ± 649.2 ng/L) and healthy controls ($n = 26$, mean ± SD: 2630.6 ± 480.3 ng/L; Welch's T-test: $P = 0.63$). Endotoxemia was not detectable in patients ($n = 29$) and controls ($n = 30$).

Expression of IL-18 mRNA in PBMC of CP patients

We investigated the IL-18 mRNA expression in PBMC of patients with chronic pancreatitis by RT-PCR to determine if the enhanced serum IL-18 levels in chronic pancreatitis represent a result of an increased gene expression in PBMC. No significant difference was found between the mRNA expression of IL-18 in patients with chronic pancreatitis ($n = 5$ patients with highest IL-18 serum levels) in comparison to healthy controls ($n = 5$ control individuals with lowest IL-18 serum levels) (Figure 1). The semiquantitative analysis of the ratio between the densitometric results from the PCR-products of IL-18 and GAPDH did not reveal a significant difference. The ratio for patients with chronic pancreatitis and healthy controls was 0.438 ± 0.074 and 0.410 ± 0.082, respectively.

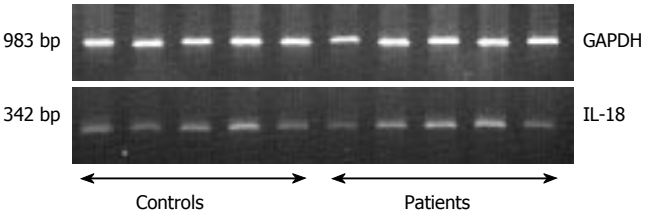

Figure 1 Analysis of RT-PCR amplification of IL-18 in PBMC of CP patients.

IL-18 protein levels in pancreatic tissues

IL-18 protein levels in pancreatic tissues were determined by ELISA technique in two patients with chronic pancreatitis, and in two individuals without pancreatic disease. The protein levels of IL-18 in chronic pancreatitis tissue ($n = 2$, 50.5 ng/L and 86.1 ng/L) were enhanced compared to pancreatic tissue from individuals without pancreatic disease ($n = 2$, 33.6 ng/L and 25.1 ng/L). In these patients and control subjects, IL-18 protein levels in the pancreas were also determined by Westernblot (Figure 2A). The IL-18 protein levels were again enhanced in patients with chronic pancreatitis compared to the control individuals (Figure 2A).

IL-18 protein levels in cell lysates of PBMC

IL-18 protein levels were determined in cell lysates of PBMC by Westernblot investigations (Figure 2B). We

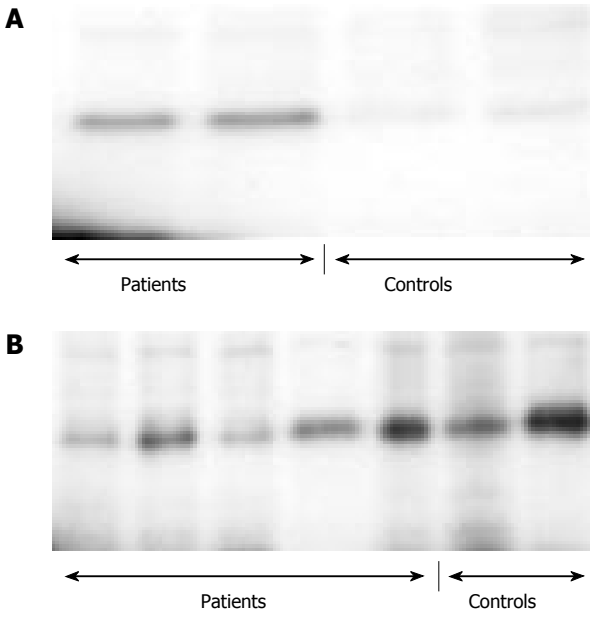

Figure 2 IL-18 in cell lysates of PBMC and in pancreatic tissue of CP patients. **A**: In the pancreatic tissue; **B**: In cell lysates of PBMC.

studied PBMC cell lysates from 5 patients with chronic pancreatitis and from 2 control individuals to determine if the elevated levels of IL-18 in patients with chronic pancreatitis result from an increased expression of IL-18 in PBMC. We confirmed IL-18 protein expression in the cell lysates of both patients and controls. However, we did not detect significant differences in IL-18 protein expression between cell lysates from patients and controls (Figure 2B).

IL-18 levels in the supernatants from PBMC after incubation with ethanol and LPS

We investigated the IL-18 levels in the supernatants of PBMC from all patients (n = 29) and all controls (n = 30). We stimulated the PBMC with ethanol and LPS to detect a possible influence of these agents on IL-18 secretion from PBMC and to reveal possible differences between PBMC from patients and control subjects. These *in vitro* studies demonstrated that the basal secretion of IL-18 from PBMC was similar in patients and control individuals.

The stimulation of PBMC with LPS (25 g/L, 250 g/L and 2500 g/L) and the stimulation with LPS (25 g/L, 250 g/L and 2500 g/L) together with ethanol (50 mmol/L) resulted in a dose related and statistically significant enhancement of IL-18 secretion from PBMC of patients and controls (T tests; $P < 0.0007$) (Figure 3A and B). However, we did not detect significant differences in the secretion patterns of IL-18 between PBMC from patients and control individuals.

The incubation with ethanol alone for 24 h did not affect basal IL-18 secretion, but ethanol significantly reduced LPS-stimulated IL-18 secretion by about 50% compared to LPS stimulation alone (T tests; $P < 0.0001$) (Figure 3A and B).

The application of the Bonferroni-Holm correction did not change the significance of previously obtained P values.

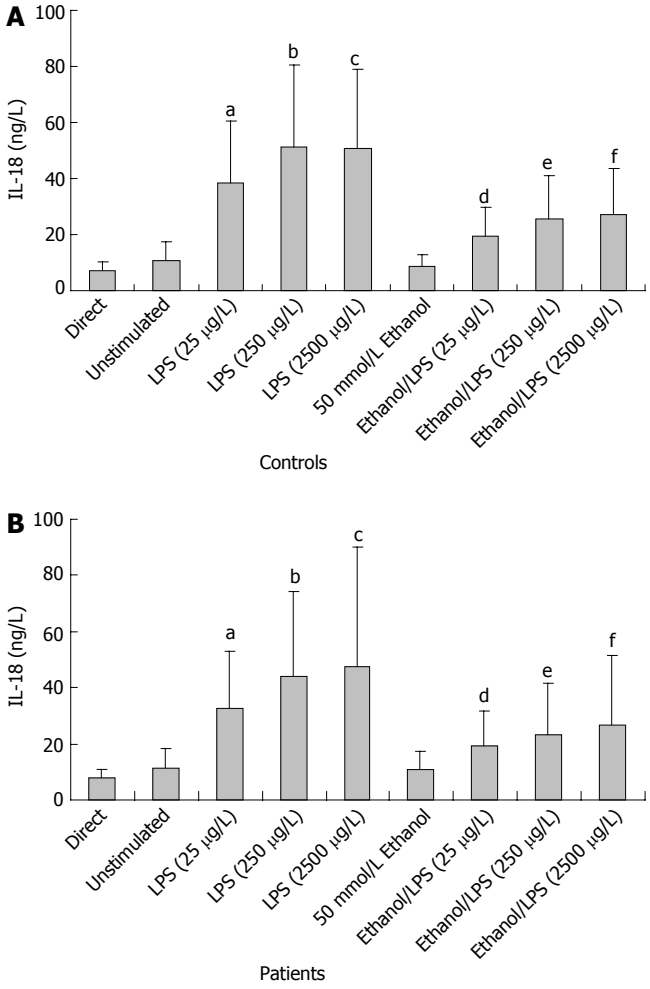

Figure 3 *In vitro* IL-18 secretion from PBMC of CP patients. [a]$P < 0.0007$ 25 μg/L LPS *vs* unstimulated; [b]$P < 0.0007$ 250 μg/L LPS *vs* unstimulated; [c]$P < 0.0007$ 2500 μg/L LPS *vs* unstimulated; [d]$P < 0.0001$ Ethanol/25 μg/L LPS *vs* 25 μg/L LPS; [e]$P < 0.0001$ Ethanol/250 μg/L LPS *vs* 250 μg/L LPS; [f]$P < 0.0001$ Ethanol/2500 μg/L LPS *vs* 2500 μg/L LPS.

Immunohistochemistry

We performed immunohistochemical investigations for IL-18 in pancreatic tissues from 6 patients with chronic pancreatitis. In 4 of these patients with chronic pancreatitis, invasive ductal adenocarcinoma of the pancreas had developed and was the reason for pancreatic surgery. In these patients, we investigated the chronic pancreatitis tissue which was free of pancreatic carcinoma tissues. The immunohistochemical analysis of the chronic pancreatitis tissue revealed that clusters of infiltrating mononuclear cells were stained positive for IL-18 by using anti-IL-18 antibody (Figure 4A). In chronic pancreatitis tissue, clusters of pancreatic acinar cells were also stained positive for the expression of IL-18 (Figure 4B). Thus, IL-18 appears to be expressed in both infiltrating inflammatory cells and pancreatic acinar cells during chronic pancreatitis. In addition, we studied sections from pancreatic carcinoma tissue, and detected positive staining for anti-IL-18 antibody in pancreatic carcinoma cells as well. In general, staining for IL-18 was more pronounced in the samples with pancreatic carcinoma than in the samples from patients with chronic pancreatitis without pancreatic carcinoma. Control investigations with mouse serum and with the IL-18-

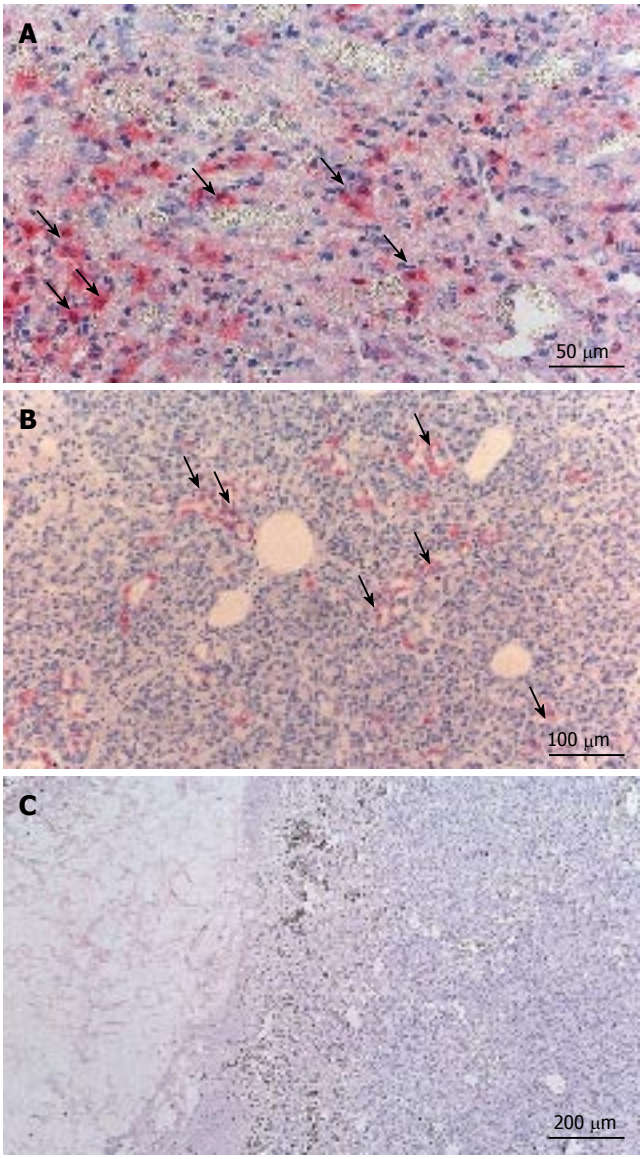

Figure 4 IL-18 expression in pancreatic tissue of CP patients. **A**: Clusters of infiltrating mononuclear cells stained positive; **B**: Clusters of pancreatic acinar cells also stained positive for the expression of IL-18; **C**: No positive staining for IL-18 in the negative control (mouse serum with IL-18 antigen-antibody mixture).

antigene-antibody-mixture revealed no positive staining for IL-18 and confirmed the accuracy of the method (Figure 4C).

DISCUSSION

The major findings of the present investigation are: (1) the fasted serum levels of IL-18 are significantly enhanced by 76% in patients with chronic pancreatitis compared to healthy control individuals; (2) the mRNA expression of IL-18, the protein levels of IL-18, and the *in vitro* secretion of IL-18 after stimulation with ethanol and endotoxin is similar in PBMC from patients with chronic pancreatitis and from healthy control subjects; (3) *in vitro*, ethanol significantly reduces endotoxin-stimulated IL-18 secretion by about 50%; (4) the IL-18 protein levels in chronic pancreatitis tissue are increased; and (5) IL-18 is expressed in pancreatic acinar cells and infiltrating inflammatory cells within the pancreas.

These results suggest that PBMC are not the main source of the enhanced serum levels of IL-18 in patients with chronic pancreatitis. It rather appears that IL-18 originates from the chronically inflammed pancreas. Indeed, there is increasing evidence that immunological mechanisms play an important role in the development and progression of chronic pancreatitis[9-13,30,31]. Cytokines, growth factors and other immunological mediators are produced by resident cells and recruited cells within the chronically inflammed pancreas and contribute to pancreatic fibrosis[14]. In addition, pancreatic acinar cells also produce, release and respond to cytokines[32]. Thus, we assume that IL-18 participates in the destruction of the pancreas during chronic pancreatitis.

Alcohol consumption represents an important risk factor for the development of chronic pancreatitis. Chronic alcohol consumption leads to an increased gut permeability with subsequent endotoxemia, and endotoxin has been reported as a mediator of alcohol induced liver damage[33]. Studies in ethanol-fed rats also suggest a role of endotoxin in the development of pancreatic injury[34]. Endotoxin strongly induced IL-18 gene expression in PBMC, and the response to endotoxin was mainly regulated by the expression of CD14[21]. In our study, we did not detect endotoxin in the plasma of patients with chronic pancreatitis, and the soluble CD14 levels were similar in patients and control individuals. However, it has to be stressed that the present study was not designed to investigate the full impact of endotoxin on the development of pancreatic damage. Enhanced endotoxin plasma levels may only be detectable for short periods of time during acute ethanol consumption in humans, whereas the patients in our study had not consumed alcohol on the day of the investigation. In addition, not all patients with alcoholic chronic pancreatitis were still abusing alcohol at the time of recruitment into the study. Therefore, it appears that the enhanced blood levels of IL-18 do not result from endotoxin-mediated mechanisms.

Interestingly, our *in vitro* studies showed that the incubation of PBMC with ethanol decreased the endotoxin-stimulated secretion of IL-18 by PBMC thereby suggesting that ethanol consumption modulates the IL-18 expression in the pancreas. However, the role of IL-18 in alcoholic pancreatitis remains speculative since only limited data are available regarding the probably pleiotropic immunological function of IL-18 in chronic pancreatitis.

IL-18 participates both in Th1 and Th2 immune responses[16]. IL-18 induces the production of cytokines such as TNF-α and IL-1 and enhances the production of further chemokines such as IL-8 and MCP-1[35] that are increased in chronic pancreatitis tissue[10,31,36]. Of note, IL-8 plays a major role in the recruitment of infiltrating neutrophils to the site of inflammation[37]. The simultaneous presence of IL-12 and IL-18 results in a marked production of nitric oxide and reactive oxygen intermediates in macrophages and neutrophils[38] which may also facilitate premature intrapancreatic trypsin activation and pancreatic autodigestion[39]. The predominantly antiinflammatory cytokine IL-10 inhibits the synthesis of

proinflammatory cytokines such as TNF-α, IL-1, IL-6 and IL-8 and may play a dominant role in protecting the pancreas during pancreatic inflammation[40,41]. There was only minimal induction of the antiinflammatory cytokines IL-1 receptor and IL-10 through IL-18[35], and IL-10 failed to inhibit IL-18 production in response to inflammatory stimuli[42]. Therefore, IL-18 may escape the influence of IL-10 during pancreatic inflammation.

Finally, among its pleiotropic effects, IL-18 strongly induces IFN-γ production[15,16]. Increased levels of IFN were reported in chronic pancreatitis tissue from humans and animals[13,43,44]. Interestingly, the fibrosis in chronic pancreatitis seems to be driven by TGF-β1[45], and IFN-γ and TGF-β1 are linked by an antagonistic relationship[46,47]. INF-γ possesses several antifibrotic characteristics, and IFN-γ has already been used therapeutically in animals and humans with fibrotic diseases[47-49]. The recruitment and activation of infiltrating cells may depend on the local production of inflammatory mediators such as TGF-β1 and IFN-γ[11,31]. Recent *in vitro* studies have demonstrated that TGF-β1 strongly suppressed the production of IFN-γ that had been induced by costimulation with IL-18 and phytohaemagglutinin, a strong stimulator of IFN-γ synthesis, or by costimulation with IL-18 and IL-12[50]. However, the role of IFN-γ in chronic pancreatitis is not completely clarified, and further studies are required to reveal the role of IL-18 during pancreatic fibrosis.

In conclusion, PSC are activated during pancreatic injury, and these cells produce and release increased amounts of extracellular matrix proteins thereby leading to the fibrotic destruction of the pancreas[6,7]. Pancreatic stellate cells are activated and regulated on exposure to various proinflammatory cytokines[9]. Thus, future studies should address the role of IL-18 in the context of PSC activation and regulation.

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REFERENCES

1 **Steer ML**, Waxman I, Freedman S. Chronic pancreatitis. *N Engl J Med* 1995; **332**: 1482-1490
2 **Niebergall-Roth E**, Harder H, Singer MV. A review: acute and chronic effects of ethanol and alcoholic beverages on the pancreatic exocrine secretion *in vivo* and *in vitro*. *Alcohol Clin Exp Res* 1998; **22**: 1570-1583
3 **Apte MV**, Wilson JS. Alcohol-induced pancreatic injury. *Best Pract Res Clin Gastroenterol* 2003; **17**: 593-612
4 **Pfutzer RH**, Whitcomb DC. SPINK1 mutations are associated with multiple phenotypes. *Pancreatology* 2001; **1**: 457-460
5 **Schneider A**, Whitcomb DC. Hereditary pancreatitis: a model for inflammatory diseases of the pancreas. *Best Pract Res Clin Gastroenterol* 2002; **16**: 347-363
6 **Bachem MG**, Schneider E, Gross H, Weidenbach H, Schmid RM, Menke A, Siech M, Beger H, Grunert A, Adler G. Identification, culture, and characterization of pancreatic stellate cells in rats and humans. *Gastroenterology* 1998; **115**: 421-432
7 **Apte MV**, Haber PS, Applegate TL, Norton ID, McCaughan GW, Korsten MA, Pirola RC, Wilson JS. Periacinar stellate shaped cells in rat pancreas: identification, isolation, and culture. *Gut* 1998; **43**: 128-133
8 **Apte MV**, Phillips PA, Fahmy RG, Darby SJ, Rodgers SC, McCaughan GW, Korsten MA, Pirola RC, Naidoo D, Wilson JS. Does alcohol directly stimulate pancreatic fibrogenesis? Studies with rat pancreatic stellate cells. *Gastroenterology* 2000; **118**: 780-794
9 **Mews P**, Phillips P, Fahmy R, Korsten M, Pirola R, Wilson J, Apte M. Pancreatic stellate cells respond to inflammatory cytokines: potential role in chronic pancreatitis. *Gut* 2002; **50**: 535-541
10 **Hunger RE**, Mueller C, Z'graggen K, Friess H, Buchler MW. Cytotoxic cells are activated in cellular infiltrates of alcoholic chronic pancreatitis. *Gastroenterology* 1997; **112**: 1656-1663
11 **Ebert MP**, Ademmer K, Muller-Ostermeyer F, Friess H, Buchler MW, Schubert W, Malfertheiner P. CD8+CD103+ T cells analogous to intestinal intraepithelial lymphocytes infiltrate the pancreas in chronic pancreatitis. *Am J Gastroenterol* 1998; **93**: 2141-2147
12 **Emmrich J**, Weber I, Nausch M, Sparmann G, Koch K, Seyfarth M, Lohr M, Liebe S. Immunohistochemical characterization of the pancreatic cellular infiltrate in normal pancreas, chronic pancreatitis and pancreatic carcinoma. *Digestion* 1998; **59**: 192-198
13 **Hasel C**, Rau B, Perner S, Strater J, Moller P. Differential and mutually exclusive expression of CD95 and CD95 ligand in epithelia of normal pancreas and chronic pancreatitis. *Lab Invest* 2001; **81**: 317-326
14 **Vyas SK**. Growth factors and cytokines in chronic pancreatitis. In: Johnson CD, Imrie CW. Pancreatic disease: towards the year 2000. London: Springer Verlag, 1999: 155-165
15 **Dinarello CA**. IL-18: A TH1-inducing, proinflammatory cytokine and new member of the IL-1 family. *J Allergy Clin Immunol* 1999; **103**: 11-24
16 **Nakanishi K**, Yoshimoto T, Tsutsui H, Okamura H. Interleukin-18 regulates both Th1 and Th2 responses. *Annu Rev Immunol* 2001; **19**: 423-474
17 **Gracie JA**, Robertson SE, McInnes IB. Interleukin-18. *J Leukoc Biol* 2003; **73**: 213-224
18 **Hanck C**, Bertsch T, Rossol S, Kurimoto M, Singer MV, Richter A. Enhanced serum levels of IL-18 in patients with severe acute pancreatitis. *Digestion* 1999; **60**: 379 (abstract)
19 **Rau B**, Baumgart K, Paszkowski AS, Mayer JM, Beger HG. Clinical relevance of caspase-1 activated cytokines in acute pancreatitis: high correlation of serum interleukin-18 with pancreatic necrosis and systemic complications. *Crit Care Med* 2001; **29**: 1556-1562
20 **Wereszczynska-Siemiatkowska U**, Mroczko B, Siemiatkowski A. Serum profiles of interleukin-18 in different severity forms of human acute pancreatitis. *Scand J Gastroenterol* 2002; **37**: 1097-1102
21 **Manigold T**, Bocker U, Traber P, Dong-Si T, Kurimoto M, Hanck C, Singer MV, Rossol S. Lipopolysaccharide/endotoxin induces IL-18 via CD14 in human peripheral blood mononuclear cells in vitro. *Cytokine* 2000; **12**: 1788-1792
22 **Hanck C**, Manigold T, Bocker U, Kurimoto M, Kolbel CB, Singer MV, Rossol S. Gene expression of interleukin 18 in unstimulated peripheral blood mononuclear cells of patients with alcoholic cirrhosis. *Gut* 2001; **49**: 106-111
23 **Rumpf HJ**, Hapke U, Hill A, John U. Development of a screening questionnaire for the general hospital and general practices. *Alcohol Clin Exp Res* 1997; **21**: 894-898
24 **Saunders JB**, Aasland OG, Babor TF, de la Fuente JR, Grant M. Development of the Alcohol Use Disorders Identification Test (AUDIT): WHO Collaborative Project on Early Detection of Persons with Harmful Alcohol Consumption--II. *Addiction* 1993; **88**: 791-804
25 **Skinner HA**, Sheu WJ. Reliability of alcohol use indices. The Lifetime Drinking History and the MAST. *J Stud Alcohol* 1982; **43**: 1157-1170
26 **Wittchen HU**, Zaudig M, Fydrich T. SKID - Strukturiertes Klinisches Interview für DSM-IV. Achse I und II. Hogrefe, Göttingen, 1997
27 **Ammann RW**. A clinically based classification system for

alcoholic chronic pancreatitis: summary of an international workshop on chronic pancreatitis. *Pancreas* 1997; **14**: 215-221

28 **Rossol S**, Marinos G, Carucci P, Singer MV, Williams R, Naoumov NV. Interleukin-12 induction of Th1 cytokines is important for viral clearance in chronic hepatitis B. *J Clin Invest* 1997; **99**: 3025-3033

29 **Feick P**, Gilhaus S, Schulz I. Pervanadate stimulates amylase release and protein tyrosine phosphorylation of paxillin and p125(FAK) in differentiated AR4-2J pancreatic acinar cells. *J Biol Chem* 1998; **273**: 16366-16373

30 **Hanck C**, Rossol S, Hartmann A, Singer MV. Cytokine gene expression in peripheral blood mononuclear cells reflects a systemic immune response in alcoholic chronic pancreatitis. *Int J Pancreatol* 1999; **26**: 137-145

31 **Saurer L**, Reber P, Schaffner T, Buchler MW, Buri C, Kappeler A, Walz A, Friess H, Mueller C. Differential expression of chemokines in normal pancreas and in chronic pancreatitis. *Gastroenterology* 2000; **118**: 356-367

32 **Gukovskaya AS**, Gukovsky I, Zaninovic V, Song M, Sandoval D, Gukovsky S, Pandol SJ. Pancreatic acinar cells produce, release, and respond to tumor necrosis factor-alpha. Role in regulating cell death and pancreatitis. *J Clin Invest* 1997; **100**: 1853-1862

33 **Thurman RG**, Bradford BU, Knecht KT, Iimuro Y, Arteel GE, Yin M, Connor HD, Wall C, Raleigh JA, von Frankenberg M, Adachi Y, Forman DT, Brenner D, Kadiiska M, Mason RP. Endotoxin, kupffer cells and alcoholic liver injury. In: Blum HE, Bode C, Bode JC, Sartor RB. Gut and the liver. Dordrecht, Boston, London: Kluwer Academic Publishers, 1998: 222-240

34 **Kono H**, Nakagami M, Rusyn I, Connor HD, Stefanovic B, Brenner DA, Mason RP, Arteel GE, Thurman RG. Development of an animal model of chronic alcohol-induced pancreatitis in the rat. *Am J Physiol Gastrointest Liver Physiol* 2001; **280**: G1178-G1186

35 **Puren AJ**, Fantuzzi G, Gu Y, Su MS, Dinarello CA. Interleukin-18 (IFNgamma-inducing factor) induces IL-8 and IL-1beta via TNFalpha production from non-CD14+ human blood mononuclear cells. *J Clin Invest* 1998; **101**: 711-721

36 **Di Sebastiano P**, di Mola FF, Di Febbo C, Baccante G, Porreca E, Innocenti P, Friess H, Buchler MW. Expression of interleukin 8 (IL-8) and substance P in human chronic pancreatitis. *Gut* 2000; **47**: 423-428

37 **Mueller C**, Saurer L. Cellular immune reactions in chronic pancreatitis. In: Büchler MW, Friess H, Uhl W, Malfertheiner P. Chronic pancreatitis. Novel concepts in biology and therapy. Berlin, Wien: Blackwell Wissenschafts-Verlag GmbH, 2002: 184-195

38 **Kashiwamura S**, Ueda H, Okamura H. Roles of interleukin-18 in tissue destruction and compensatory reactions. *J Immunother* 2002; **25** Suppl 1: S4-S11

39 **Gukovskaya AS**, Vaquero E, Zaninovic V, Gorelick FS, Lusis AJ, Brennan ML, Holland S, Pandol SJ. Neutrophils and NADPH oxidase mediate intrapancreatic trypsin activation in murine experimental acute pancreatitis. *Gastroenterology* 2002; **122**: 974-984

40 **Pezzilli R**, Billi P, Miniero R, Barakat B. Serum interleukin-10 in human acute pancreatitis. *Dig Dis Sci* 1997; **42**: 1469-1472

41 **Demols A**, Van Laethem JL, Quertinmont E, Degraef C, Delhaye M, Geerts A, Deviere J. Endogenous interleukin-10 modulates fibrosis and regeneration in experimental chronic pancreatitis. *Am J Physiol Gastrointest Liver Physiol* 2002; **282**: G1105-G1112

42 **Zediak VP**, Hunter CA. IL-10 fails to inhibit the production of IL-18 in response to inflammatory stimuli. *Cytokine* 2003; **21**: 84-90

43 **Sparmann G**, Behrend S, Merkord J, Kleine HD, Graser E, Ritter T, Liebe S, Emmrich J. Cytokine mRNA levels and lymphocyte infiltration in pancreatic tissue during experimental chronic pancreatitis induced by dibutyltin dichloride. *Dig Dis Sci* 2001; **46**: 1647-1656

44 **Xie MJ**, Motoo Y, Su SB, Sawabu N. Expression of tumor necrosis factor-alpha, interleukin-6, and interferon-gamma in spontaneous chronic pancreatitis in the WBN/Kob rat. *Pancreas* 2001; **22**: 400-408

45 **van Laethem JL**, Deviere J, Resibois A, Rickaert F, Vertongen P, Ohtani H, Cremer M, Miyazono K, Robberecht P. Localization of transforming growth factor beta 1 and its latent binding protein in human chronic pancreatitis. *Gastroenterology* 1995; **108**: 1873-1881

46 **Ulloa L**, Doody J, Massague J. Inhibition of transforming growth factor-beta/SMAD signalling by the interferon-gamma/STAT pathway. *Nature* 1999; **397**: 710-713

47 **Sime PJ**, O'Reilly KM. Fibrosis of the lung and other tissues: new concepts in pathogenesis and treatment. *Clin Immunol* 2001; **99**: 308-319

48 **Oldroyd SD**, Thomas GL, Gabbiani G, El Nahas AM. Interferon-gamma inhibits experimental renal fibrosis. *Kidney Int* 1999; **56**: 2116-2127

49 **Menke A**, Vogelmann R, Bachem M, Adler G. Mechanisms of fibrosis and potential antifibrotic agents. In: Lankisch PG, DiMagno EP. Pancreatic disease. Berlin, Heidelberg, New York: Springer Verlag, 1999: 132-139

50 **Kampfer H**, Paulukat J, Muhl H, Wetzler C, Pfeilschifter J, Frank S. Lack of interferon-gamma production despite the presence of interleukin-18 during cutaneous wound healing. *Mol Med* 2000; **6**: 1016-1027

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RAPID COMMUNICATION

Strong prognostic value of nodal and bone marrow micro-involvement in patients with pancreatic ductal carcinoma receiving no adjuvant chemotherapy

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Abstract

AIM: To study the prognostic value of adjuvant chemotherapy in patients with pancreatic, ductal adenocarcinoma.

METHODS: Lymph nodes from 106 patients with resectable pancreatic ductal adenocarcinoma were systematically sampled. A total of 318 lymph nodes classified histopathologically as tumor-free were examined using sensitive immunohistochemical assays. Forty-three (41%) of the 106 patients were staged as $pT_{1/2}$, 63 (59%) as $pT_{3/4}$, 51 (48%) as pN_0, and 55 (52%) as pN_1. The study population included 59 (56%) patients exhibiting $G_{1/2}$, and 47 (44%) patients with G_3 tumors. Patients received no adjuvant chemo- or radiation therapy and were followed up for a median of 12 (range: 3.5 to 139) mo.

RESULTS: Immunostaining with Ber-EP4 revealed nodal microinvolvement in lymph nodes classified as "tumor free" by conventional histopathology in 73 (69%) out of the 106 patients. Twenty-nine (57%) of 51 patients staged histopathologically as pN_0 had nodal microinvolvement. The five-year survival probability for pN0-patients was 54% for those without nodal microinvolvement and 0% for those with nodal microinvolvement. Cox-regression modeling revealed the independent prognostic effect of nodal microinvolvement on recurrence-free (relative risk 2.92, P = 0.005) and overall (relative risk 2.49, P = 0.009) survival.

CONCLUSION: The study reveals strong and independent prognostic significance of nodal microinvolvement in patients with pancreatic ductal adenocarcinoma who have received no adjuvant therapy. The addition of immunohistochemical findings to histopathology reports may help to improve risk stratification of patients with pancreatic cancer.

Key words: Pancreatic ductal adenocarcinoma; Nodal microinvolvement; Micrometastases

Yekebas EF, Bogoevski D, Bubenheim M, Link BC, Kaifi JT, Wachowiak R, Mann O, Kutup A, Cataldegirmen G, Wolfram L, Erbersdobler A, Klein C, Pantel K, Izbicki JR. Strong prognostic value of nodal and bone marrow micro-involvement in patients with pancreatic ductal carcinoma receiving no adjuvant chemotherapy. *World J Gastroenterol* 2006; 12(40): 6515-6521

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INTRODUCTION

Pancreatic adenocarcinoma is the fifth leading cause of death among all malignancies[1], leading to approximately 40 000 deaths each year in Europe[2]. Reported probabilities of five-year survival after curative surgery are still below 10 percent[3]. Stage, grade and resection margin status are currently accepted as the most accurate pathologic variables predicting survival[4-10]. Pathologic staging only insufficiently reflects the individual risk to develop tumor recurrence which is even high in early tumor stages. Thus, effort continues to identify new prognosticators of tumor

relapse that indicate the need of adjuvant therapy.

Occult residual tumor disease is suggested when either bone marrow or lymph nodes from which tumor relapse may originate are affected by micrometastatic lesions undetectable by conventional histopathology[11]. The clinical significance of antibodies against tumor-associated targets both in lymph nodes[12-15] and in bone marrow[15,16] is still controversial[16-26]. Various monoclonal antibodies are in use for micrometastatic detection, thus contributing to the incongruity of data and validity of results. These assays have been rarely used in patients with pancreatic carcinoma[27-31]. Recently, our group showed that immunohistochemical staining with the monoclonal antibody Ber-EP4 is a sensitive and specific method for detecting isolated or clusters of tumor cells in lymph nodes from patients with lung[13], esophageal[25], or pancreatic carcinomas[28]. Ber-EP4 is an antibody against two glycopolypeptides of 34 and 49 kD on the surface and in the cytoplasm of all epithelial cells (except parietal cells, hepatocytes, and the superficial layers of squamous epithelium).

The present study was to increase our knowledge gained in the previous studies on lymph node micrometa stasis[25,28,31,32]. In non-small cell lung carcinoma[32], the risk to develop tumor relapse in pN1 patients is overall greater than in pN0 patients. However, we have shown that further risk stratification for patients with histopathological involvement may be performed according to their immunohistochemical status. Therefore, here we have extended our previously published study on lymph node-negative patients with pancreatic cancer[31] with patients staged as pN1 through conventional histology as well as the bone marrow data from those patients that gave us written consent. The primary aim of this study was to assess the role of immunohistochemically detectable micrometastases in lymph nodes of an unselected group of patients with "curatively" resected pancreatic ductal adenocarcinoma. The secondary aim was to assess whether lymph node microinvolvement is correlated to bone marrow micrometastasis and which of these two sites is a better indicator for tumor cell dissemination in pancreatic cancer.

MATERIALS AND METHODS

Patients and study design

The local ethical committee of Hamburg approved this study. Informed consent was obtained from all patients before inclusion in the study. Tumor samples, lymph nodes, and bone marrow aspirates of the upper iliac crest were collected from 487 patients with pancreatic and periampullary malignancies. Out of these patients, 171 (35%) had carcinomas of the papilla of Vater, 47 (10%) exhibited carcinoma of the distal common bile duct, and 269 (55%) had pancreatic carcinoma. Out of these 269 patients, 49 (18%) had neuroendocrine tumors and 220 (82%) had true pancreatic carcinoma.

Our study population included 106 patients with resectable pancreatic ductal adenocarcinoma who had undergone curative surgery and had given informed consent for immunohistochemical analysis of lymph nodes. Patients with cystic malignancies (IPMN, cystadenocarcinoma), acinar cell and squamous cell carcinomas were not considered for this study. The most frequent surgical procedure was pancreatoduodenectomy. Lymph node dissection was performed as previously described by Pedrazzoli *et al*[33]. A total of 1643 lymph nodes were removed with a median number of 16 (range 7 to 38) lymph nodes per patient. Among all histopathologically negative lymph nodes, 318 were selected in a representative fashion as described most recently for subsequent immunohistochemical screening[31]. Tumor stage and grade were classified according to the 6th edition of the tumor-node-metastasis classification (TNM) of the International Union against Cancer[34] by investigators unaware of the immunohistochemical findings.

Follow-up evaluations at three-month intervals included a physical examination, abdominal ultrasonography, computed tomography of the abdomen and studies of tumor markers, i.e. carcinoembryonic antigen and CA 19-9. Out of all 106 patients studied, the vital status in 89 patients could be determined at the end of the study. Seventeen patients were excluded from the survival analysis because they were either censored or died within 90 d after surgery. From 3 patients, only information about the date of death but not of recurrence was available

Tissue preparation and immunohistochemical analysis

Lymph nodes were divided into two parts, one for conventional histopathology, the other was snap-frozen in liquid nitrogen within three hours after their removal and stored at -80℃ until use. Only histopathologically "tumor-free" lymph nodes were screened by immunohistochemistry with the anti-epithelial-cell monoclonal antibody Ber-EP4 (IgG1; Dako, Hamburg, Germany) as described previously[14]. Ber-EP4 is an antibody against two glycopolypeptides of 34 and 49 kD on the surface and in the cytoplasm of all epithelial cells (except parietal cells, hepatocytes, and the superficial layers of squamous epithelium). The antibody does not react with mesenchymal tissue, including lymphoid tissue[15,23].

Cryostat sections (5 to 6 μm thick) were cut at three different levels in each node and transferred onto glass slides treated with 3-triethoxysilylpropylamin (Merck, Darmstadt, Germany). One section of the sample obtained at each level was stained by the alkaline phosphatase-antialkaline phosphatase technique combined with the new fuchsine stain (Sena, Heidelberg, Germany) for the visualization reaction[23].

In 16 control patients with nonepithelial tumors or inflammatory diseases, lymph nodes were consistently stained negative. Sections of normal colon served as positive staining controls and isotype-matched, irrelevant murine monoclonal antibodies served as negative controls (purified immunoglobulin mouse myeloma protein for IgG1; Sigma, Deisenhofen, Germany).

The slides were evaluated in a blinded fashion by two observers working independently (D.B., J.T.K.). Minimal tumor cell involvement in a lymph node that was considered to be tumor-free by conventional histopathological staining was defined as the presence of

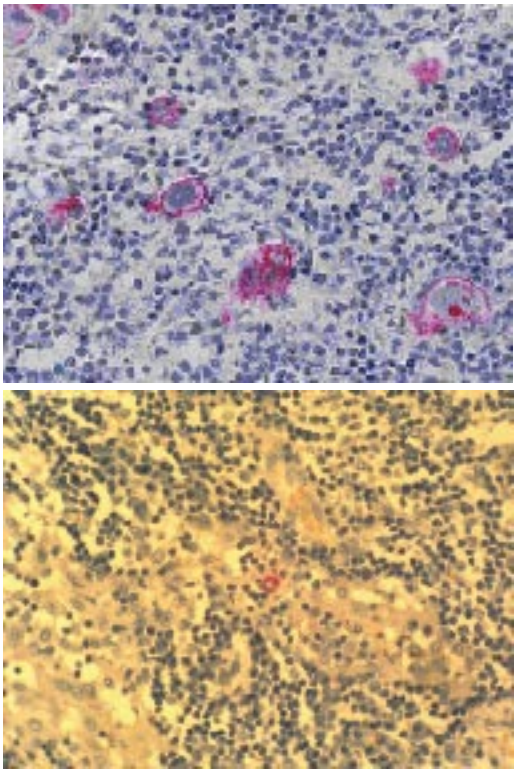

Figure 1 Immunohistochemically detectable nodal microinvolvement with monoclonal antibody Ber EP4 (×400).

one to ten positive cells in the body of the node (Figure 1). If more than 10 cells were detected (2 lymph nodes in two patients), a HE re-staining was conducted. Under routine histology both lymph nodes were judged as negative.

Aspirates of 4 to 8 mL of bone marrow from the iliac crest were obtained from those patients who gave additional written consent for sampling bone marrow (59 patients) and were processed as previously described[16]. The specimens were collected in heparin, and mononuclear cells isolated by density-gradient centrifugation through Ficoll-Hypaque (Pharmacia, Freiburg, Germany) at 400 r/min for 30 min, were deposited onto glass slides by cytocentrifugation at 150 r/min for 3 min. To detect tumor cells in bone marrow (Figure 2), we used the monoclonal antibody A45-B/B3 (IgG1; Micromet, Munich, Germany) that detects an epitope on a variety of cytokeratin components, including cytokeratins[14,15,18,20].

Statistical analysis

All statistical calculations concerning survival (overall and recurrence-free survival) were based on the group of 89 patients who were available for follow-up. The primary outcome measure was the five-year survival probability. Secondary outcomes were the incidence of local recurrence and distant metastases of the disease. Survival was calculated from the date of resection until the date of death from any cause. For patients lost to follow-up, data were censored on the date the patient was last seen alive. Associations between categorical variables were assessed using Fisher's exact test. Survival estimates were derived using the method proposed by Kaplan and Meier[35] and the log-rank test was used to assess differences in survival

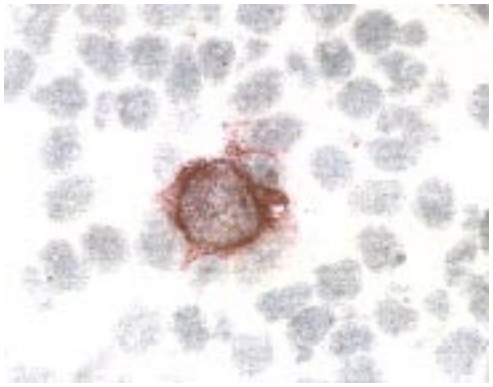

Figure 2 Immunohistochemically detectable bone marrow microinvolvement with monoclonal antibody AE1/AE3 (×400).

estimates among the groups. Point and interval estimates of the survival probabilities at 60 mo were calculated. For comparison purposes, log-rank test and exact stratified log-rank test were performed. Cox proportional-hazards modeling[36] was used to investigate and adjust the major prognostic and stratification factors. $P < 0.05$ was considered statistically significant.

Since this analysis was intended to be explorative, no adjustment for multiple testing was carried out.

RESULTS

Characteristics of patients and comparison of staining procedures

One hundred and six patients [47 (44%) women and 59 (56%) men] with pancreatic ductal adenocarcinoma were included in the study. Their mean age was 61 years (range 32 to 83 years, median 61 years). Table 1 shows the characteristics of patients and tumors.

A total of 318 lymph nodes classified to be "tumor-free" by conventional histopathology were analyzed. Positive cells in the sinuses, the lymphoid interstitium, or in both locations were found in 132 lymph nodes (42%). These 132 positive lymph nodes were found from 73 (69%) of the 106 patients by immunostaining. Whereas the presence of Ber-EP4 cells was significantly associated with nodal metastases (pN1) identified through conventional histopathology ($P = 0.012$), no correlation between tumor stage and tumor grade was found.

Survival

After an average observation period of 18 mo (range 3 to 137 mo, median 12 mo), the presence of nodal microinvolvement was associated with significantly reduced recurrence-free and overall survival probabilities. The Kaplan-Meyer overall survival curve for all patients who were stratified according to the presence or absence of occult tumor cells in lymph nodes showed a significant survival benefit for patients negative in immunohistochemistry (median not yet reached-NYR *vs* 13 mo; 2- year survival 66% *vs* 20%; 5-year survival 50% *vs* 0%) irrespective of the histopathological classification (pN0/pN1) of lymph nodes (log-rank test; $P < 0.0001$, Figure 3).

Table 1 Characteristics of patients and tumors

Variable	*n*	Ber-EP4 positive cells in lymph nodes (*n*)
All patients	106	73
Male	59	42
Female	47	31
Primary Tumor		
Carcinoma in situ	1	0
pT1	6	3
pT2	36	26
pT3	59	40
pT4	4	4
Nodal status		
Negative (pN0)	51	29*
Positive (pN1)	55	44*
Tumor Grade		
Well differentiated (G1)	5	4
Moderately differentiated (G2)	54	35
Poorly differentiated (G3)	47	34
Resection margin		
Negative (R0)	90	59
Positive (R1)	16	14
Tumor cells in Bone marrow		
Yes	14	9
No	45	31
Not analysed	47	33

Nodal status was detected both histopathologically and immunohistochemically. Bone marrow micrometastases were detected by immunohistochemistry. $P = 0.035$.

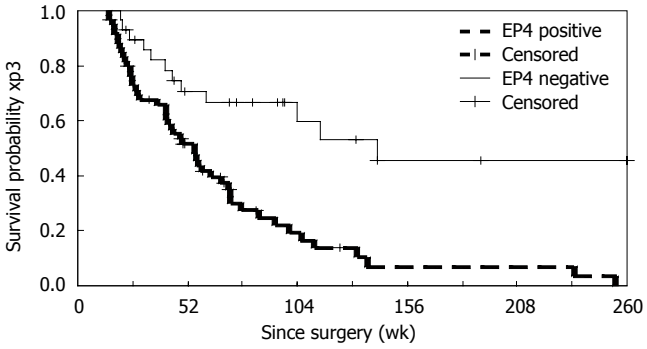

Figure 3 Overall survival according to the presence or absence of nodal microinvolvement in immunohistochemistry for patients alive at least 13 wk after surgery. Median: Not yet reached-NYR *vs* 13 mo; Mean: 81 (SD 15, 95% CI 52-109) *vs* 16 (SD 2, 95% CI 12-20); 2-yr overall survival 66% *vs* 20%; 5-yr overall survival 50% *vs* 0%.

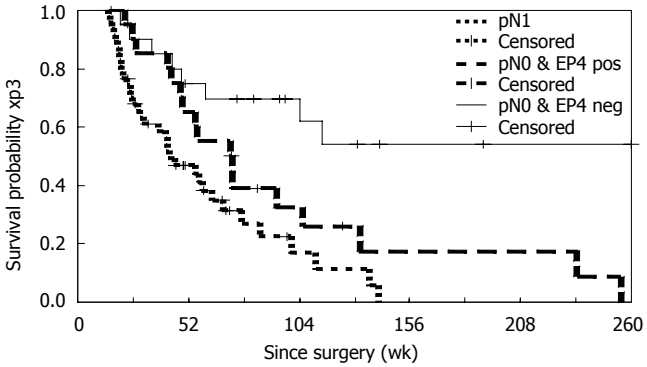

Figure 4 Overall survival according to the presence or absence of nodal metastases in conventional histopathology and immunohistochemistry for patients alive at least 13 wk after surgery. Median: Not yet reached-NYR *vs* 13 mo *vs* 10 mo; Mean: 90 (SD 15, 95% CI 60-119) *vs* 17 (SD 3, 95% CI 11-23) *vs* 14 (SD 2, 95% CI 7-13); 2-yr overall survival 66% *vs* 20%; 5-yr overall survival 50% *vs* 0%.

The analysis of the subset of patients who were staged pN0 in conventional histopathology revealed significantly better survival rates in patients without occult tumor cells as compared with those with nodal microinvolvement (median NYR *vs* 17 mo; 2-year survival 70% *vs* 34%; 5-year survival 61% *vs* 0%; log-rank test; P=0.012, Figure 4). Patients without any nodal involvement, as excluded by both conventional histopathology and immunohistochemistry, had a five-year overall survival probability of 61% (standard error: 13%). In contrast, the five-year survival probability of pN0-patients with nodal microinvolvement resembled that of pN1-patients (log-rank test; $P = 0.059$, Figure 4) and in both groups no patient was still alive 5 years after surgery.

The predictive value of nodal microinvolvement was strengthened by the finding that pN1-patients who additionally had disseminated tumor cells in other lymph nodes classified as tumor-free by histopathology had shorter recurrence-free and overall survival probabilities than pN1-patients without occult tumor cells in immunohistochemistry (median survival 33 *vs* 10 mo; 2-year survival 69% *vs* 10%; 5-year survival 69% *vs* 0%; log rank test: $P = 0.004$ and $P = 0.049$, respectively, data not shown).

Although no statistical significance was reached, pN1-patients *without* nodal microinvolvement had better overall survival probabilities (median survival = 33 mo) than pN0-patients *with* nodal microinvolvement (median survival = 17 mo). This could in part confirm the hypothesis that nodal microinvolvement in patients not burdened with nodal metastases detected through routine histology (pN0) might literally reflect systemic disease, whereas pN1-patients without nodal microinvolvement could be in fact treated as locally advanced disease. This should be discussed very cautiously since only 8 patients were included in the group of pN1-patients without nodal microinvolvement, but all of them were alive at the last follow-up.

Influence of resection margins

A total of 16 patients among the entire study population had positive resection margins (R1-status), only 2 of them belonged to the subset of pN0-patients without nodal microinvolvement. The remaining 14 patients with positive resection margins were either staged pN1 or had nodal microinvolvement which was as reported above, per se associated with a significantly worse prognosis. Due to this distribution of patients with R1-status, log-rank test might not have the sufficient power to assess the influence of the resection margins on overall survival. Therefore, no significant differences were found between patients with positive resection margins, as compared with those with negative resection margins ($P = 0.976$, data not shown).

Table 2 Cox regression analysis showing the presence of occult tumor cells in lymph nodes, tumor grade, and histopathologically detectable lymph node involvement as independent prognostic factors for disease-free and overall survival

Prognostic factor	Reference group	Relative risk	*P*	95% confidence interval	
				Lower bound	Upper bound
Recurrence-free (*n* = 86)					
Age at surgery		1.01	0.706	0.98	1.03
Sex	Male	1.42	0.223	0.81	2.49
Stage	pT1-pT2	0.87	0.652	0.48	1.57
Grade	G I - GII	3.14	0.000	1.74	5.68
Nodal involvement	pN0	1.66	0.112	0.89	3.10
Nodal microinvolvement (Ber-EP4)	Negative	2.92	0.005	1.39	6.13
Overall survival (*n* = 89)					
Age at surgery		1.00	0.996	0.97	1.03
Sex	Male	1.47	0.172	0.85	2.55
Stage	pT1-pT2	0.93	0.801	0.53	1.64
Grade	G I - GII	3.36	0.000	1.90	5.94
Nodal involvement	pN0	2.18	0.012	1.19	4.00
Nodal microinvolvement (Ber-EP4)	Negative	2.49	0.009	1.25	4.96

Influence of bone marrow micrometastases

Occult tumor cells were detected in 14 of 59 patients who were evaluated with respect to bone marrow micrometastases. No association was found between bone marrow and nodal microinvolvement (Fisher' s exact test, $P = 0.75$). Among the 14 patients with bone marrow micrometastases, 9 (64%) had also nodal microinvolvement, whereas 5 (36%) did not. In turn, Ber-EP4-positive cells in lymph nodes were detected in 31 (69%) of the 45 patients with negative bone marrow findings (Table 1). Neither bone marrow micrometastases nor nodal microinvolvement was found in 14 patients. Here, we want to stress the finding (although again not significant due to the small number of patients) that those patients without any nodal involvement (negative both in histopathology and IHC) who had bone marrow micrometastases seemed to have considerably worse overall prognosis (mean survival time: 8 mo) than patients without (mean survival time: 75 mo). This also has to be discussed with considerable caution since we identified only 3 patients with bone marrow micrometastases (without any nodal involvement), although they all died within the first year unlike the other 8 patients without bone marrow micrometastases who were all alive on the last follow-up.

As regards the influence of bone marrow micrometastases alone, no significant differences were found with respect to relapse-free time ($P = 0.55$) and overall survival ($P = 0.14$), respectively. However, these results might be biased by the small number of patients analyzed.

Multivariate analysis

Apart from nodal involvement assessed either by histopathology or immunohistochemistry, the comparison of survival curves revealed also significant differences with respect to grading when $G_{1,2}$ tumors were compared to G_3 tumors (median survival time: 28 mo *vs* 12 mo; log-rank test: $P < 0001$).

Cox regression analysis identified the presence of occult tumor cells in lymph nodes, tumor grade, and histopathologically detectable lymph node involvement as independent prognostic factors for disease-free and overall survival (Table 2). With respect to 5-year recurrence-free time, nodal microinvolvement had a relative risk of 2.92 (95% confidence interval: 1.39 to 6.13; $P = 0.005$), as compared with negative findings in immunohistochemistry. G3 tumors had a relative risk of 3.14 as compared with G1/2 tumors (95% confidence interval: 1.74 to 5.68; $P = 0.000$) with respect to recurrence-free time. A pN1-stage, as compared to pN0 patients, carried a relative risk of 2.18 (95% confidence interval: 1.19 to 4.0; $P = 0.012$) as to overall survival time, but had no significant influence on recurrence-free time (relative risk of 1.66; 95% confidence interval: 0.89 to 3.10; $P = 0.112$). Age, sex and tumor stage had no independent prognostic influence on recurrence-free or overall survival.

The analysis of the interaction between pN-status, nodal microinvolvement and grading did not reveal that the proportional assumption was violated. Hence, the Cox model appeared appropriate and grading followed by nodal microinvolvement remained the two most important prognostic variables also in the subset of pN0-patients.

DISCUSSION

The key finding of this investigation is that isolated tumor cells, detectable in lymph nodes by immunohistochemical analysis, are strong independent prognosticators in pancreatic ductal adenocarcinoma irrespective of the histopathological N-status. We analyzed patients who suffered from pancreatic ductal adenocarcinoma and did not receive any adjuvant chemoradiation or chemotherapy. Two subsets of patients could be identified and were classified as pN0 in conventional histopathology. One subset with a poor five-year survival probability of 0% which was close to that of patients with overt nodal involvement (pN_1), the other subset had a much better prognosis with a five-year survival probability of over 50% without nodal microinvolvement, suggesting that immunohistochemistry can confirm the cardinal importance of occult tumor cells for the separation of the

respective survival curves in pN0-patients.

Even in patients who were staged as pN1, the detection of occult tumor cells in "tumor-free" lymph nodes had prognostic significance. This finding is consistent with previous observations of our group showing that survival is significantly worsened in both esophageal[25] and non-small cell lung carcinoma[32] when histopathological pN1-status is accompanied with nodal microinvolvement. Basically, pN1-status in solid tumors is considered as a local disease which can be potentially cured with surgery, although it generally carries a higher risk of systemic dissemination than pN0-status. Therefore, the finding that pN1 patients with additional nodal microinvolvement in "tumor-free" lymph nodes apart from overt lymph node metastases had significantly shorter recurrence-free survival as compared with pN1-patients without occult tumor cells suggests that immunohistochemistry may help to identify different risk profiles in these patients.

The reliability of these immunohistochemical assays used so far for detection of nodal microinvolvement is questioned[16-26] and could be also hampered by a sampling error[25,37]. The sampling error might be influenced by the number of lymph nodes dissected during the course of pancreatoduodenectomy and assessed by immunohistochemistry, as well as the number of lymph node sections and the level of these sections within the lymph nodes. In our present study, the lymph node dissection was performed as previously described by Pedrazzoli *et al*[33]. A total of 1643 lymph nodes were removed with a median number of 16 (range 7 to 38) lymph nodes per patient. Thus, the first possible cause of a sampling error was diminished, considering the high median lymph node yield of 16 per patient. In another study (oesophagus carcinoma, not yet published) all lymph nodes dissected in the course of esophagectomy were immunohistochemically stained, unlike in this and some other previously published studies[25] where only 20% of all lymph nodes were stained, showing comparable results in terms of impact on overall and relapse- free survival. We also believe that 6 sections cut from 3 different levels of each lymph node are enough for proper access to the nodal microinvolvement. Analyzing more than 3 sections would not be routinely feasible, and the positive correlation between the result of our assay and clinical outcome indicates that examining 3 lymph node levels are sufficient. The results from this study are in the same line with those published earlier from our group[25], thus confirming that the random selection of lymph nodes for IHC is enough for access to the nodal microinvolvement.

The strong adverse influence of nodal microinvolvement on outcome was most likely the reason for the lack of prognostic significance of resection margins in this series. In the ESPAC-1 trial, the resection margin status ceased to be an independent, prognostic factor for overall survival when tumor grading and pN-status were co-variables in the regression modeling[38], suggesting that the unfavorable outcome linked with poorly differentiated tumors with nodal metastases can hardly be impaired by further adverse variables, e.g. R1-status. We therefore assume that the characteristics of patients with R1-status are considerably biased. This hypothesis is strengthened by our observation that out of the 16 patients with R1-status all except two had occult tumor cells in immunohistochemistry.

Our data also indicate primary dissemination of tumor cells into lymph nodes before blood-borne spread occurs, because only 22% of the patients with nodal microinvolvement had identifiable tumor cells in their bone marrow. The lack of a significant difference in recurrence-free time between patients with nodal microinvolvement alone and those with additional involvement of the bone marrow suggests that the key event in pancreatic cancer progression is the spread of tumor cells to the regional lymph nodes. Nodal microinvolvement seems to indicate a systemic disease in pancreatic carcinoma much more accurately than occult tumor cells in bone marrow. However, these results have to be interpreted cautiously because bone marrow findings may have been biased by the fact that only a subset of patients was analyzed. Therefore, the influence of bone marrow microinvolvement on the outcome of patients with pancreatic carcinoma needs to be clarified in future studies.

Although chemoradiation and/or chemotherapy for adjuvant treatment of pancreatic carcinoma may have severe side effects[39], in common clinical practice, it is in most instances applied irrespective of tumor stage. This reflects the distrust in the value of conventional tumor-staging nomenclature in terms of reliably predicting the risk of tumor relapse even in patients with early pancreatic cancer (T_1, N_0). Our data indicate that immunohistochemical assessment of lymph nodes can be used to refine the staging system for pancreatic ductal adenocarcinoma and might help us to identify patients who could not be cured by surgery alone and need adjuvant therapy. In turn, patients who are true node-negative both in histopathology and in immunohistochemistry have an excellent five-year survival probability of nearly 60%, even without chemotherapy. Whether this prognosis can be further improved by adjuvant therapy needs further study.

REFERENCES

1 **Greenlee RT**, Hill-Harmon MB, Murray T, Thun M. Cancer statistics, 2001. *CA Cancer J Clin* 2001; **51**: 15-36

2 **Parkin DM**, Muir CS. Cancer Incidence in Five Continents. Comparability and quality of data. *IARC Sci Publ* 1992; **120**: 45-173

3 **Kuhlmann KF**, de Castro SM, Wesseling JG, ten Kate FJ, Offerhaus GJ, Busch OR, van Gulik TM, Obertop H, Gouma DJ. Surgical treatment of pancreatic adenocarcinoma; actual survival and prognostic factors in 343 patients. *Eur J Cancer* 2004; **40**: 549-558

4 **Allison DC**, Piantadosi S, Hruban RH, Dooley WC, Fishman EK, Yeo CJ, Lillemoe KD, Pitt HA, Lin P, Cameron JL. DNA content and other factors associated with ten-year survival after resection of pancreatic carcinoma. *J Surg Oncol* 1998; **67**: 151-159

5 **Allema JH**, Reinders ME, van Gulik TM, Koelemay MJ, Van Leeuwen DJ, de Wit LT, Gouma DJ, Obertop H. Prognostic factors for survival after pancreaticoduodenectomy for patients with carcinoma of the pancreatic head region. *Cancer* 1995; **75**: 2069-2076

6 **Yeo CJ**, Abrams RA, Grochow LB, Sohn TA, Ord SE, Hruban RH, Zahurak ML, Dooley WC, Coleman J, Sauter PK, Pitt HA, Lillemoe KD, Cameron JL. Pancreaticoduodenectomy

for pancreatic adenocarcinoma: postoperative adjuvant chemoradiation improves survival. A prospective, single-institution experience. *Ann Surg* 1997; **225**: 621-633; discussion 633-636
7 **Nitecki SS**, Sarr MG, Colby TV, van Heerden JA. Long-term survival after resection for ductal adenocarcinoma of the pancreas. Is it really improving? *Ann Surg* 1995; **221**: 59-66
8 **Mosca F**, Giulianotti PC, Balestracci T, Di Candio G, Pietrabissa A, Sbrana F, Rossi G. Long-term survival in pancreatic cancer: pylorus-preserving versus Whipple pancreatoduodenectomy. *Surgery* 1997; **122**: 553-566
9 **Trede M**, Schwall G, Saeger HD. Survival after pancreatoduodenectomy. 118 consecutive resections without an operative mortality. *Ann Surg* 1990; **211**: 447-458
10 **Geer RJ**, Brennan MF. Prognostic indicators for survival after resection of pancreatic adenocarcinoma. *Am J Surg* 1993; **165**: 68-72; discussion 72-73
11 **Pantel K**, Brakenhoff RH. Dissecting the metastatic cascade. *Nat Rev Cancer* 2004; **4**: 448-456
12 **Byrne J**, Waldron R, McAvinchey D, Dervan P. The use of monoclonal antibodies for the histopathological detection of mammary axillary micrometastases. *Eur J Surg Oncol* 1987; **13**: 409-411
13 **Passlick B**, Izbicki JR, Kubuschok B, Nathrath W, Thetter O, Pichlmeier U, Schweiberer L, Riethmuller G, Pantel K. Immunohistochemical assessment of individual tumor cells in lymph nodes of patients with non-small-cell lung cancer. *J Clin Oncol* 1994; **12**: 1827-1832
14 **Raymond WA**, Leong AS. Immunoperoxidase staining in the detection of lymph node metastases in stage I breast cancer. *Pathology* 1989; **21**: 11-15
15 **Latza U**, Niedobitek G, Schwarting R, Nekarda H, Stein H. Ber-EP4: new monoclonal antibody which distinguishes epithelia from mesothelial. *J Clin Pathol* 1990; **43**: 213-219
16 **Pantel K**, Izbicki JR, Angstwurm M, Braun S, Passlick B, Karg O, Thetter O, Riethmuller G. Immunocytological detection of bone marrow micrometastasis in operable non-small cell lung cancer. *Cancer Res* 1993; **53**: 1027-1031
17 **Bussolati G**, Gugliotta P, Morra I, Pietribiasi F, Berardengo E. The immunohistochemical detection of lymph node metastases from infiltrating lobular carcinoma of the breast. *Br J Cancer* 1986; **54**: 631-636
18 **Chen ZL**, Perez S, Holmes EC, Wang HJ, Coulson WF, Wen DR, Cochran AJ. Frequency and distribution of occult micrometastases in lymph nodes of patients with non-small-cell lung carcinoma. *J Natl Cancer Inst* 1993; **85**: 493-498
19 **de Mascarel I**, Bonichon F, Coindre JM, Trojani M. Prognostic significance of breast cancer axillary lymph node micrometastases assessed by two special techniques: reevaluation with longer follow-up. *Br J Cancer* 1992; **66**: 523-527
20 **Martini N**, Flehinger BJ, Zaman MB, Beattie EJ Jr. Results of resection in non-oat cell carcinoma of the lung with mediastinal lymph node metastases. *Ann Surg* 1983; **198**: 386-397
21 **Casson AG**, Rusch VW, Ginsberg RJ, Zankowicz N, Finley RJ. Lymph node mapping of esophageal cancer. *Ann Thorac Surg* 1994; **58**: 1569-1570
22 **Momburg F**, Moldenhauer G, Hammerling GJ, Moller P. Immunohistochemical study of the expression of a Mr 34,000 human epithelium-specific surface glycoprotein in normal and malignant tissues. *Cancer Res* 1987; **47**: 2883-2891
23 **Pantel K**, Schlimok G, Angstwurm M, Weckermann D, Schmaus W, Gath H, Passlick B, Izbicki JR, Riethmuller G. Methodological analysis of immunocytochemical screening for disseminated epithelial tumor cells in bone marrow. *J Hematother* 1994; **3**: 165-173
24 **Kasper M**, Stosiek P, Typlt H, Karsten U. Histological evaluation of three new monoclonal anti-cytokeratin antibodies. 1. Normal tissues. *Eur J Cancer Clin Oncol* 1987; **23**: 137-147
25 **Izbicki JR**, Hosch SB, Pichlmeier U, Rehders A, Busch C, Niendorf A, Passlick B, Broelsch CE, Pantel K. Prognostic value of immunohistochemically identifiable tumor cells in lymph nodes of patients with completely resected esophageal cancer. *N Engl J Med* 1997; **337**: 1188-1194
26 **Z'graggen K**, Centeno BA, Fernandez-del Castillo C, Jimenez RE, Werner J, Warshaw AL. Biological implications of tumor cells in blood and bone marrow of pancreatic cancer patients. *Surgery* 2001; **129**: 537-546
27 **Kanemitsu K**, Hiraoka T, Tsuji T, Inoue K, Takamori H. Implication of micrometastases of lymph nodes in patients with extended operation for pancreatic cancer. *Pancreas* 2003; **26**: 315-321
28 **Hosch SB**, Knoefel WT, Metz S, Stoecklein N, Niendorf A, Broelsch CE, Izbicki JR. Early lymphatic tumor cell dissemination in pancreatic cancer: frequency and prognostic significance. *Pancreas* 1997; **15**: 154-159
29 **Vogel I**, Kruger U, Marxsen J, Soeth E, Kalthoff H, Henne-Bruns D, Kremer B, Juhl H. Disseminated tumor cells in pancreatic cancer patients detected by immunocytology: a new prognostic factor. *Clin Cancer Res* 1999; **5**: 593-599
30 **Milsmann C**, Fuzesi L, Werner C, Becker H, Horstmann O. Significance of occult lymphatic tumor spread in pancreatic cancer. *Chirurg* 2005; **76**: 1064-1072
31 **Bogoevski D**, Yekebas EF, Schurr P, Kaifi JT, Kutup A, Erbersdobler A, Pantel K, Izbicki JR. Mode of spread in the early phase of lymphatic metastasis in pancreatic ductal adenocarcinoma: prognostic significance of nodal microinvolvement. *Ann Surg* 2004; **240**: 993-1000; discussion 1000-1001
32 **Kubuschok B**, Passlick B, Izbicki JR, Thetter O, Pantel K. Disseminated tumor cells in lymph nodes as a determinant for survival in surgically resected non-small-cell lung cancer. *J Clin Oncol* 1999; **17**: 19-24
33 **Pedrazzoli S**, Beger HG, Obertop H, Andren-Sandberg A, Fernandez-Cruz L, Henne-Bruns D, Luttges J, Neoptolemos JP. A surgical and pathological based classification of resective treatment of pancreatic cancer. Summary of an international workshop on surgical procedures in pancreatic cancer. *Dig Surg* 1999; **16**: 337-345
34 **Hermanek P**, Sobin LH. TNM classification of malignant tumors. 4th ed. New York: Springer, 1992
35 **Klein JP**, Moeschberger ML. SURVIVAL ANALYSIS Techniques for Censored and Truncated Data. New York: Springer, 1997
36 **Cox DR**. Regression Models and Life Tables (with discussion). *J R Statist Soc* 1972; **34**: 187-220
37 **Knisely AS**. Cryptic tumor cells in lymph nodes of patients with esophageal cancer. *N Engl J Med* 1998; **338**: 550
38 **Neoptolemos JP**, Stocken DD, Dunn JA, Almond J, Beger HG, Pederzoli P, Bassi C, Dervenis C, Fernandez-Cruz L, Lacaine F, Buckels J, Deakin M, Adab FA, Sutton R, Imrie C, Ihse I, Tihanyi T, Olah A, Pedrazzoli S, Spooner D, Kerr DJ, Friess H, Buchler MW. Influence of resection margins on survival for patients with pancreatic cancer treated by adjuvant chemoradiation and/or chemotherapy in the ESPAC-1 randomized controlled trial. *Ann Surg* 2001; **234**: 758-768
39 **Neoptolemos JP**, Stocken DD, Friess H, Bassi C, Dunn JA, Hickey H, Beger H, Fernandez-Cruz L, Dervenis C, Lacaine F, Falconi M, Pederzoli P, Pap A, Spooner D, Kerr DJ, Buchler MW. A randomized trial of chemoradiotherapy and chemotherapy after resection of pancreatic cancer. *N Engl J Med* 2004; **350**: 1200-1210

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RAPID COMMUNICATION

Phase Ⅱ study of protracted irinotecan infusion and a low-dose cisplatin for metastatic gastric cancer

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Abstract

AIM: To test protracted irinotecan infusion plus a low-dose cisplatin in this Phase Ⅱ trial to decrease its toxicity.

METHODS: The eligibility criteria were: (1) histologically proven measurable gastric cancer; (2) performance status of 0 or 1; (3) no prior chemotherapy or completion of prior therapy at least 4 wk before enrollment; (4) adequate function of major organs; (5) no other active malignancy; and (6) written informed consent. The regimen consisted of irinotecan (60 mg/m^2) on d 1 and 15 by 24-h infusion and cisplatin (10 mg/m^2) on d 1, 2, 3, 15, 16, and 17. Treatment was repeated every 4 wk.

RESULTS: Thirty-one patients were registered between April 2000 and January 2001. The response rate for all 31 patients, 20 patients without prior chemotherapy, and 11 patients with prior chemotherapy was 52% (16/31), 60% (12/20), and 36% (4/11), respectively. The median survival time was 378 d. The median number of courses given to all patients was 2. Grade 4 neutropenia occurred in 11 (35%) patients, while grade 3 to 4 diarrhea or nausea occurred in 1 (3%) and 3 (10%) patients, respectively. Fatigue was minimal as grade 1 fatigue was found only in 3 (10%) patients. Other adverse events were mild and no treatment-related deaths occurred.

CONCLUSION: This regimen showed a high level of activity and acceptable toxicity in patients with metastatic gastric cancer.

Key words: Gastric cancer; CPT-11; CDDP; Protracted Irinotecan; Chemotherapy

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INTRODUCTION

In Japan, for advanced gastric cancer, surgery is still the most effective treatment and good survival can be achieved if the tumor is resectable. On the contrary, unresectable advanced or recurrent gastric cancer still has a poor prognosis and chemotherapy is the most important treatment for survival prolongation. To date, combination chemotherapy with 5-fluorouracil (5-FU) and cisplatin has been used most widely. This two-drug regimen showed superior response rate in comparison with single-agent 5FU regimen, however, failed to demonstrate survival prolongation[1-3]. This regimen has response rates ranging from 10% to 35%, and the median survival time (MST) from 6 to 8 mo with around 10% in a 2-year survival[4]. Advent of new active agents is awaited.

Among new drugs, irinotecan, a derivative of camptothecin, has strong antitumor activity through inhibition of DNA topoisomerase I[5]. It has a single agent activity with a response rate of 23.3%[6]. As the response rate is not satisfactory, irinotecan was first investigated in combination with cisplatin. Phase I study was conducted for metastatic gastric cancer and treatment regimen of irinotecan (70 mr/m^2, d 1 and 15) and cisplatin (80 mg/m^2, d 1), every 4 wk, was recommended[7]. Boku's group tested this regimen for metastatic gastric cancer[8]. The overall response rate was 48% and the median survival time was 272 d. Although no treatment-related deaths occurred, adverse events were severe, including grade 4 neutropenia in

57% of the patients and grade 3 or 4 diarrhea in 20%. As for renal toxicity, they found a total of 34% (grade 1: 23%, grade 2: 11%) serum creatinine increase. This combination study was followed with modification of weekly schedule to reduce toxicity in western countries. Ajani *et al*[9] conducted phase Ⅱ study with irinotecan (65 mg/m^2) with cisplatin (30 mg/m^2), both administered intravenously 1 d per week for 4 consecutive weeks, followed by two weeks recovery period. Of the 36 patients registered, 21 achieved complete or partial response with response rate of 58%. They had one treatment-related death of neutropenic sepsis with multiple organ failure. Major toxic effects were diarrhea, neutropenia, and fatigue. The incidence of grade 3 or 4 neutropenia was 37%, however, grade 3 or 4 diarrhea was still found in 22% of patients. Surprisingly, grade 3 or 4 fatigue was 41%, a cause of delays or cancellation of drugs. To decrease adverse events, Fujitani *et al*[10] conducted a pharmacokinetic study of continuous infusion of irinotecan for 24 h combined with infusion of cisplatin over 90 min, and demonstrated only mild hematological and nonhematological adverse reactions, while protracted infusion of irinotecan increased the area under the concentration *vs* time curve (AUC) of SN-38.

Accordingly, we further investigated the safety and efficacy of this combined protracted infusion of irinotecan (to maintain a high AUC of SN-38) with a low-dose cisplatin to determine whether this regimen can improve response rate and reduce toxicity.

MATERIALS AND METHODS

Eligibility criteria

Patients were required to satisfy the following eligibility criteria; (1) histologically proven gastric cancer; (2) measurable metastatic lesions; (3) Eastern Clinical Oncology Group scale performance status of 0 or 1; (4) no prior chemotherapy or completion of therapy at least 4 wk before entry; (5) adequate function of the bone marrow WBC count $\geqslant 4 \times 10^9$ and $\leqslant 12 \times 10^9$, platelet count $\geqslant 100 \times 10^9$, and hemoglobin $\geqslant$ 95 g/L), liver (serum bilirubin $\leqslant$ 25.6 μmol/L and serum transaminases $\leqslant$ 1667 nkat/L), and kidneys (serum creatinine $\leqslant$ 133 μmol/L); (6) normal cardiac function; (7) no other severe medical conditions; (8) no other active malignancy; and (9) ability to give written informed consent. This study was approved by the institutional review boards of all participating hospitals.

Treatment schedule

On d 1, irinotecan (60 mg/m^2) was administered as a 24-h infusion; the drug was diluted in 500 mL of saline or 50 g/L glucose and was protected from the light. Cisplatin (10 mg/m^2) was administered as a 60-min intravenous infusion with adequate hydration on d 1, 2, and 3. The same doses of irinotecan and cisplatin were repeated on d 15 and 15-17, respectively, to complete one course. Treatment was repeated every 4 wk until the occurrence of disease progression, patient refusal, or unacceptable adverse reactions. On d 15, if the patient had leucopenia or thrombocytopenia of grade 2 or higher, diarrhea of grade 1 or higher, or fever (a temperature > 38℃) due to infection, administration of the second dose of irinotecan was delayed for one week. If recovery from the adverse reaction did not occur after one week, the second dose was skipped. If a grade 4 hematologic adverse event, grade 3 or 4 diarrhea, fever associated with infection, or omission of the second dose occurred, the dose of irinotecan for the second course was reduced to 50 mg/m^2. The antiemetic granisetron was given before cisplatin administration. Granulocyte colony-stimulating factor (G-CSF) was used when grade 4 leucopenia and/or neutropenia occurred. If the patient stopped treatment due to toxicity or tumor progression, other chemotherapy or surgery was offered.

Evaluation

The National Cancer Institute Common Toxicity Criteria (Version 2.0) were applied for the assessment of adverse events. The objective response of measurable lesions was evaluated by standard World Health Organization criteria. Both patient eligibility and the response to treatment were reviewed extramurally.

Statistical analysis

The expected efficacy rate of this regimen was hypothesized to be 50%, so the required number of patients was 25 when the 95% confidence interval was set at ± 20%. Because some patients might be excluded from analysis, the target number of patients for this study was set at 30. Analysis was performed on an intent-to-treat basis. The percentage of patients with complete remission (CR) plus partial remission (PR) among all treated patients was defined as the response rate. The 95% confidence interval (CI) of the response rate was calculated using the normal approximation and precision method. The Kaplan-Meier method was used to calculate the survival period.

RESULTS

Patients characteristics

Thirty-one patients were enrolled from April 2000 to January 2001. One patient was found to be ineligible because the performance status was 2. As analysis was performed on an intent-to-treat basis, this patient was also analyzed for efficacy and safety, so all 31 patients were assessed for efficacy and safety. Their clinical characteristics are shown in Table 1. The median age was 60.5 (range: 26-75 years) and 21 patients (68%) had performance status of 0. Sixteen patients (52%) had intestinal type adenocarcinoma and 14 patients had diffuse tumors. The measurable metastatic lesions were located in the lymph nodes in 20 patients (26 lesions), liver in 13 patients, peritoneum in 3 patients, ovary in 2 patients, and the skin, lung, and rectum in one patient each, respectively. Twenty patients (65%) had not received prior chemotherapy, while the other 11 patients had undergone chemotherapy with 5-FU, cisplatin plus 5-FU, or other drugs. All of the patients who had received prior therapy showed progressive disease before enrollment. The total number of treatment courses was 84 with a median number of 2 cycles per patient (range 1-5). A total of 149 of 168 planned CPT-11 administration was carried out. While a total of 112 of 122 (92%) planned CPT-11 was administered in the first and second cycle, a

Table 1 Patient profile

	n	Site of metastases	
Sex			
Female	20	Lymph nodes	26
Male	11	Perigastric	6
PS		Para-aortic	6
0	21	Virchow's	3
1	9	Neck	2
2	1	Mediastinum	1
Histology		Others	8
Intestinal	16	Liver	13
Diffuse	14	Peritoneal	3
Others	1	Skin	1
Prior chemotherapy		Lung	1
No	20	Ovary	2
Yes	11	Rectum	1

PS: Performance status.

Table 2 Response to therapy *n* (%)

	CR	PR	NC	PD	RR	95% CI
Overall	0	16 (52)	11 (35)	4 (13)	16 (52)	33-70
Prior chemotherapy						
Yes	0	4 (36)	4 (27)	3 (27)	4 (36)	11-69
No	0	12 (60)	7 (35)	1 (5)	12 (60)	36-81
Liver	2 (15)	5 (39)	5 (39)	1 (8)	7 (54)	
Lymph Nodes	2 (8)	12 (46)	12 (46)	0	14 (54)	
Peritoneal	0	1 (33)	1 (33)	1 (33)	1 (33)	
Ovary	0	0	1 (50)	1 (50)	0	
Lung	0	1 (100)	0	0	1 (100)	
Others	0	1 (50)	1 (50)	0	1 (50)	

CR: Complete remission; PR: Partial response; NC: No change; PD: Progressive disease; RR: Response rate.

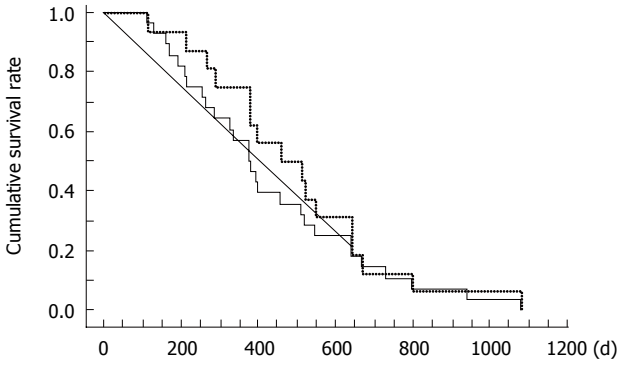

Figure 1 Survival curves of all patients and patients without prior chemotherapy. —: Survival curve of all patients (median survival time, 378 d). ...: Survival curve of patients without priorchemotherapy (median survival time, 509 d).

Table 3 Adverse reactions

	Grade (%)			
	1	2	3	4
Hematologic				
Leukopenia	1 (3)	14 (45)	8 (26)	3 (10)
Neutropenia	1 (3)	2 (6)	13 (42)	11 (35)
Thrombocytopenia	8 (25)	1 (3)	1 (3)	1 (3)
Anemia	5 (16)	13 (42)	7 (23)	2 (6)
Nonhematologic				
Fatigue	3 (10)	0	0	0
Nausea	12 (39)	8 (26)	3 (10)	0
Diarrhea	9 (29)	4 (13)	1 (3)	0
Abdo pain	2 (6)	2 (6)	1 (3)	0
Alopecia	11 (35)	10 (30)	1 (3)	0
AST	1 (3)	0	0	0
T. bilirubin	1 (3)	0	0	0
Cr	8 (26)	0	0	0

Abdo pain: Abdominal pain.

total of 37 of 46 (80%) planned CPT-11 administration in the third to fifth cycles. Dose reduction was not required in the first cycle, but it was needed in 8 patients in the second cycle and one patient in the third cycle. Causes of dose reduction were grade 4 neutropenia in 5 patients, grade 4 leukopenia in one patient, and second dose skip in 3 patients. Treatment was stopped due to tumor progression in 8 patients, refusal to continue in 9 patients, and insufficient recovery from adverse reactions in 3 patients. Median duration between the beginning and end of treatment was 75 d. The actual administered dose of CPT-11 in the all courses was 25.7 mg/m^2 per week and that of cisplatin was 13.0 mg/m^2 per week, which corresponded to 85.5% and 86.4% of the planned doses.

Response and survival

There were no complete remissions, but partial remission was achieved in 16 patients for a response rate of 52% (16/31 patients, 95% CI: 33% to 70%). The response rate was 60.0% for the 20 patients without prior chemotherapy (12/20 patients, 95% CI: 36% to 81%), while the rate for the 11 patients with prior chemotherapy was only 36% (4/11 patients, 95% CI: 11% to 69%). The response rate of patients with lymph node, liver, and peritoneal metastasis were 54% (14/26 patients), 54% (7/13 patients) and 33% (1/3 patients), respectively (Table 2). The median duration of response for all the patients was 218 d. The median survival time of all patients was 378 d, while the median survival time of the 20 patients without prior chemotherapy was 509 d. The 1-year and 2-year survival rates were 57% (95% CI: 37%-72%) and 9% (95% CI: 2% -23%), respectively (Figure 1).

Adverse reactions

Adverse reactions to this regimen are shown in Table 3. Grade 4 neutropenia, leukopenia, anemia, and thrombocytopenia were observed in 11 (35%), 3 (10%), 2 (6%), and 1 (3%) of the patients, respectively. The median nadir of the neutrophil count was seen on d 8 (range: d 2-19). G-CSF was administered to 15 patients (48%) during 28 courses. Non-hematologic adverse reactions (nausea, diarrhea, increased AST, and increased bilirubin) were all moderate, with grade 3 nausea (10%) and grade 3 diarrhea (3%) being the maximal reactions. Loperamide or other mild anti-diarrheal medicines were effective for diarrhea, while granisetron and other common anti-emetic medicines were effective for nausea without additional hydration. Renal

toxicity determined by the level of serum creatinine was also mild. We found 8 patients (26%) with only grade 1 abnormal creatinine level. Fatigue was found in only 3 patients and all grade 1. The non-hematologic reactions did not disrupt the treatment schedule and there were no treatment-related deaths.

DISCUSSION

The aim of this phase Ⅱ study was to confirm the antitumor effects and safety of combined chemotherapy of protracted infusion of irinotecan and low-dose cisplatin[10]. Protracted infusion of irinotecan significantly increases the AUC of active metabolite, SN-38[10], suggesting it has the potential to maximize the effect of irinotecan. The response rate to irinotecan as a single agent for gastric cancer was reported to be 23.3%[6] and that for cisplatin was 20%[11]. The overall response rate of the 31 patients in the present series was 52% and that of 20 patients without any prior chemotherapy was 60%, with both rates being better than for single agent therapy. These data are consistent with a previous phase Ⅱ study of cisplatin and irinotecan therapy[8,9], and confirms the activity of combined therapy of irinotecan and cisplatin.

Diarrhea is a serious toxicity of irinotecan therapy. The incidence of grade 3 or 4 diarrhea was reported to be 18.8% or 35.6% with irinotecan alone and 21.9% or 35.6% with bolus combined administration[12-15]. In the previous phase Ⅱ studies of irinotecan and cisplatin, diarrhea was still one of the major cause that could affect treatment schedule and the incidence of grade 3 or 4 diarrhea was around 20%[8,9]. In the present study, grade 4 diarrhea did not occur and only one patient (3%) suffered from grade 3 diarrhea that did not necessitate a change of schedule.

The incidence of grade 3 or 4 neutropenia (77%) is still high in this study, and Boku *et al*[8] found the incidence of grade 3 or 4 neutropenia was 89%. Ajani *et al*[9] demonstrated that 37% grade 3 or 4 neutropenia by weekly administration. Sakaki *et al*[16] found a positive correlation between the AUC of irinotecan and the decrease of the white cell count, however, diarrhea has a stronger correlation with the AUC of SN-38 than with that of irinotecan. As this treatment regimen has greater AUC of SN-38 while AUC of irinotecan was same in comparison with 90-min infusion of irinotecan[10], we expected to find more hematological toxicities than diarrhea. To the contrary, we found significantly less common severe diarrhea, and high incidence of grade 3 or 4 neutropenia that was comparable to the previous Japanese trial[8]. The target dose of irinotecan for this study (60 mg/m^2 × 2) was lower than that used by Boku (70 mg/m^2 × 2)[8] or that used to treat lung cancer (60 mg/m^2 × 3)[17,18]. Actual dose of CPT-11 administered in the Boku's study was 28.5 mg/m^2 per week, and that in our study was 25.7 mg/m^2 per week, so our regimen seemed to reduce the incidence and grade of diarrhea while achieving a similar response rate with a lower dose of irinotecan.

Nausea and renal toxicity is a common problem with cisplatin therapy, so we administered a low dose of 10 mg/m^2 on six occasions to achieve a total dose of 60 mg/m^2. Grade 3 nausea occurred in 3 patients (10%) and there was no grade 4 nausea. Low-dose, repeated administration has already been reported to decrease the incidence and grade of nausea due to cisplatin[19-22]. Some Japanese authors have reported that repeated cisplatin administration at a low dose reduces the incidence of nausea and allows outpatient treatment, and that this method achieves a high response rate and longer survival when combined with irinotecan without the need for 24-h infusion of the latter drug[9,23-25]. As for renal toxicity, Boku *et al* with high-dose cisplatin administration[8] found grade 1 or 2 serum creatinine increase in 23 and 11 % of patients, respectively. We found only grade 1 abnormality, and the incidence of 26%, demonstrating that renal toxicity with this regimen is minimal. As hydration is not required to prevent renal toxicity with low-dose therapy[20], continuous infusions are not necessary after finishing irinotecan administration on d 2, 3, 16, and 17.

In order to complete this regimen, hospitalization is required to receive 24-h irinotecan infusions and 6 divided dose of cisplatin. Therefore, this regimen is not applicable for out-patient basis; however, as the incidence of fatigue of this regimen was 10% (only grade 1), severe diarrhea was rare, and hydration was not necessary, it is possible to treat patients on an out-patient basis between infusions. Ajani et al demonstrated very high incidence (41%) of grade 3 or 4 fatigue with weekly irinotecan and cisplatin administration, and they discussed cisplatin might contribute to excessive fatigue, thus either the dose of cisplatin might be reduced, or cisplatin might be replaced by other agents. Our results with 10% grade 1 fatigue suggested that low dose cisplatin is a practical alternative for reducing the fatigue.

In conclusion, though hospitalization is required at this time, 24-h infusion of irinotecan combined with a low-dose, repeated administration of cisplatin achieved a high response rate and prolonged the survival of patients with metastatic gastric cancer. This regimen also reduces non-hematologic adverse reactions and thus shortens the time in hospital.

REFERENCES

1 **Cullinan SA**, Moertel CG, Wieand HS, O'Connell MJ, Poon MA, Krook JE, Mailliard JA, Tschetter LK. Controlled evaluation of three drug combination regimens versus fluorouracil alone for the therapy of advanced gastric cancer. North Central Cancer Treatment Group. *J Clin Oncol* 1994; **12**: 412-416

2 **Kim NK**, Park YS, Heo DS, Suh C, Kim SY, Park KC, Kang YK, Shin DB, Kim HT, Kim HJ. A phase III randomized study of 5-fluorouracil and cisplatin versus 5-fluorouracil, doxorubicin, and mitomycin C versus 5-fluorouracil alone in the treatment of advanced gastric cancer. *Cancer* 1993; **71**: 3813-3818

3 **Ohtsu A**, Shimada Y, Shirao K, Boku N, Hyodo I, Saito H, Yamamichi N, Miyata Y, Ikeda N, Yamamoto S, Fukuda H, Yoshida S. Randomized phase III trial of fluorouracil alone versus fluorouracil plus cisplatin versus uracil and tegafur plus mitomycin in patients with unresectable, advanced gastric cancer: The Japan Clinical Oncology Group Study (JCOG9205). *J Clin Oncol* 2003; **21**: 54-59

4 **Ohtsu A**. Current status and future prospects of chemotherapy for metastatic gastric cancer: a review. *Gastric Cancer* 2005; **8**: 95-102

5 **Kunimoto T**, Nitta K, Tanaka T, Uehara N, Baba H, Takeuchi M, Yokokura T, Sawada S, Miyasaka T, Mutai M. Antitumor activity of a new camptothecin derivative, SN-22, against

various murine tumors. *J Pharmacobiodyn* 1987; **10**: 148-151
6 **Futatsuki K**, Wakui A, Nakao I, Sakata Y, Kambe M, Shimada Y, Yoshino M, Taguchi T, Ogawa N. Late phase II study of irinotecan hydrochloride (CPT-11) in advanced gastric cancer. CPT-11 Gastrointestinal Cancer Study Group. *Gan To Kagaku Ryoho* 1994; **21**: 1033-1038
7 **Shirao K**, Shimada Y, Kondo H, Saito D, Yamao T, Ono H, Yokoyama T, Fukuda H, Oka M, Watanabe Y, Ohtsu A, Boku N, Fujii T, Oda Y, Muro K, Yoshida S. Phase I-II study of irinotecan hydrochloride combined with cisplatin in patients with advanced gastric cancer. *J Clin Oncol* 1997; **15**: 921-927
8 **Boku N**, Ohtsu A, Shimada Y, Shirao K, Seki S, Saito H, Sakata Y, Hyodo I. Phase II study of a combination of irinotecan and cisplatin against metastatic gastric cancer. *J Clin Oncol* 1999; **17**: 319-323
9 **Ajani JA**, Baker J, Pisters PW, Ho L, Mansfield PF, Feig BW, Charnsangavej C. CPT-11 plus cisplatin in patients with advanced, untreated gastric or gastroesophageal junction carcinoma: results of a phase II study. *Cancer* 2002; **94**: 641-646
10 **Fujitani K**, Tsujinaka T, Hirao M. Pharmacokinetic study of two infusion schedules of irinotecan combined with cisplatin in patients with advanced gastric cancer. *Oncology* 2003; **64**: 111-115
11 **Lacave AJ**, Izarzugaza I, Anton Aparicio LM, Valle Pereda M, Gracia Marco JM, Buesa JM. Phase II clinical trial of cis-dichlorodiammineplatinum in gastric cancer. *Am J Clin Oncol* 1983; **6**: 35-38
12 **Rothenberg ML**, Kuhn JG, Burris HA 3rd, Nelson J, Eckardt JR, Tristan-Morales M, Hilsenbeck SG, Weiss GR, Smith LS, Rodriguez GI. Phase I and pharmacokinetic trial of weekly CPT-11. *J Clin Oncol* 1993; **11**: 2194-2204
13 **de Forni M**, Bugat R, Chabot GG, Culine S, Extra JM, Gouyette A, Madelaine I, Marty ME, Mathieu-Boue A. Phase I and pharmacokinetic study of the camptothecin derivative irinotecan, administered on a weekly schedule in cancer patients. *Cancer Res* 1994; **54**: 4347-4354
14 **Rowinsky EK**, Grochow LB, Ettinger DS, Sartorius SE, Lubejko BG, Chen TL, Rock MK, Donehower RC. Phase I and pharmacological study of the novel topoisomerase I inhibitor 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin (CPT-11) administered as a ninety-minute infusion every 3 weeks. *Cancer Res* 1994; **54**: 427-436
15 **Abigerges D**, Chabot GG, Armand JP, Herait P, Gouyette A, Gandia D. Phase I and pharmacologic studies of the camptothecin analog irinotecan administered every 3 weeks in cancer patients. *J Clin Oncol* 1995; **13**: 210-221
16 **Sasaki Y**, Yoshida Y, Sudoh K, Hakusui H, Fujii H, Ohtsu T, Wakita H, Igarashi T, Itoh K. Pharmacological correlation between total drug concentration and lactones of CPT-11 and SN-38 in patients treated with CPT-11. *Jpn J Cancer Res* 1995; **86**: 111-116
17 **Negoro S**, Fukuoka M, Masuda N, Takada M, Kusunoki Y, Matsui K, Takifuji N, Kudoh S, Niitani H, Taguchi T. Phase I study of weekly intravenous infusions of CPT-11, a new derivative of camptothecin, in the treatment of advanced non-small-cell lung cancer. *J Natl Cancer Inst* 1991; **83**: 1164-1168
18 **Fukuda M**, Nishio K, Kanzawa F, Ogasawara H, Ishida T, Arioka H, Bojanowski K, Oka M, Saijo N. Synergism between cisplatin and topoisomerase I inhibitors, NB-506 and SN-38, in human small cell lung cancer cells. *Cancer Res* 1996; **56**: 789-793
19 **Kobayashi K**, Kudoh S, Takemoto T, Hino M, Hayashihara K, Nakahiro K, Ando M, Niitani H. In vitro investigation of a combination of two drugs, cisplatin and carboplatin, as a function of the area under the c/t curve. *J Cancer Res Clin Oncol* 1995; **121**: 715-720
20 **Kurihara N**, Kubota T, Hoshiya Y, Otani Y, Ando N, Kumai K, Kitajima M. Pharmacokinetics of cis-diamminedichloroplatinum (II) given as low-dose and high-dose infusions. *J Surg Oncol* 1996; **62**: 135-138
21 **Schellens JH**, Ma J, Planting AS, van der Burg ME, van Meerten E, de Boer-Dennert M, Schmitz PI, Stoter G, Verweij J. Relationship between the exposure to cisplatin, DNA-adduct formation in leucocytes and tumour response in patients with solid tumours. *Br J Cancer* 1996; **73**: 1569-1575
22 **Ikeda K**, Terashima M, Kawamura H, Takiyama I, Koeda K, Takagane A, Sato N, Ishida K, Iwaya T, Maesawa C, Yoshinari H, Saito K. Pharmacokinetics of cisplatin in combined cisplatin and 5-fluorouracil therapy: a comparative study of three different schedules of cisplatin administration. *Jpn J Clin Oncol* 1998; **28**: 168-175
23 **Sato A**, Kurihara M, Matsukawa M, Shimada K, Yamazaki T, Nakamachi M, Koda T. Preliminary study of fortnightly irinotecan hydrochloride plus cisplatin therapy in patients with advanced gastric and colorectal cancer. *Cancer Chemother Pharmacol* 2001; **47**: 380-384
24 **Ajani JA**, Baker J, Pisters PW, Ho L, Feig B, Mansfield PF. Irinotecan plus cisplatin in advanced gastric or gastroesophageal junction carcinoma. *Oncology* (Williston Park) 2001; **15**: 52-54
25 **Shimada S**, Yagi Y, Kuramoto M, Aoki N, Ogawa M. Second-line chemotherapy with combined irinotecan and low-dose cisplatin for patients with metastatic gastric carcinoma resistant to 5-fluorouracil. *Oncol Rep* 2003; **10**: 687-691

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RAPID COMMUNICATION

Gallbladder carcinoma associated with pancreatobiliary reflux

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Abstract

AIM: To detect the patients with and without pancreaticobiliary maljunction who had pancreatobiliary reflux with extremely high biliary amylase levels.

METHODS: Ninety-six patients, who had diffuse thickness (> 3 mm) of the gallbladder wall and were suspected of having a pancreaticobiliary maljunction on ultrasonography, were prospectively subjected to endoscopic retrograde cholangiopancreatography, and bile in the common bile duct was sampled. Among them, patients, who had extremely high biliary amylase levels (>10 000 IU/L), underwent cholecystectomy, and the clinicopathological findings of those patients with and without pancreaticobiliary maljunction were examined.

RESULTS: Seventeen patients had biliary amylase levels in the common bile duct above 10 000 IU/L, including 11 with pancreaticobiliary maljunction and 6 without pancreaticobiliary maljunction. The occurrence of gallbladder carcinoma was 45.5% (5/11) in patients with pancreaticobiliary maljunction, and 50% (3/6) in those without pancreaticobiliary maljunction.

CONCLUSION: Pancreatobiliary reflux with extremely high biliary amylase levels and associated gallbladder carcinoma could be identified in patients with and without pancreaticobiliary maljunction, and those patients might be detected by ultrasonography and bile sampling.

Key words: Amylase; Bile; Gallbladder carcinoma; Pancreatobiliary reflux; Pancreaticobiliary maljunction; Diagnosis

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INTRODUCTION

It is well known that pancreatobiliary reflux is an important risk factor for the carcinogenesis of the biliary system in patients with pancreaticobiliary maljunction (PBM)[1,2], which is a congenital anomaly defined as an abnormal union of the common bile duct and pancreatic duct that is located outside the duodenal wall where a sphincter system is not present and pancreatic juice freely regurgitates into the biliary tract through the communication[3].

Recently, we reported that pancreatobiliary reflux with extremely high biliary amylase levels can occur not only in patients with PBM, but also in those without PBM[4,5], although the latter condition is not well known yet. In the present study, we tried to detect the patients, who had pancreatobiliary reflux with extremely high biliary amylase levels, and examined the clinicopathological findings of those patients.

MATERIALS AND METHODS

Subjects

Between March 2002 and February 2006, 96 patients, who had diffuse thickness of the gallbladder wall above 3 mm and were suspected of having pancreaticobiliary maljunction on ultrasonography, were prospectively subjected to endoscopic retrograde cholangiopancreatography (ERCP). Bile in the common bile duct was sampled as follows and biliary amylase levels were measured in those patients. After selective insertion of an ERCP catheter into the bile duct, bile was aspirated from the common bile duct at a depth of 5 cm. To avoid contamination with pancreatic juice, the first 5 mL of the aspirated bile was not used to measure biliary amylase levels, and another sample of bile was obtained for the measurement. In all patients, serum amylase was measured within 48 h before cholecystectomy. Amylase in the serum and bile was measured by an enzymatic method using 3-ketobutylidene-2-chloro-4-nitrophenyl-maltopentaoside (Diacolor Neonate, Toyobo, Osaka, Japan) as the substrate[6]. The normal range for serum amylase in our institution is 130 to 400 IU/L. We defined extremely high biliary amylase levels as those above 10 000 IU/L, and patients with extremely high biliary amylase levels were indicated for cholecystectomy. Patients having bile duct stenosis, filling defect in the bile duct, including choledocholithiasis on ERCP, were excluded

Table 1A Clinical characteristics of patients with non-pancreaticobiliary maljunction

	Age	Sex	Biliary amylase (IU/L)	Common channel length (mm)	Pathology	Depth of invasion	GB stone	Choledocal cyst
1	56	M	47190	7	Hyperplasia		+	-
2	69	F	27038	8	Hyperplasia		+	-
3	63	F	61710	4	Hyperplasia		-	-
4	53	F	52600	8	ca	m	-	-
5	68	F	84700	9	ca	m	-	-
6	70	F	64260	4	ca	ss	-	-

GB: Gallbladder; m: Tumor invades the mucosa; ss: Tumor invades the subserosa; ca: Adenocarcinoma.

Table 1B Clinical characteristics of patients with pancreaticobiliary maljunction

	Age	Sex	Biliary amylase (IU/L)	Common channel length (mm)	Pathology	Depth of invasion	GB stone	Choledocal cyst
1	65	F	12030	18	chr-itis		+	+
2	74	F	153000	16	chr-itis		+	-
3	63	M	136400	48	ADM		-	+
4	34	F	145000	15	Hyperplasia		+	-
5	46	F	103500	26	Hyperplasia		-	-
6	40	F	126900	28	Hyperplasia		+	+
7	47	M	95400	19	ca	m	-	-
8	54	F	132180	27	ca	ss	-	-
9	64	F	177520	24	ca	ss	-	-
10	75	F	26510	20	ca	ss	-	-
11	57	F	21100	19	ca	se	-	-

GB: Gallbladder; m: Tumor invades the mucosa; ss: Tumor invades the subserosa; se: Tumor perforates serosa; chr-it is: Chronic cholecystitis; ADM: Adenomyomatosis; ca: Adenocarcinoma.

from the present study, because their biliary amylase levels in the common bile duct would not correctly reflect pancreatobiliary reflux.

Patients were divided into those with PBM and without PBM (non-PBM). On ERCP images, the two ducts were always communicated in PBM patients, but not in non-PBM patients due to the sphincter contraction. The length of the common channel was measured on ERCP images corrected for magnification using the diameter of the endoscope as a reference.

Informed consent was obtained from all the patients. The study protocol was approved by the ethics committee of our institution.

Tissue specimens and histologic analysis

All specimens were fixed in 10% buffered formalin. The gallbladder tissues embedded in paraffin were cut into 4-μm serial sections, and the gallbladder mucosa was histologically examined using sections stained with hematoxylin and eosin (HE). All specimens were diagnosed based on light microscopic findings. Carcinoma of the gallbladder was diagnosed according to the criteria reported by Albores-Saavedra *et al*[7].

RESULTS

Serum amylase was within 600 IU/L in all patients. Among the 96 patients who underwent ERCP, bile was successfully sampled in 87 patients; four patients underwent unsuccessful ERCP, two had bile duct stenosis, three had choledocholithiasis, and these patients were excluded from the present study. Consequently, 17 patients had extremely high biliary amylase levels in the common bile duct above 10000 IU/L, including 11 PBM patients and 6 non-PBM patients. Among the PBM patients, 4 had a choledocal cyst. All patients with biliary amylase levels above 10000 IU/L underwent cholecystectomy. The main clinical characteristics of PBM and non-PBM patients are shown in Tables 1 and 2. The occurrence of gallbladder carcinoma was 45.5% (5/11) in PBM patients, and 50% (3/6) in non-PBM patients. PBM patients included 1 carcinoma limited within the mucosa, while non-PBM patients included 2 (Table 1, Figure 1).

DISCUSSION

Gallbladder carcinoma is known to carry a poor prognosis[8]. It is therefore essential to identify patients at a high risk for developing gallbladder carcinoma[9]. The risk of gallbladder carcinoma associated with PBM is substantial; it was reported that the occurrence of biliary cancer in 388 PBM patients without biliary dilatation was 37.9%, including 93.2% with gallbladder carcinoma and 6.8% with bile duct cancer, while that in 1239 PBM

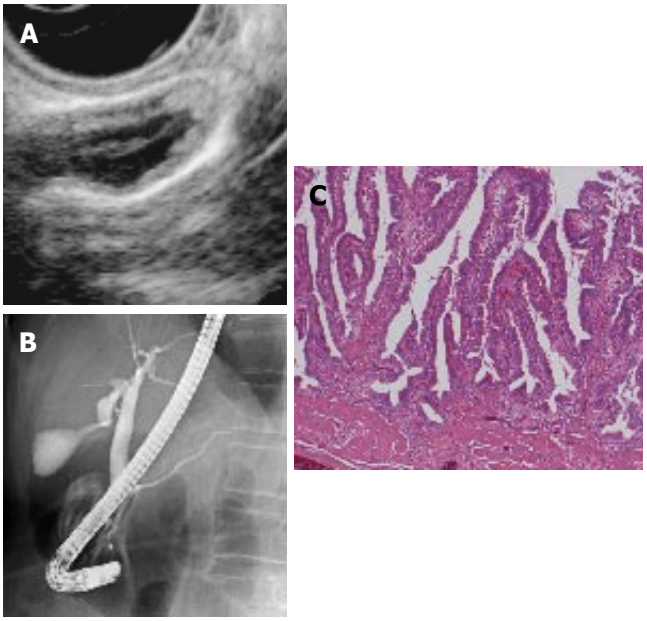

Figure 1 The 52-year old woman who had extremely high biliary amylase level without pancreaticobiliary maljunction and gallbladder carcinoma limited within the mucosa. **A**: Ultrasonography, showing diffuse thickness of the gallbladder wall above 3 mm; **B**: ERCP shows no pancreaticobiliary maljunction and common channel of 7 mm. Biliary amylase levels in the common bile duct was 52 600 IU/L; **C**: Gallbladder carcinoma limited within the mucosa (HE, x 40).

patients with choledocal cyst was 10.6%, including 33.6% with extrahepatic bile duct cancer and 64.9% with gallbladder carcinoma[10]. Numerous studies have shown that pancreatobiliary reflux is a major risk factor for biliary carcinogenesis in patients with PBM; the mixture of bile and pancreatic juice can induce chronic inflammation and genetic alterations and increase cellular proliferation of the biliary tract epithelium, leading to hyperplasia, dysplasia and ultimately carcinoma of the biliary tract mucosa[11,12].

Pancreatobiliary reflux with extremely high biliary amylase levels was also identified in non-PBM patients in the present study, and 50% of those patients had gallbladder carcinoma. Furthermore it included gallbladder carcinoma limited within the mucosa, and thus detection of pancreatobiliary reflux with extremely high biliary amylase levels might allow management for gallbladder carcinoma at an early stage.

In order to identify pancreatobiliary reflux pre-operatively, secretin-injection magnetic resonance cholangiopancreatography (MRCP) is an option that has proved useful as we have previously reported[4,5], although high amylase levels in the bile sampled during ERCP is a direct evidence of pancreatobiliary reflux. One of the characteristic findings of ultrasonography associated with pancreaticobiliary maljunction was reported as diffuse thickness of the gallbladder wall above 3 mm, that might reflect mucosal change associated with pancreatobiliary reflux including chronic inflammation and increased cellular proliferation of the gallbladder mucosa[13]. In the present study, ultrasonographic finding also proved useful to diagnose non-PBM patients with extremely high biliary amylase levels, although the finding was non-specific and was seen in other diseases including chronic cholecystitis with or without gallstone, adenomyomatosis, and liver cirrhosis[13].

Table 2 Clinicopathological findings in patients with and without pancreaticobiliary maljunction

	non-PBM (n = 6)	PBM (n = 11)
Age (mean ± SD)	63.2 ± 7.2	54.1 ± 12
Female (number)	5/6 (83%)	9/11 (82%)
CBD-Amy (mean ± SD) IU/L	56 250 ± 19 246	102 686 ± 57 660
Cholecystolithiasis (number)	2/6 (33%)	4/11 (36%)
Length of the common channel (mean ± SD) mm	6.7 ± 2.2	23.6 ± 9.2
Gallbladder carcinoma (number)	3/6 (50%)	5/11 (46%)

PBM: Pancreaticobiliary maljunction; CBD-Amy: Amylase levels in the common bile duct; SD: Standard deviation.

Reflux of pancreatic juice into the biliary tract is influenced by the function of Oddi's sphincter and the form of the junction of the pancreaticobiliary duct[14]. One of the mechanisms of pancreatobiliary reflux in non-PBM patients could be a long common channel. Actually, 67% (4/6) of non-PBM patients had a long common channel more than 7 mm in the present study. Misra *et al* reported that a common channel more than 8 mm in length was seen more frequently in patients with gallbladder carcinoma (38%) compared with normal subjects (3%) or patients with gallstones (1%)[15]. Kamisawa *et al* also reported that the occurrence of gallbladder carcinoma in non- PBM patients with a common channel of more than 6 mm in length was 12%, being significantly higher than that in controls[16]. They speculated that the longer common channel could be associated with a higher occurrence and a more significant degree of pancreatobiliary reflux, which might be the cause of gallbladder carcinoma, although they did not measure the biliary amylase levels in their study. Another mechanism of pancreatobiliary reflux in non-PBM patients, especially in our two patients with a common channel of 4 mm, might be the dysfunction of the sphincter of Oddi, however, the precise mechanism of pancreatobiliary reflux in non-PBM patients should be further clarified in future studies.

In conclusion, pancreatobiliary reflux with extremely high biliary amylase levels and associated gallbladder carcinoma could be identified in patients with and without pancreaticobiliary maljunction, and those patients might be detected by ultrasonography and bile sampling.

REFERENCES

1 **Kimura K**, Ohto M, Saisho H, Unozawa T, Tsuchiya Y, Morita M, Ebara M, Matsutani S, Okuda K. Association of gallbladder carcinoma and anomalous pancreaticobiliary ductal union. *Gastroenterology* 1985; **89**: 1258-1265

2 **Nagata E**, Sakai K, Kinoshita H, Kobayashi Y. The relation between carcinoma of the gallbladder and an anomalous connection between the choledochus and the pancreatic duct. *Ann Surg* 1985; **202**: 182-190

3 The Japanese Study Group on Pancreaticobiliary Maljunction. Diagnostic criteria of pancreaticobiliary maljunction. *J Hep Bil Pancr Surg* 1994; **1**: 219-221

4 **Sai JK**, Ariyama J, Suyama M, Kubokawa Y, Sato N. Occult regurgitation of pancreatic juice into the biliary tract: diagnosis with secretin injection magnetic resonance cholangiopancreato graphy. *Gastrointest Endosc* 2002; **56**: 929-932

5 **Sai JK**, Suyama M, Kubokawa Y, Tadokoro H, Sato N, Maehara T, Iida Y, Kojima K. Occult pancreatobiliary reflux in patients with a normal pancreaticobiliary junction. *Gastrointest Endosc* 2003; **57**: 364-368

6 **Teshima S**, Hayashi Y, Emi S, Ishimaru K. Determination of alpha-amylase using a new blocked substrate (3-ketobutylidene beta-2-chloro-4-nitrophenyl-maltopentaoside). *Clin Chim Acta* 1991; **199**: 23-31

7 **Albores-Saavedra J**, Henson DE, Klimstra DS. Tumors of the gallbladder, extrahepatic bile duct, and ampulla of Vater. Atlas of tumor pathology. 3rd series, fascicle 27. Washington, D.C.: Armed Forces Institute of Pathology; 2000: 51-113

8 **Bengmark S**, Jeppsson B. Tumors of the gallbladder. In: Textbook of Gastroenterology, 2nd ed. T Yamada ed. Philadelphia, JB Lippincott, 1995: 2739-2744

9 **Sheth S**, Bedford A, Chopra S. Primary gallbladder cancer: recognition of risk factors and the role of prophylactic cholecystectomy. *Am J Gastroenterol* 2000; **95**: 1402-1410

10 **Tashiro S**, Imaizumi T, Ohkawa H, Okada A, Katoh T, Kawaharada Y, Shimada H, Takamatsu H, Miyake H, Todani T. Pancreaticobiliary maljunction: retrospective and nationwide survey in Japan. *J Hepatobiliary Pancreat Surg* 2003; **10**: 345-351

11 **Hanada K**, Itoh M, Fujii K, Tsuchida A, Hirata M, Ishimaru S, Iwao T, Eguchi N, Kajiyama G. Pathology and cellular kinetics of gallbladder with an anomalous junction of the pancreaticobiliary duct. *Am J Gastroenterol* 1996; **91**: 1007-1011

12 **Matsubara T**, Sakurai Y, Sasayama Y, Hori H, Ochiai M, Funabiki T, Matsumoto K, Hirono I. K-ras point mutations in cancerous and noncancerous biliary epithelium in patients with pancreaticobiliary maljunction. *Cancer* 1996; **77**: 1752-1757

13 **Yamao K**, Mizutani S, Nakazawa S, Inui K, Kanemaki N, Miyoshi H, Segawa K, Zenda H, Kato T. Prospective study of the detection of anomalous connections of pancreatobiliary ducts during routine medical examinations. *Hepatogastroenterology* 1996; **43**: 1238-1245

14 **Boyden EA**. The anatomy of the choledochoduodenal junction in man. *Surg Gynecol Obstet* 1957; **104**: 641-652

15 **Misra SP**, Gulati P, Thorat VK, Vij JC, Anand BS. Pancreaticobiliary ductal union in biliary diseases. An endoscopic retrograde cholangiopancreatographic study. *Gastroenterology* 1989; **96**: 907-912

16 **Kamisawa T**, Amemiya K, Tu Y, Egawa N, Sakaki N, Tsuruta K, Okamoto A, Munakata A. Clinical significance of a long common channel. *Pancreatology* 2002; **2**: 122-128

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RAPID COMMUNICATION

Signaling pathway of insulin-like growth factor-Ⅱ as a target of molecular therapy for hepatoblastoma

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Abstract

AIM: To address the possibility that insulin-like growth factor (IGF)-Ⅱ is a growth factor and its signaling pathway so as to develop a molecular therapy for hepatoblastoma.

METHODS: Huh-6 and HepG2, human hepatoblastoma cell lines, were used. IGF-Ⅱ was added to the medium deprived of serum. Western blot analysis was performed to clarify the expression of IGF-I receptor (IGF-IR). Inhibitors of IGF-IR (picropodophyllin, PPP), phosphatidyl-inositol (PI) 3-kinase (LY294002 and Wortmannin), or mitogen-activated protein (MAP) kinase (PD98059) were added to unveil the signaling pathway of IGF-Ⅱ. Cells were analyzed morphologically with hematoxylin-eosin staining to reveal the mechanism of suppression of cell proliferation.

RESULTS: IGF-Ⅱ stimulated cells proliferated to 2.7 (269% ± 76%) (mean ± SD) (Huh-6) and 2.1 (211% ± 85%) times (HepG2). IGF-IR was expressed in Huh-6 and HepG2. PPP suppressed the cell number to 44% ± 11% (Huh-6) and 39% ± 5% (HepG2). LY294002 and Wortmannin suppressed the cell number to 30% ± 5% (Huh-6), 44% ± 0.4% (HepG2), 49% ± 1.0% (Huh-6) and 46% ± 1.1% (HepG2), respectively. PD98059 suppressed the cell number to 33% ± 11% for HepG2 but not for Huh-6. When cell proliferation was prohibited, many Huh-6 and HepG2 cells were dead with pyknotic or fragmented nuclei, suggesting apoptosis.

CONCLUSION: IGF-Ⅱ was shown to be a growth factor of hepatoblastoma *via* IGF-I receptor and PI3 kinase which were good candidates for target of molecular therapy.

INTRODUCTION

Hepatoblastoma (HBL) arises in the liver of infants younger than 3 years of age[1]. The growth factor and its signaling pathway could be revealed so as to develop a novel molecular therapy for HBL.

Insulin-like growth factor (IGF)-Ⅱ is a hormone that plays an important role in fetal growth and development. IGF-Ⅱ is abundantly expressed in fetus, with its concentration decreasing after birth[2]. Interestingly, IGF-Ⅱ is detected at high levels in the serum of HBL patients[3]. Moreover, the expression levels of IGF-Ⅱ in tumor tissues are higher than those of surrounding non-tumor tissues in surgical specimens from HBL patients due to biallelic expression of the gene by loss of the methylated status of the promoter[4]. These facts indicate that IGF-Ⅱ is deeply involved in the carcinogenesis and progression of HBL.

IGF-Ⅱ binds to insulin receptor (IR), IGF-I receptor (IGF-IR), and IGF-Ⅱ receptor[5]. With the binding of IGF-Ⅱ, IR mediates glucose metabolism, such as insulin. IGF-ⅡR mediates the degradation of IGF-Ⅱ, acting as a tumor-suppressor gene. It was expected that IGF-IR mediated the stimulation of proliferation by IGF-Ⅱ. Indeed, antibody to IGF-IR successfully suppressed the proliferation of HepG2[6]. Once IGF-Ⅱ binds to IGF-IR, the receptor autophosphorylates and activates phosphatidyl-inositol (PI) 3-kinase or mitogen-activated protein (MAP) kinase. Further down-stream pathways modulate the gene expression to perform the role of IGF-Ⅱ.

However, it is not clear whether IGF-Ⅱ promotes the cell proliferation of HBL, or the signaling pathway of IGF-Ⅱ to mediate the stimulation of cell proliferation.

Here, we tried to address the possibility of IGF-Ⅱ in HBL as a growth factor. We also tried to clarify its signaling pathway with inhibitors to signaling pathways, pursuing a potential application for a molecular therapy.

MATERIALS AND METHODS

Cell culture

Huh-6 and HepG2 hepatoblastoma cell lines, were purchased from RIKEN Cell Bank (Tsukuba, Japan) and cultured in Dulbecco's Minimum Essential Medium (Sigma, St. Louis, MO) supplemented with 100 g/L fetal bovine serum (FBS) (Trace Scientific, Melbourne, Australia) in 50 mL/L at 37℃ in a humidified chamber. Both cell lines are IGF-IR positive[7]. For hematoxylin-eosin (H&E) staining, cells were spread onto chamber slides.

Cell viability assay

Freshly thawed cells were seeded onto 10 cm dishes (Asahi Techno Glass, Funabashi, Japan). When they reached sub-confluence, they were trypsinized, harvested, and spread onto 96-well flat-bottom plates with (Asahi Techno Glass) at a density of 1000 cells per well. Following 24 h of culture under DMEM with 100 g/L FBS, medium was changed to DMEM without FBS to quench the FBS effects. After 24 h of culture under DMEM without FBS, IGF-Ⅱ (Wako Pure Chemicals, Osaka, Japan) was added to the medium. Seventy-two hours later, 3- (4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay was performed according to the manufacturer's instructions (Promega Corporation, Tokyo, Japan). MTS is bioreduced by cells into a colored formazan product that reduces absorbance at 490 nm. The absorbance was analyzed with a multiple plate reader at a wavelength of 490 nm with a BIO-RAD Model 550 microplate reader (Bio-RAD, Hercules, CA). When LY290042 (Wako Pure Chemicals), PD98059 (Wako Pure Chemicals), Wortmannin (Wako Pure Chemicals), and picropodophyllin (PPP) (Wako Pure Chemicals) were used, each was added to the medium 30 min prior to the addition of IGF-Ⅱ.

Western blot analysis

Twenty microgram of protein isolated from cultured cells was subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis, and transferred to a nylon filter. Primary antibodies were polyclonal rabbit anti-IGF-IR antibody (Cell Signaling Technology, Danvers, MA) and mouse monoclonal anti-Tubulin-α antibody (Lab Vision, Fremont, CA). Second antibodies were horseradish peroxidase (HRP)-linked anti-rabbit antibody (Amersham Bioscience, Tokyo, Japan) and HRP-linked anti-mouse antibody (Amersham Bioscience). Dilutions were 1:500 for primary antibodies, and 1:1000 for second antibodies. The filter was reprobed with anti-Tubulin-α antibody. The specific antigen-antibody complexes were visualized by enhanced chemiluminescence (GE Healthcare Bio-Sciences Corp, Piscataway, NJ).

Statistical analysis

Cell proliferation, demonstrated by MTS assay, was analyzed statistically by one-factor analysis of variance. Statistical analysis was performed with JMP5.0J (SAS Institute Japan, Tokyo, Japan). $P < 0.05$ was accepted as statistically significant.

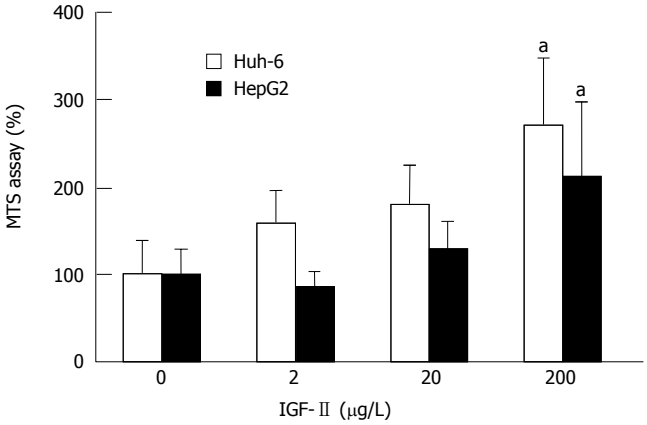

Figure 1 IGF-Ⅱ stimulated proliferation of hepatoblastoma cells. IGF-Ⅱ was added to the medium without serum, followed by MTS assay, a modified method of MTT assay ($^aP < 0.05$).

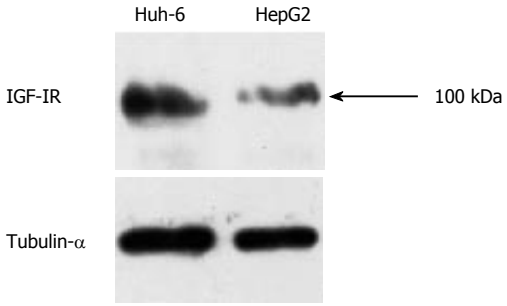

Figure 2 Western blot analysis clearly shows specific bands to IGF-IR. Protein was isolated 72 h after stimulation with IGF-Ⅱ (200 μg/L). The same membrane was reprobed with anti-Tubulin-α antibody to confirm an equal amount of protein loadings.

RESULTS

IGF-Ⅱ stimulated proliferation

Huh-6 and HepG2 proliferated as the concentration of IGF-Ⅱ increased (Figure 1). When IGF-Ⅱ was 200 μg/L, cell proliferation of Huh-6 and HepG2 were 2.7 times (269% ± 76%) (mean ± SD) and 2.1 times (211% ± 85%) higher than those at 0 μg/L of IGF-Ⅱ, respectively ($P < 0.05$, $n = 3$).

IGF-IR expressed in Huh-6 and HepG2

Western blot analysis was performed to analyze expression of IGF-IR in Huh-6 and HepG2 since IGF-IR mediates proliferation activity of IGF-Ⅱ. Protein was isolated from Huh-6 and HepG2 72 h after stimulation with IGF-Ⅱ (200 μg/L). IGF-IR was expressed in Huh-6 and HepG2 (Figure 2). Tubulin-α was expressed to confirm that equal amount of protein was loaded (Figure 2).

PPP suppressed the stimulation of IGF-Ⅱ

PPP, a selective inhibitor of IGF-IR, was used to show

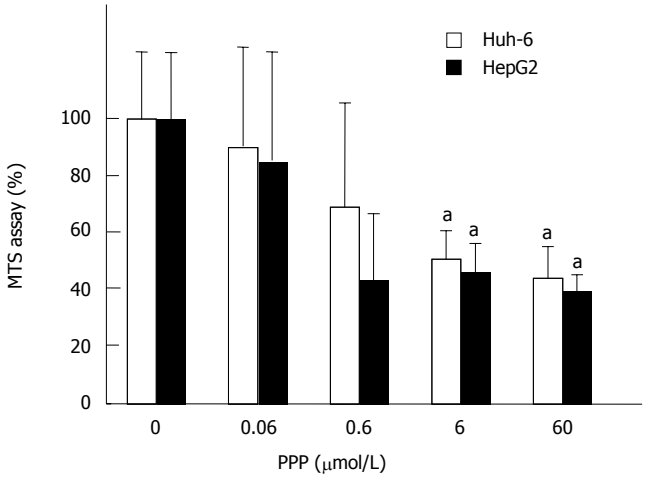

Figure 3 PPP was added to the medium 30 min prior to the stimulation with IGF-II (200 μg/L) and suppressed proliferation of Huh-6 and HepG2 ($^aP < 0.05$).

that IGF-IR mediated the signal of IGF-Ⅱ (Figure 3). PPP at 60 μmol/L suppressed the cell number of Huh-6 and HepG2 stimulated with 200 μg/L of IGF-Ⅱ to 44% ± 10% and 39% ± 5%, respectively ($P < 0.05$, $n = 3$).

Stimulation of IGF-Ⅱ was suppressed by LY294002 and Wortmannin

LY294002 and Wortannin, specific inhibitors of PI3 kinase, were used to reveal that PI3 kinase mediated the signal of IGF-Ⅱ (Figure 4). LY294002 at 50 μmol/L and Wortmannin at 200 μmol/L suppressed the cell numbers of Huh-6 and HepG2 stimulated with 200 μg/L of IGF-Ⅱ to 30% ± 5% and 44% ± 0.4% (Figure 4A) ($P < 0.05$, $n = 3$), and 49% ± 1.0% and 46% ± 1.1% (Figure 4B) ($P < 0.05$, $n = 3$), respectively. PD98059, a specific inhibitor of MAP kinase, was used to clarify whether MAP kinase mediated the signal of IGF-Ⅱ. PD98059 did not suppress the proliferation of Huh-6 even at 20 μmol/L, while it suppressed that of HepG2 to 33% ± 11%, which was statistically significant ($P < 0.05$, $n = 3$) (Figure 4C).

Suppression of cell proliferation was due to apoptosis

Cultured cells were HE stained to analyze the morphological change 72 h after addition of inhibitors, Huh-6 with PPP, LY294002, and Wortmannin but not with PD98059 while HepG2 with PPP, LY294002, Wortmannin, and PD98059 (Figure 5). Most of the dead cells had pyknotic or fragmented nuclei, indicating apoptosis.

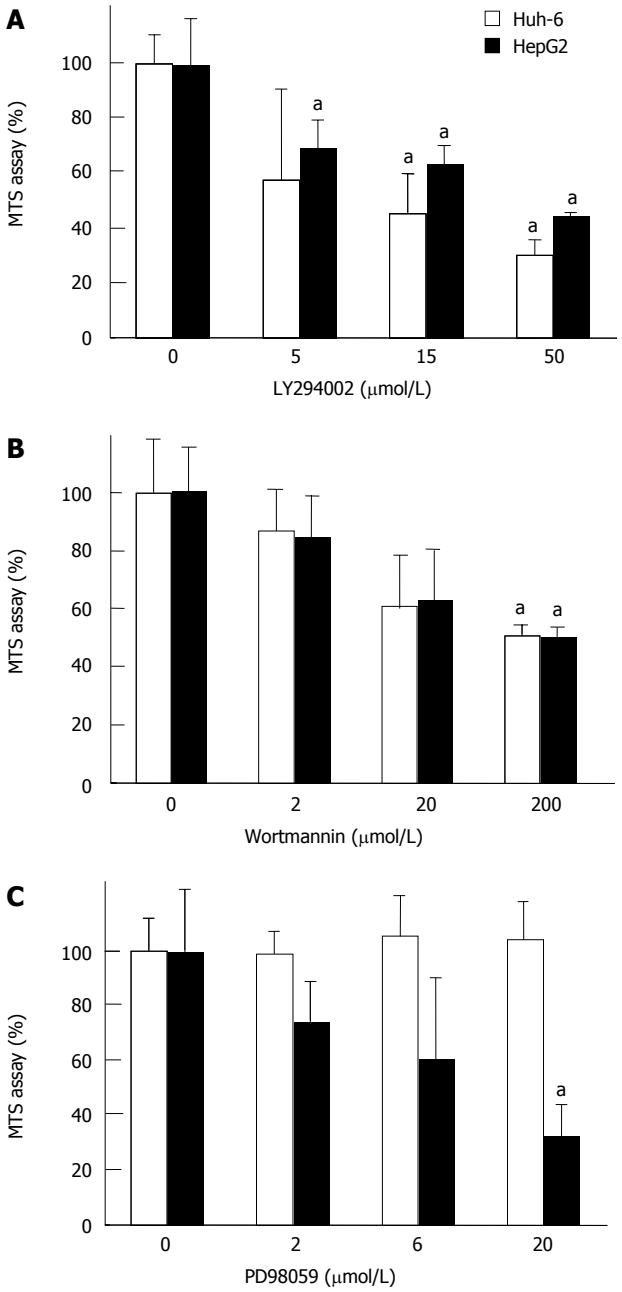

Figure 4 LY294002 or Wortmannin, selective inhibitors of PI3 kinase, or PD98059, a selective inhibitor of MAP kinase, was added to the medium 30 min prior to the stimulation with IGF-II (200 μg/L). LY294002 (**A**) and Wortmannin (**B**) suppressed the proliferation of Huh-6 and HepG while PD98059 suppressed HepG2 (**C**) ($^aP < 0.05$).

DISCUSSION

The existence of a growth factor in HBL has not been confirmed. It is reported that the expression of IGF-Ⅱ is elevated in tumor tissues and serum of HBL patients, but its exact role is not clear in terms of carcinogenesis[8]. In this study, we demonstrated that IGF-Ⅱ stimulated the proliferation of HBL cell lines. A previous report showed that IGF-Ⅱ does not stimulate the proliferation of HepG2[6]. They added IGF-Ⅱ at a concentration of 200 μg/L as well as fetal bovine serum. We added 200 μg/L of IGF-Ⅱ to the medium deprived of serum. Serum stimulates the proliferation of HepG2 to obscure the effect of IGF-Ⅱ. Moreover, we analyzed Huh-6, another human hepatoblastoma cell line, and revealed that IGF-Ⅱ stimulated the proliferation of Huh-6[9]. Our data clearly demonstrated that IGF-Ⅱ stimulated the proliferation of hepatoblastoma cell lines. Interestingly, HepG2 produces IGF-Ⅱ and antisense oligonucleotides of IGF-Ⅱ suppress the proliferation. It may be safe to conclude that IGF-Ⅱ acts as a growth factor for HBL by autocrine action[6,10].

The previous report suggested that IGF-Ⅱ stimulates proliferation *via* IGF-IR[6]. Our data clearly showed that IGF-IR was expressed in Huh-6 and HepG2. Since IGF-IR mediates proliferation activity of IGF-Ⅱ, it was expected

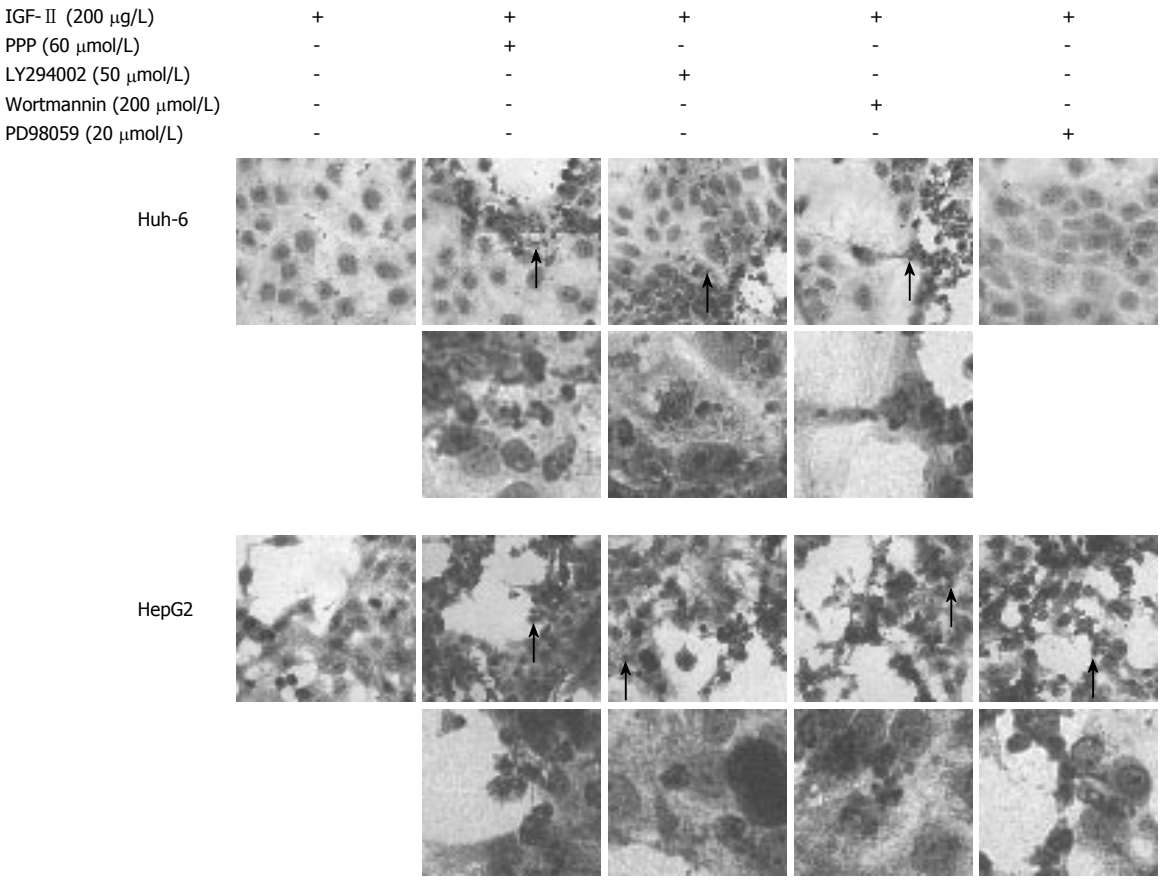

Figure 5 Huh-6 and HepG2 dead due to apoptosis. HE staining was performed to analyze morphological changes after addition of inhibitors. Many Huh-6 cells were dead treated with PPP (60 μmol/L), LY294002 (50 μmol/L), or Wortmannin (200 μmol/L) while HepG2 with PPP, LY294002, Wortmannin, or PD98059 (20 μmol/L). Most of the dead cells had pyknotic or fragmented nuclei (arrows), suggesting apoptosis. Areas indicated by arrows were magnified (x 400).

that an inhibitor of IGF-IR suppressed proliferation[5]. NVP-AEW541, a tyrosine kinase inhibitor, suppresses the proliferation of HepG2[7]. We used the commercially available PPP, another tyrosine kinase inhibitor, and it successfully suppressed the proliferation of Huh-6 and HepG2[11]. Our data on HepG2 was consistent with that of the previous report, and thus was strong evidence that IGF-Ⅱ mediated the signaling pathway of IGF-Ⅱ. Since IGF-IR is not involved in glucose metabolism, an inhibitor of IGF-Ⅱ would be a good candidate for molecular therapy for hepatoblastoma.

PI3 kinase and MAP kinase are the main downstream molecules of IGF-IR[5]. It was reported that the proliferation of HepG2 cells is suppressed by LY294002 and PD98059[12], and our results confirmed this. Our results also showed that LY294002 suppressed the proliferation of Huh-6, but PD98059 did not. One may speculate that the stimulation of IGF-Ⅱ was transmitted *via* PI3 kinase and MAP kinase in HepG2, but only *via* PI3 kinase in Huh-6. Wortmannin, another inhibitor of PI3 kinase, suppressed the proliferation of Huh-6 and HepG2 stimulated by IGF-Ⅱ, confirming the results of LY294002.

MTS assay measured cell viability, representing cell proliferation. The mechanism of suppression of cell proliferation, however, was not known. To unveil the mechanism, morphological analysis was performed with HE staining. Many of Huh-6 cells were dead with PPP, LY294002, and Wortmannin while HepG2 with PPP, LY294002, Wortmannin, and PD98059. Nuclei of dead cells were pyknotic or fragmented, indicating apoptosis. It is reported that DNA ladder formation is observed when HepG2 is treated with LY294002, Wortmannin, or PD98059[5]. It was suggested that Huh-6 and HepG2 were dead due to apoptosis when IGF-I signaling pathway was inhibited. It may be concluded that PI3 kinase would be a better target than MAP kinase for the molecular therapy of hepatoblastoma.

REFERENCES

1 **Ishak KG**, Glunz PR. Hepatoblastoma and hepatocarcinoma in infancy and childhood. Report of 47 cases. *Cancer* 1967; **20**: 396-422

2 **Brown AL**, Graham DE, Nissley SP, Hill DJ, Strain AJ, Rechler MM. Developmental regulation of insulin-like growth factor II mRNA in different rat tissues. *J Biol Chem* 1986; **261**: 13144-13150

3 **Dong ZZ**, Yao DF, Yao DB, Wu XH, Wu W, Qiu LW, Jiang DR, Zhu JH, Meng XY. Expression and alteration of insulin-like growth factor II-messenger RNA in hepatoma tissues and peripheral blood of patients with hepatocellular carcinoma. *World J Gastroenterol* 2005; **11**: 4655-4660

4 **Poirier K**, Chalas C, Tissier F, Couvert P, Mallet V, Carrie A, Marchio A, Sarli D, Gicquel C, Chaussade S, Beljord C, Chelly J, Kerjean A, Terris B. Loss of parental-specific methylation

at the IGF2 locus in human hepatocellular carcinoma. *J Pathol* 2003; **201**: 473-479

5 **LeRoith D**, Roberts CT Jr. The insulin-like growth factor system and cancer. *Cancer Lett* 2003; **195**: 127-137

6 **Lund P**, Schubert D, Niketeghad F, Schirmacher P. Autocrine inhibition of chemotherapy response in human liver tumor cells by insulin-like growth factor-II. *Cancer Lett* 2004; **206**: 85-96

7 **Hopfner M**, Huether A, Sutter AP, Baradari V, Schuppan D, Scherubl H. Blockade of IGF-1 receptor tyrosine kinase has antineoplastic effects in hepatocellular carcinoma cells. *Biochem Pharmacol* 2006; **71**: 1435-1448

8 **Gray SG**, Eriksson T, Ekstrom C, Holm S, von Schweinitz D, Kogner P, Sandstedt B, Pietsch T, Ekstrom TJ. Altered expression of members of the IGF-axis in hepatoblastomas. *Br J Cancer* 2000; **82**: 1561-1567

9 **Tokiwa T**, Doi I, Sato J. Preparation of single cell suspensions from hepatoma cells in culture. *Acta Med Okayama* 1975; **29**: 147-150

10 **Lin SB**, Hsieh SH, Hsu HL, Lai MY, Kan LS, Au LC. Antisense oligodeoxynucleotides of IGF-II selectively inhibit growth of human hepatoma cells overproducing IGF-II. *J Biochem (Tokyo)* 1997; **122**: 717-722

11 **Vasilcanu D**, Girnita A, Girnita L, Vasilcanu R, Axelson M, Larsson O. The cyclolignan PPP induces activation loop-specific inhibition of tyrosine phosphorylation of the insulin-like growth factor-1 receptor. Link to the phosphatidyl inositol-3 kinase/Akt apoptotic pathway. *Oncogene* 2004; **23**: 7854-7862

12 **Mitsui H**, Takuwa N, Maruyama T, Maekawa H, Hirayama M, Sawatari T, Hashimoto N, Takuwa Y, Kimura S. The MEK1-ERK map kinase pathway and the PI 3-kinase-Akt pathway independently mediate anti-apoptotic signals in HepG2 liver cancer cells. *Int J Cancer* 2001; **92**: 55-62

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RAPID COMMUNICATION

A *p53* genetic polymorphism of gastric cancer: Difference between early gastric cancer and advanced gastric cancer

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Abstract

AIM: To investigate the role of the polymorphism of *p53* codon 72 in early gastric cancer (EGC) and advanced gastric cancer (AGC) in Korean patients.

METHODS: DNA was extracted from blood samples of gastric cancer patients (n = 291) and controls (n = 216). In the *p53* codon 72 genotypes were determined by PCR-RFLP.

RESULTS: Patients with gastric cancer had a significantly higher frequency of the homozygous proline (Pro) allele than the control (P = 0.032). Patients with AGC had a significantly higher frequency of the *Arg/Arg* (arginine) allele (P = 0.038) than EGC and a similar *Pro/Pro* allele. The signet ring cell type had a higher frequency of the *Pro/Pro* allele than other types (P = 0.031). The *Pro/Pro* genotype carries a 3.9-fold increased risk of developing gastric cancer (95% CI, 1.3-15.4, P = 0.039) when compared to *Arg/Arg* and *Arg/Pro* genotypes and to develop EGC is a 5.25 fold increased risk (95% CI, 1.8-19.6, P = 0.021).

CONCLUSION: The *Pro/Pro* genotype of the *p53* codon 72 polymorphism carries a higher risk for gastric cancer in general and is also associated with a much higher risk for EGC than AGC.

Key words: *p53* gene; Polymorphism; Gastric cancer

Yi SY, Lee WJ. A *p53* genetic polymorphism of gastric cancer: Difference between early gastric cancer and advanced gastric cancer. *World J Gastroenterol* 2006; 12(40): 6536-6539

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INTRODUCTION

Gastric cancer is one of the most common malignancies worldwide, although the overall incidence of gastric cancer has been decreasing over the past few decades. Chronic *H pylori* infection and dietary factors, such as those high in salt or nitrate, and nutritional deficiencies have been associated with gastric cancer[1]. Gastric carcinogenesis is a complex, multistep, and multifactorial process, in which many factors are implicated. The majority of gastric cancers are thought to be caused by environmental factors that result in damage to the mucosa and that inhibit its ability to repair itself[2]. This response is regulated, in part, by inhibitory and stimulatory factors that are products of proto-oncogenes and tumor suppressor genes[3].

TP53 gene, an important tumor suppressor gene, encoded *p53* protein. The *p53* tumor suppressor protein was initially isolated in 1979 as a 53 kDa protein that was associated with SV 40 large T antigen[4]. It was a decade before *p53* was recognized as an important tumor suppressor because of its frequent mutation in human cancers. A large number of human cancers show the evidence of inactivation of the *p53* pathway, suggesting that malignant transformation requires reduction or elimination of *p53*'s function as "guardian of the genome". It is estimated that up to 50% of human cancers carry a mutation of the *p53* gene[5,6].

Germ line polymorphism of genes involved in multiple steps of carcinogenesis may also account for genetic difference in stomach cancer susceptibility. The *p53* gene is the most intensively studied human gene because of its role as a central tumor suppressor, and has been widely studied in gastric cancer. However, although more than 75% of gastric cancer showed p53 overexpression, less than 30% had mutation in this gene[7]. The codon 72 polymorphism is located in exon 4 of the *p53* gene, a region involving very few mutations[8]. At least two forms of wild-type *p53* protein exist among major human populations; these forms are ascribed to amino acid replacement at codon 72 of *Arg* (CGC) by *Pro* (CCC) in the domain of transactivation of the *p*53 protein, though the functional difference between them is unknown. *Pro* variant allele of this *p*53 polymorphism has been studied as a potential risk factor for cancer of the lung, breast,

and large bowel, with inconsistent results. Shepherd *et al*[8]. examined the relationship between codon 72 polymorphism and their susceptibilities to gastric cancer in a group of American gastric cancer patients.

In this study, we examined the genotypic frequency of codon 72 in early gastric cancer (EGC) and advanced gastric cancer (AGC) in 292 Korean patients to investigate the role of the *p*53 polymorphism.

MATERIALS AND METHODS

Patients

Two hundred twenty two diagnosed gastric cancers were recruited from Ewha Womans University Mokdong Hospital from 2001 to 2005. Their mean age was 56 years (range, 26-88 years); 171 were males and 121 were females. Of the 292 patients, 189 (64.7%) showed advanced gastric cancer, 103 (35.3%) showed early gastric cancer. Two hundred sixteen controls were randomly selected from subjects attending routine medical check-ups (mean age 58 years; range 24-85 years; 128 males and 88 females) who were not affected with stomach cancer by endoscopy. Their details are presented in Table 1. Blood specimens, including serum, plasma, and white blood cells, from the study subjects were also obtained and frozen at -70℃ for subsequent analysis. Informed written consent was obtained from all the enrolled patients.

Table 1 Demographic characteristics of patients with stomach cancer (*n* = 292)

	n
Median age	56 (22 -88)
Gender	
Male	171
Female	121
Type of stomach cancer	
Early cancer (EGC)	103
Advanced cancer (AGC)	189
Codon 72 polymorphism	
Arg/Arg	101
Arg/Pro	126
Pro/Pro	65
Laurence classification	
Intestinal type	140
Diffuse type	152
Differentiation	
Well/Moderate	114
Poor	132
Signet ring cell	46

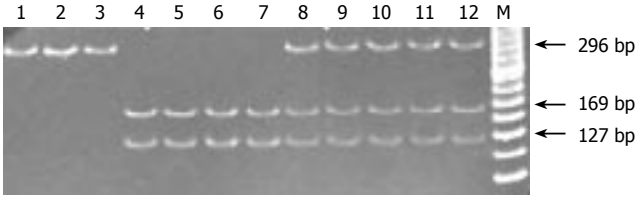

Figure 1 Restriction fragment length polymorphism of PCR-amplified fragment by BstUI. The Lane 1-3 showed only a single undigested band at 296 bp (homozygote of *Pro*), the fragment from lane 4-7 gave two bands at 169 and 127 bp (homozygote of *Arg*), while the fragment from lane 8-12 showed three bands at 296, 169 and 127 bp (heterozygote of *Arg/Pro*). M was a DNA marker.

DNA extraction

Genomic DNAs were isolated from peripheral whole blood, 200 μL (which has been treated with EDTA) by a DNA purifying kit (QIAamp DNA kit Blood Mini Kit, Qiagen, Germany, Hilden) according to the manufacture's instructions.

p53 codon 72 polymorphism

Genotyping of *p*53 at codon 72 in exon 4 [M22884. Human phosphoprotein (gi:189467)] was carried out by a polymerase chain reaction (PCR) amplification procedure using primers (*p*53-S: 5'-ATC TAC AGT CCC CCT TGC CG-3 and *p*53-AS: 5'-GCA ACT GAC CGT GCA AGT CA-3'). The amplification reaction was performed in a 6 μL (0.1 μg/μL) genomic DNA template, 0.1 μL (10 nmol/mL) of each primer, 1.6 μL (5 mmol/L per mL) dNTP, 0.1 μL (0.5 U/μL) Taq polymerase (Promega, Madison, WI, USA), and 2 μL of 10X reaction buffer (200 mmol/L per mL Tris-HCL (pH8.3), 500 mmol/L per mL KCL, and 30 mmol/L per mL $MgCl_2$). PCR was carried out by 30 cycles under the following conditions: 1 min at 95℃ for denaturation, 1 min at 62℃ for primer annealing, and 1 min at 72℃ for primer extension, using the GeneAmp PCR System 9600 (Applied Biosystem, Foster City, CA). The PCR product was visualized on a 2% agarose gel by electrophoresis, followed by ethidium bromide staining. This generates a 296-base pair fragment. The restriction enzyme BstUI (10 unit, New England Biolabs, Berverly, MA) digests (for 3 h at 60℃) within the sequence corresponding to the *Arg* codon (CGC) at position 72 to generate two visible fragments of 169 bp and 127 bp and leaves the *Pro* allele uncut (Figure 1).

Statistical analysis

Frequency tables were constructed using the SPSS (11.0 version) statistical package with statistical significance using the χ^2 test. The odd ratios and 95% Confidence interval (CI) were calculated as an approximation of relative risk and adjusted for confounding factors such as age and gender using a logistic regression model.

RESULTS

Distribution of the three genotypes of the p53 gene

We determined the frequency of the three phenotypes of the *p53* gene in the patients with stomach cancer and controls (Table 2). Genotypes *Arg/Arg, Arg/Pro*, and *Pro/Pro* were found 41.2%, 47.7%, and 11.1% in individual controls and 34.5%, 43.1%, and 22.3% in the patients of stomach cancer, respectively. Distribution patterns of the germ line *p53* polymorphism of the patients with stomach cancer included EGC and AGC showed in Table 2. We observed a dramatically increased frequency of *Pro/Pro* allelotype in stomach cancer patient, especially in the patients of EGC.

A logistic regression analysis suggests that the homozygous 72 *Pro* genotype carries a 3.9-fold increased risk of developing gastric cancer (95% CI, 1.3-15.4, *P* =

Table 2 Frequency of the codon 72 genotype

	n	*Arg/Arg* (%)	*Arg/Pro* (%)	*Pro/Pro* (%)
Control	216	89/216 (41.2)	103/216 (47.7)	24/216 (11.1)
Stomach cancer	292	101/292 (34.5)	126/292 (43.1)	65/292 (22.3)[a,1]
AGC	189	75/189 (39.7)[d]	76/189 (39.9)	38/189 (20.4)[b]
EGC	103	26/103 (25.8)	50/103 (48.1)	27/103 (26.1)[c,2]

[a]P = 0.032, control *vs* stomach cance; [b]P = 0.029, control *vs* AGC; [c]P = 0.027, control *vs* EGC; [d]P = 0.038, AGC *vs* EGC; [1]OR = 3.9 (95% CI, 1.3-15.4, P = 0.039); [2]OR = 5.25 (95% CI, 1.8-19.6, P = 0.021); OR: Odd ratio; CI: Confidence interval.

0.039) when compared to *Arg* homozygous and *Arg/Pro* heterozygous. The risk for 72 *Pro* homozygous patients to develop early gastric cancer is 5.35 (95% CI, 1.8-19.6, P = 0.021).

p53 polymorphism and histology grading of stomach cancer

We examined the frequency difference in each genotype of *p53* by histological type of all stomach cancers. Germ line *p53* polymorphism was associated with stomach cancer, especially the signet ring cell type of adenocarcinoma (P = 0.031, Table 2). There was no relationship between patient gender, tumor stage, the depth of invasion in the wall, histologic type of cancer (Laurence classification: intestinal and diffuse types) and the distribution of codon 72 genotypes.

DISCUSSION

The identification of genes involved in cancer development is critical for uncovering the molecular basis of cancer. The *p53* tumor suppressor protein is essential in the control of cell growth, apoptosis and the maintenance of genomic stability. Loss of *p53* function caused by genomic alterations or interaction with environmental and bacterial products has been suggested as a critical step in multistage human carcinogenesis[9].

The *p53* gene consists of 11 exons; exons 2-11 code for the protein of 393 aminoacids. The majority of *p53* mutations identified have been found in exons 5-8. However, mutations outside exons 5-8 may occur and they were chiefly observed in exons 4 and 10[10,11]. At least 10 different polymorphisms have been detected in the human genomic *p53*[12]. The functional significance of these polymorphisms is currently unknown. The hypothesized relationship between the codon 72 *p53* polymorphism and cancer susceptibility dose not have any mechanistic basis. The *Pro* variant allele of the *p53* polymorphism at codon 72 may not directly affect the *p53* function. It may be in linkage disequilibrium with an as-yet-unidentified functional polymorphism. However, the single-codon difference of the *p53* gene has been demonstrated to result in structurally different proteins[13]. The polymorphism is localized within a region of polypeptide that was lacking in a deletion mutant of mouse *p53* that had an enhanced ability to immortalize primary rat cells[14,15].

In a literature review, there was little report about investigation of *p53* gene polymorphism between AGC and EGC comparing with control. We have observed that TP53 codon 72 genotype in stomach cancer and control subjects. Our data further suggest that a *p53* genetic polymorphism was associated with the susceptibility for stomach cancer, especially advanced stomach cancer.

Table 3 Distribution of *p53* genotypes and histopathological classification among the patients with gastric cancer

Histopathological classification (*n*)	*p53* genotypes		
	Arg/Arg	*Arg/Pro*	*Pro/Pro*
Control (216)	89/216(41.2)	103/216(47.7)	24/216(11.1)
Differentiation			
Well to poorly (246)	88/292 (30.1)	151/292 (51.7)	53/292 (18.2)
Signet ring cell (46)	17/46 (37.0)	11/46 (23.0)	18/46 (39.1)[a,b]
Tumor histologic type			
Intestinal type (140)	48/140 (34.2)	60/140 (42.8)	31/140 (22.1)
Diffuse type (152)	53/152 (34.8)	66/152 (43.4)	34/152 (22.4)

[a]P = 0.031, Well to poorly differentiation *vs* Signet ring cell; [b]P = 0.019, Control *vs* Signet ring cell.

There were several studies conducted to investigate the association between the codon 72 *p53* polymorphism and lung cancer or gastric cancer. The risk increased approximately twofold for smoking-related lung cancer among individuals carrying the *Pro/Pro* genotype compared with those with other genotypes of the codon 72 *p53* polymorphism[16-18]. A recent study in the patients of gastric cancer showed the significant difference from healthy control, with 48.6% *Arg/Arg* and 3.6% *Pro/Pro* in gastric cancer patients compared with 41.5% and 10.9% in healthy controls[19]. They showed an increased frequency of *Arg/Arg* genotype in cancer patients at age 75 or more than at a younger age. They suggested the prognosis in patients with *Pro* allele (proline homozygote or *Pro/Arg* heterozygotes) was worse than that those with *Arg/Arg* genotype. They also showed that preferential frequency of codon 72 *Arg p53* acts as a survival factor in gastric cancer patients who have homozygous *Arg* alleles which confer a late start of gastric cancer when compared with those with the *Pro* allele.

In this study we found that the distribution of genotypes had significant difference between the patients of stomach cancer and controls, with 34.5% *Arg/Arg* and 22.3% *Pro/Pro* in stomach cancer patients compared with 41.2% and 11.1% in controls (P = 0.032). We observed a significantly higher distribution of *Pro/Pro* genotype in the patient with stomach cancer, especially in the patients with EGC than control. Also in comparison between AGC and EGC, the frequency of *Arg/Arg* genotype was higher than the frequency of AGC, statistically (P = 0.038).

The reason for the tissue-specific difference of the germ line *p53* polymorphism was unknown, though an association with histopathologic grading was suggested for gastric cancer[16]. In this study, we divided two groups whether signet ring cell type or not. Generally, we knew that the stomach cancer with signet ring type was highly malignant and had worse prognosis than other cell types. There was statistically significant difference that the patients with signet ring cell type had a higher ratio of *Pro/Pro* genotype than non-signet ring cell type (P = 0.031, Table 3). So the association with histopathologic grading

may suggest that germ line *p53* polymorphism is involved in survival as a clinical prognostic factor as well as cancer susceptibility. Further studies are needed to examine this possibility.

There were several explanations about the role of different genotypes. First, transcriptional properties are different. It has been shown that the *Arg*/*Arg* and *Pro*/*Pro* variants differ in binding activity at transcription, to activate transcription, and to induce apoptosis or cell cycle arrest[20]. The *p53 Arg*/*Arg* variant induces apoptosis with faster kinetics more efficiently than the *p53 Pro*/*Pro* variant and the *p53 Arg*/*Arg* variant is a better inducer of transcription[19]. Namely, if there was a high proportion of *Arg*/*Arg* genotype in stomach cancer, it induced the efficient apoptosis or cell cycle arrest, and then it could be better prognosis. Second, wild-type *p53* protein is rapidly degraded, and has a short half-life and low intracellular levels. Stabilization of the protein following an appropriate stimulus such as DNA damage is a physical regulation to increase function[6]. Different structure of *p53* proteins resulting from a substitution of *Pro* for *Arg* at codon 72 may have different functions in the responsiveness to different stimuli caused by diverse carcinogens such as *H pylori*, dietary, or nutritional deficiency. The reaction of *Pro* genotype and various carcinogens may have more carcinogenic properties. *H pylori* is the well-known cause of chronic gastritis, gastro-duodenal ulcer, and gastric cancer. Some reports studied the relationship between *p53* polymorphism and *H pylori* and found the genotypic frequency of *p53* was similar between cases and controls[21,22]. We cannot confirm results of *H pylori* status of all patients with stomach cancer in this study, we cannot represent the relationship between *H pylori* and *p53* polymorphism.

In conclusion, the *Pro*/*Pro* genotype of the *p53* codon 72 polymorphism carries a higher risk for gastric cancer in general (3.9-fold) and is also associated with a higher risk for EGC (5.25-fold) than AGC. Although this finding is provocative, it should be considered preliminary because of the limited sample size. Clearly, a well-designed follow-up study with a larger number of samples is needed to confirm these findings.

REFERENCES

1 **Stadtlander CT**, Waterbor JW. Molecular epidemiology, pathogenesis and prevention of gastric cancer. *Carcinogenesis* 1999; **20**: 2195-2208
2 **Hotz J**, Goebell H. Epidemiology and pathogenesis of gastric cancer. In: H.J. Meyer and H.J. Schmoll. Gastric Carcinoma. New York: Springer-Verlag, 1989: 3-15
3 **Tahara E**. Molecular mechanism of stomach carcinogenesis. *J Cancer Res Clin Oncol* 1993; **119**: 265-272
4 **Lane DP**, Crawford LV. T antigen is bound to a host protein in SV40-transformed cells. *Nature* 1979; **278**: 261-263
5 **Lane DP**. Cancer. p53, guardian of the genome. *Nature* 1992; **358**: 15-16
6 **Brachmann RK**. p53 mutants: the achilles' heel of human cancers? *Cell Cycle* 2004; **3**: 1030-1034
7 **Zhang ZW**, Farthing MJ. Molecular mechanisms of H. pylori associated gastric carcinogenesis. *World J Gastroenterol* 1999; **5**: 369-374
8 **Shepherd T**, Tolbert D, Benedetti J, Macdonald J, Stemmermann G, Wiest J, DeVoe G, Miller MA, Wang J, Noffsinger A, Fenoglio-Preiser C. Alterations in exon 4 of the p53 gene in gastric carcinoma. *Gastroenterology* 2000; **118**: 1039-1044
9 **van Oijen**, Slootweg PJ. Gain-of-function mutations in the tumor suppressor gene p53. *Clin Cancer Res* 2000; **6**: 2138-2145
10 **Yu MW**, Yang SY, Chiu YH, Chiang YC, Liaw YF, Chen CJ. A p53 genetic polymorphism as a modulator of hepatocellular carcinoma risk in relation to chronic liver disease, familial tendency, and cigarette smoking in hepatitis B carriers. *Hepatology* 1999; **29**: 697-702
11 **Milner J**. Flexibility: the key to p53 function? *Trends Biochem Sci* 1995; **20**: 49-51
12 **Hoe YJ**, Cho HM, Chin HM, Kim W, Jeon HM. Gastric cancer susceptibility in the p53 codon 72 polymorphism. *J Korean Surg Soc* 2005; **69**: 24-30
13 **Bae DH**, Kim JS, Choi SD, Sunwoo JG, Nam KH, Kim CJ. Human papillomavirus infection and polymorphism of p53 codon 72 in the uterine cervical neoplasia. *Korean J Gynecol Oncol* 2003; **14**: 281-289
14 **Perez-Perez GI**, Bosques-Padilla FJ, Crosatti ML, Tijerina-Menchaca R, Garza-Gonzalez E. Role of p53 codon 72 polymorphism in the risk of development of distal gastric cancer. *Scand J Gastroenterol* 2005; **40**: 56-60
15 **Marin MC**, Jost CA, Brooks LA, Irwin MS, O'Nions J, Tidy JA, James N, McGregor JM, Harwood CA, Yulug IG, Vousden KH, Allday MJ, Gusterson B, Ikawa S, Hinds PW, Crook T, Kaelin WG Jr. A common polymorphism acts as an intragenic modifier of mutant p53 behaviour. *Nat Genet* 2000; **25**: 47-54
16 **Bong JG**, Lee MH, Song KE, Kim T, Yu W. p53 gene mutation, tumor p53 protein overexpression, and serum and-p53 antibody in patients with gastric cancer. *J Korean Gastric Cancer Assoc* 2003; **3**: 206-213
17 **Wang YC**, Chen CY, Chen SK, Chang YY, Lin P. p53 codon 72 polymorphism in Taiwanese lung cancer patients: association with lung cancer susceptibility and prognosis. *Clin Cancer Res* 1999; **5**: 129-134
18 **Wang YC**, Lee HS, Chen SK, Chang YY, Chen CY. Prognostic significance of p53 codon 72 polymorphism in lung carcinomas. *Eur J Cancer* 1999; **35**: 226-230
19 **Zhang ZW**, Newcomb P, Hollowood A, Feakins R, Moorghen M, Storey A, Farthing MJ, Alderson D, Holly J. Age-associated increase of codon 72 Arginine p53 frequency in gastric cardia and non-cardia adenocarcinoma. *Clin Cancer Res* 2003; **9**: 2151-2156
20 **Thomas M**, Kalita A, Labrecque S, Pim D, Banks L, Matlashewski G. Two polymorphic variants of wild-type p53 differ biochemically and biologically. *Mol Cell Biol* 1999; **19**: 1092-1100
21 **Wu MT**, Chen MC, Wu DC. Influences of lifestyle habits and p53 codon 72 and p21 codon 31 polymorphisms on gastric cancer risk in Taiwan. *Cancer Lett* 2004; **205**: 61-68
22 **Hiyama T**, Tanaka S, Kitadai Y, Ito M, Sumii M, Yoshihara M, Shimamoto F, Haruma K, Chayama K. p53 Codon 72 polymorphism in gastric cancer susceptibility in patients with Helicobacter pylori-associated chronic gastritis. *Int J Cancer* 2002; **100**: 304-308

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RAPID COMMUNICATION

Molecular analysis of hepatitis B virus isolates in Mexico: Predominant circulation of hepatitis B virus genotype H

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Abstract

AIM: To determine the genotypes in Mexican hepatitis B virus (HBV) isolates and characterize their precore and core promoter mutations.

METHODS: Forty-nine HBV isolates of Mexico obtained from sera of 15 hepatitis patients, 6 hemodialysis patients, 20 men seeking HIV testing, and 8 AIDS patients were analyzed. HBV isolates were amplified by PCR, and genotyped by line probe assay (INNO-LiPA HBV Genotyping; INNOGENETICS N V, Ghent, Belgium). HBV genotype confirmation was performed by DNA sequencing part of the sAg region. Precore and core promoter mutation characterization was performed by line probe assay (INNO-LiPA HBV PreCore; INNOGENETICS N V, Ghent, Belgium).

RESULTS: Overall, HBV genotype H was found in 37 (75.5%) out of the 49 isolates studied. HBV genotypes G, A, and D were found in 5 (10.2%), 4 (8.2%), and 3 (6.1%) isolates, respectively. HBV genotype H was predominant in isolates from hemodialysis patients (100%), hepatitis patients (80%), and men seeking HIV testing (75%), and accounted for half of infections in AIDS patients (50%). Six (12.2%) out of the 49 HBV isolates showed both wild type and mutant populations at precore codon 28. These mixed wild type and precore mutant populations were observed in one HBV genotype A isolate and in all HBV genotype G isolates. A dual variant core promoter mutation was observed in 1 (2%) of the isolates, which was genotype H.

CONCLUSION: HBV genotype H is highly predominant in HBV isolates of Mexico followed by genotypes G, A and D. A low frequency of precore and core promoter mutations is observed in HBV Mexican isolates.

Key words: Hepatitis B virus; Genotypes; Mutations; Molecular epidemiology

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INTRODUCTION

Hepatitis B virus (HBV) is an important cause of morbidity and mortality worldwide. It is estimated that 2 billion people are infected with HBV and 350 million individuals suffer from chronic HBV infection in the world[1,2]. Chronic HBV infection may lead to hepatic fibrosis, cirrhosis and hepatocellular carcinoma (HCC)[3]. In addition, HBV infection is the 10th leading cause of death worldwide[1]. HBV is a hepadnavirus that possesses a double stranded DNA genome[4]. The genetic organization of HBV has been described elsewhere[4]. Briefly, HBV genome consists of four partly overlapping open reading frames: *S* that encodes for envelope proteins (HBsAg), *C* for core protein (HBcAg) and e antigen (HBeAg), *P* for polymerase protein and *X* for transcriptional transactivator protein[4]. Based on sequence divergence of the entire genome of > 8%, HBV genomes have been classified into 8 genotypes designated A to H[5, 6]. The distribution of HBV genotypes is geographically restricted. In the American continent, HBV genotypes A and C are common in the USA[7,8], while genotype F is predominant in Central American countries[9]. In Europe, HBV genotype D is prevalent in Mediterranean Europe[10], while genotype A is frequent in Northwest Europe[11]. In the African continent, HBV genotype E is common in the Sub-Saharan Africa[12], and genotype A in South Africa[13]. In the Asian continent, HBV genotypes B and C predominate in south east countries[14-16], while genotype D is prevalent in central Asia[17]. Reports indicate that HBV genotypes are related with the severity of liver disease. HBV genotype C has

been associated with the development of liver cirrhosis and hepatocellular carcinoma[14,18-21]. In addition, HBV genotypes are related with the response to antiviral therapy. Asian studies have shown that compared to HBV genotype B, genotype C has a lower response to interferon alpha (IFN-α) therapy[22,23]. A recent European randomized trial showed that patients infected with HBV genotypes A and B respond significantly better to pegylated IFN-α-2b alone or in combination with lamivudine than patients infected with HBV genotypes C and D[24]. Mutations in the precore and core promoter regions of HBV have been also related with genotypes. Precore and core promoter mutations have been observed mainly in HBV genotypes D and B[25-27], as well as A and C[25,28,29], respectively.

Little is known about the molecular epidemiology of HBV in Mexico. A previous study on HBV genotypes published in 1998 showed that HBV genotype F is predominant in Mexico[30]. However, more recently it was discovered that HBV genotype F is divergent[31], to the extent that a new genotype is split off from genotype F, this new genotype has been designated as HBV genotype H[9]. Thus some divergent strains formerly classified as HBV genotype F could now be classified as HBV genotype H. Therefore, in order to obtain an updated classification we sought to determine the HBV genotypes and characterize the precore and core promoter mutations in HBV isolates of Mexico.

MATERIALS AND METHODS

HBV isolates

Forty-nine HBV isolates of Mexico were analyzed. HBV isolates were obtained from serum samples of 15 hepatitis patients, 6 hemodialysis patients, 20 men seeking HIV antibody testing, and 8 AIDS patients. All samples were collected in Mexico City and Guadalajara City; both cities are located in central Mexico. Participants were of Mestizo ethnicity. All samples were HBsAg positive. HBeAg, anti-HBe, and HBV DNA levels were not determined. Men seeking HIV antibody testing came from a high risk population for sexually transmitted infection acquisition. Seropositivity for anti-HBc among them (30.4%) was associated to men who had sex with men exclusively and seropositivity for both HIV and herpes simplex virus type 2[32].

HBV DNA detection and genotyping

HBV DNA was extracted from 200 μL of serum by using the high pure PCR template preparation kit (Roche Applied Science, Penzberg, Germany) as recommended by the manufacturer. HBV DNA was amplified with HBV pol gene domain B and C primers (INNO-LiPA HBV DR amplification, INNOGENETICS N. V., Ghent, Belgium) by nested PCR as described in the product insert. Primer sequences were used as previously described[33]. PCR conditions were as follows: 40 cycles at 94℃ for 30 s, at 45℃ for 30 s and at 72℃ for 30 s. HBV genotyping was performed by INNO-LiPA HBV genotyping (INNOGENETICS N. V., Ghent, Belgium) following the manufacturer's instructions.

Sequence analysis was done directly on the second round PCR products using the big dye terminator V3.1 cycle sequencing kit of Applied Biosystems. Sequencing PCR was performed for 25 cycles (at 95℃ for 10 s, at 50℃ for 5 s, at 60℃ for 4 min) and purified on a sephadex column. Three μL dextran blue/deionised formamide loading buffer was added to the dried pellet and 1 μL was then loaded on a 4.5% acrylamide slab gel. After electrophoresis of 14 h on the automated sequencer ABI377, data analysis was done using software sequencher 4.1.2.

HBV precore and core promoter mutation characterization

HBV DNA was additionally amplified with basal core promoter and precore primers (INNO-LiPA HBV PreCore amplification, INNOGENETICS N. V., Ghent, Belgium) by nested PCR as recommended by the manufacturer. Primer sequences were used as previously described [25]. PCR conditions were as follows: 40 cycles at 94℃ for 30 s, at 50℃ for 30 s and at 72℃ for 30 s. Characterization of precore mutations was performed by INNO-LiPA HBV precore (INNOGENETICS N. V., Ghent, Belgium) according to the manufacturer's instructions. This method could identify nucleotide polymorphism at nt 1762 and nt 1764 in the basal core promoter and at codon 28 in the precore region of HBV[34].

RESULTS

HBV genotyping by INNO-LiPA

For all the 49 HBV isolates, INNO-LiPA HBV genotyping assay was able to provide a genotype result. HBV genotype H was found in 36 (73.5%) out of the 49 isolates studied, while HBV genotypes G, A, D and F were found in 5 (10.2%), 4 (8.2%), 3 (6.1%) and 1 (2.0%) isolates, respectively.

HBV genotyping by DNA sequencing

Thirty-two out of the 36 isolates of HBV genotype H, all 5 isolates of genotype G, and only 1 isolate of genotype F genotyped by INNO-LiPA were selected for DNA sequencing confirmation. All 32 isolates of genotype H and all 5 isolates of genotype G were confirmed by phylogenetic analysis of the HBsAg sequences. However, sequencing and subsequent phylogenetic analysis of the HBV genotype F showed a genotype H. This was caused by a single nucleotide mismatch with the probe on line 11 of the INNO-LiPA HBV genotyping, abolishing the reaction with this probe. This in turn led to a single reactivity with the genotype F probe on line 15. When we compared the HBV genotype H sequences obtained in this study with other published sequences by means of the phylogenetic program PHYLIP, we found that our sequences formed two separate branches within genotype H (Figure 1).

Thus, the final adjusted prevalences of HBV genotypes were 37 isolates (75.5%) of genotype H, 5 isolates (10.2%) of genotype G, 4 isolates (8.2%) of genotype A, and 3 isolates (6.1%) of genotype D. HBV genotype H was predominant in the hepatitis patients, men seeking HIV

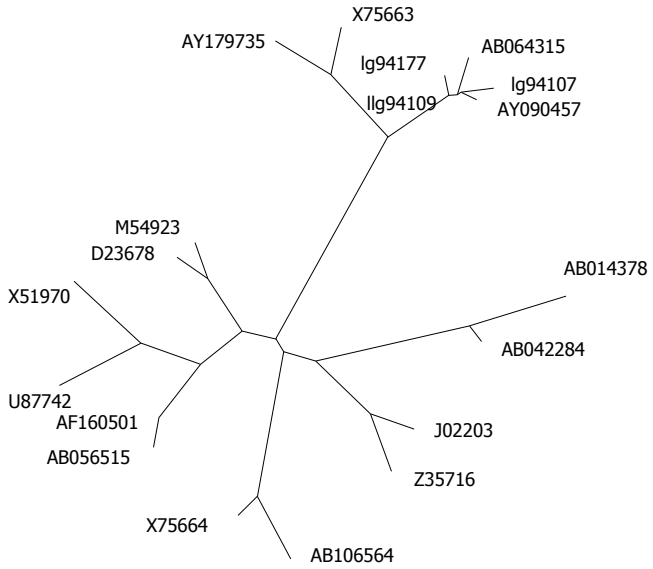

Figure1 Phylogenetic tree constructed with HBV sequences representative of the eight major genotypes, established using neighbor joining based on Kimura 2 parameter method. The three HBV genotype H sequences obtained in this study are shown and have been assigned as IG94107 (Genbank accession number: DQ990454), IG94109 (Genbank accession number: DQ990455) and IG94177 (Genbank accession number: DQ990456).

Table 1 Distribution of HBV genotypes in the populations studied *n* (%)

Group of subjects	*n*	Genotypes, *n* (%)			
		A	D	G	H
Hepatitis patients	15	1 (6.7)	2 (13.3)	0	12 (80)
Men seeking HIV testing	20	3 (15)	1 (5)	1 (5)	15 (75)
AIDS Patients	8	0	0	4 (50)	4 (50)
Hemodialysis patients	6	0	0	0	6 (100)
Total	49	4 (8.2)	3 (6.1)	5 (10.2)	37 (75.5)

testing, and hemodialysis patients, and accounted for half of infections in AIDS patients (Table 1). Since the number of HBV isolates analyzed in some groups was very small, we were unable to provide statistical evidence for the distribution of HBV genotypes within the groups.

HBV precore mutations

In the precore region, 41 (83.7%) isolates showed wild type (G) sequence at nucleotide 1896, 6 (12.2%) had a mixture of wild type and $G_{1896}A$ mutation, and 2 had an indeterminate sequence (Table 2). Mixed wild type and precore mutant populations were observed in only one isolate of HBV genotype A but in all isolates of HBV genotype G. Statistical analysis showed that the frequency of mixed wild type and precore mutant populations was significantly higher ($P = 0.04$) in isolates of HBV genotype G than in isolates of HBV genotype A. Mixed wild type and precore mutant populations were observed in 10% of isolates from men seeking HIV testing and in 50% of isolates from AIDS patients only. The frequency of these mixed wild type and precore mutant populations in AIDS patients was significantly higher than that in isolates from hepatitis patients ($P = 0.007$), and men seeking HIV testing ($P = 0.03$). This frequency of mixed wild type and precore mutant populations in AIDS patients was also higher but not statistically significant ($P = 0.06$) than that in isolates from hemodialysis patients.

Table 2 Prevalence of precore and core promoter variants and its correlation with genotypes found in Mexican HBV isolates

Region and variant	*n* (%)	Genotypes			
		A	D	G	H
		(*n* = 4)	(*n* = 3)	(*n* = 5)	(*n* = 37)
Precore (nt 1896)					
Wild type (G)	41 (83.7)	3	2		36
Wild type + variant (G + A)	6 (12.2)	1		5	
Indeterminate	2 (4.1)		1		1
Core promoter (nt 1762 and 1764)					
Wild type (AG)	44 (89.8)	4	2	5	33
Dual variant (TA)	1 (2)				1
Indeterminate	4 (8.2)		1		3

In the core promoter region, 44 (89.8%) isolates had wild type sequence (A at nucleotide 1762 and G at nucleotide 1764), 1 (2%) showed the classical dual variant ($A_{1762}T$, $G_{1764}A$), and 4 (8.2%) had an indeterminate sequence. The only one dual variant was found in a sample of a hepatitis patient infected with HBV genotype H. For its part, all 4 samples with indeterminate sequences were found in hepatitis patients too. Three of them were found in isolates of HBV genotype H, and in one isolate of HBV genotype D.

DISCUSSION

In this work, we found a predominant frequency of HBV genotype H in Mexican isolates. The extremely high frequency of this HBV genotype H in Mexico has not been reported before. A previous study in Mexican isolates from blood donors and patients with liver disease showed infections with only 3 HBV genotypes: A, D and F in both populations[30]. Nevertheless, in the present study we have found 4 HBV genotypes. It was known that HBV genotype F was predominant in Mexico [30], but also it was observed that the predominant genotype F in Mexico is divergent[31]. These results contrast with those found in the present study since HBV genotype H was predominant whilst HBV genotype F was not present anymore. Genotype H which was discovered recently is closely related with genotype F, while genotype H is most likely split off from genotype F[9]. Therefore, a number of HBV isolates previously classified as genotype F are now classified as genotype H. Thus, it explains the remarkable change in HBV genotype distribution in Mexico with a predominant circulation of genotype H. The high

frequency of HBV genotype H in this study is the highest reported worldwide. Indeed, this genotype has been rarely found or not reported at all in most countries[35-39]. HBV genotype H has been found only in HBV isolates of Nicaragua, Mexico, USA[9,31,35], and Japan[37,39]. In our study, HBV genotype H was predominant in the hepatitis patients, men seeking HIV testing, and hemodialysis patients. In addition, genotype H was responsible of 50% of infections in AIDS patients. Reports on genotypes found in hepatitis patients from other countries remark the differences in geographical distribution of HBV genotypes in the world. In the USA and other countries a high frequency of HBV genotype B and C has been found in hepatitis patients[40,41]. HBV genotypes B and C have been linked to Asian ethnicity[7,35], and the absence of these genotypes in our populations studied reflects the absence of HBV isolates of Asian origin. Frequencies of HBV genotypes found in men seeking HIV antibody testing are difficult to compare since there are not further reports for the comparison. Concerning hemodialysis patients, HBV genotype H was responsible for all infections in our study. This prevalence is clearly different from those found in hemodialysis patients of other countries. For instance, in a Turkish study all isolates of hemodialysis patients were genotype D[42], in an Indonesian study all isolates were genotype B[43], and in a Brazilian study genotypes A and D were the most frequently found[44]. These HBV genotypes reported in hemodialysis patients certainly reflect the predominant HBV genotypes found in their respective countries. For its part, HBV genotype G is responsible for half of infections in AIDS patients. HBV genotype G has been discovered in USA and France isolates by Stuyver *et al*[45]. Interestingly, all HBV genotype G isolates were found as single isolates (no co-infections) in our study. This finding is unexpected, since HBV genotype G is frequently found as a co-infection with HBV genotype A[46,47]. The HBV genotype frequencies in AIDS patients found in our study differ substantially from those found in a Spanish study where researchers found a predominant HBV genotype A followed by genotype D among the AIDS patients studied[48]. The low frequency of precore mutants in Mexican isolates found in the present study confirms a previous observation[30]. In addition, this frequency of precore mutations contrasts with a higher frequency observed in the USA and Hong Kong[7,34,49]. The distribution of precore mutations in HBV genotypes found in the present study differs substantially from those found in other studies. While other researchers have found precore mutations in HBV genotypes B, C[50,51], and D[27], we have found the mutations only in HBV genotypes G and A, being mutations largely predominant in HBV genotype G (all HBV genotype G isolates in this study). The existence of precore mutations in HBV genotype G has been reported in patients chronically infected with HBV[45]. Remarkably, all mutated HBV genotype G isolates in this study came from samples of AIDS patients and one of the men seeking HIV testing. No similar findings are reported in AIDS patients. Concerning mutations in the core promoter region, it is remarkable that the classical dual variant ($A_{1762}T$, $G_{1764}A$), and indeterminate sequences were observed only in hepatitis patients. The prevalence of core promoter mutations and its distribution in HBV genotypes found in this study also differ from those reported in other countries. A large study with Hong Kong and USA samples showed that the frequency of dual variant is 20% and 30.2%, respectively[34], which is significantly higher than that found in our study. HBV core promoter mutations have been found in HBV genotypes A, B C and D[25,29,34]. Nevertheless, these mutations were found mainly in HBV genotype H followed by HBV genotype D in the present study. Indeterminate sequences in precore and core promoter regions as well as the change in genotype F in LiPA to genotype H found in the Mexican isolates are most probably due to mismatching of the sequences with the probes used. A single mismatch is sufficient to abolish hybridization to a probe under the conditions of the assay as previously described[34].

The INNO-LiPA HBV genotyping and precore assays have proved to be rapid, sensitive and reliable for the detection of HBV genotypes and precore promotor/precore mutations.

The genetic variability of HBV has implications on the sensitivity of immunologic and molecular based assays[5]. In addition, HBV genotyping has shown its utility not only in epidemiology studies but also in predicting prognosis and therapeutic response[6,52,53].

In conclusion, HBV circulates in at least 4 different genotypes in Mexico. HBV genotype H is highly predominant in HBV isolates of Mexico followed by genotypes G, A and D. The frequency of precore mutations in Mexican HBV isolates is low and associated mainly with HBV genotype G. Core promoter mutations seem to be rare in HBV isolates of Mexico.

REFERENCES

1 **Lavanchy D**. Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. *J Viral Hepat* 2004; **11**: 97-107

2 **Custer B**, Sullivan SD, Hazlet TK, Iloeje U, Veenstra DL, Kowdley KV. Global epidemiology of hepatitis B virus. *J Clin Gastroenterol* 2004; **38**: S158-S168

3 **Thomson EC**, Main J. Advances in hepatitis B and C. *Curr Opin Infect Dis* 2004; **17**: 449- 459

4 **Zuckerman JN**, Zuckerman AJ. Infectious Diseases. Barcelona, Spain: Mosby, 2000: 4.1-4.12

5 **Weber B**. Genetic variability of the S gene of hepatitis B virus: clinical and diagnostic impact. *J Clin Virol* 2005; **32**: 102-112

6 **Kramvis A**, Kew M, Francois G. Hepatitis B virus genotypes. *Vaccine* 2005; **23**: 2409-2423

7 **Chu CJ**, Keeffe EB, Han SH, Perrillo RP, Min AD, Soldevila-Pico C, Carey W, Brown RS Jr, Luketic VA, Terrault N, Lok AS. Hepatitis B virus genotypes in the United States: results of a nationwide study. *Gastroenterology* 2003; **125**: 444-451

8 **Moriya T**, Kuramoto IK, Yoshizawa H, Holland PV. Distribution of hepatitis B virus genotypes among American blood donors determined with a PreS2 epitope enzyme-linked immunosorbent assay kit. *J Clin Microbiol* 2002; **40**: 877-880

9 **Arauz-Ruiz P**, Norder H, Robertson BH, Magnius LO. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. *J Gen Virol* 2002; **83**: 2059-2073

10 **Echevarria JM**, Avellon A, Magnius LO. Molecular epidemiology of hepatitis B virus in Spain: identification of viral genotypes and prediction of antigenic subtypes by limited sequencing. *J Med Virol* 2005; **76**: 176-184

11 **Bielawski KP**, Dybikowska A, Lisowska-Charmuszko U, Stalke P, Gregorowicz K, Trocha H, Podhajska A. Distribution

of HBV genotypes and mutants among hepatitis B infected patients from northern Poland. *Int J Mol Med* 2004; **14**: 301-304

12 **Mulders MN**, Venard V, Njayou M, Edorh AP, Bola Oyefolu AO, Kehinde MO, Muyembe Tamfum JJ, Nebie YK, Maiga I, Ammerlaan W, Fack F, Omilabu SA, Le Faou A, Muller CP. Low genetic diversity despite hyperendemicity of hepatitis B virus genotype E throughout West Africa. *J Infect Dis* 2004; **190**: 400-408

13 **Kimbi GC**, Kramvis A, Kew MC. Distinctive sequence characteristics of subgenotype A1 isolates of hepatitis B virus from South Africa. *J Gen Virol* 2004; **85**: 1211-1220

14 **Zhu B**, Luo K, Hu Z. Establishment of a method for classification of HBV genome and it's application. *Zhonghua Shiyan He Linchuang Bingduxue Zazhi* 1999; **13**: 309-313

15 **Liu CJ**, Kao JH, Chen PJ, Lai MY, Chen DS. Molecular epidemiology of hepatitis B viral serotypes and genotypes in taiwan. *J Biomed Sci* 2002; **9**: 166-170

16 **Ishikawa K**, Koyama T, Masuda T. Prevalence of HBV genotypes in asymptomatic carrier residents and their clinical characteristics during long-term follow-up: the relevance to changes in the HBeAg/anti-HBe system. *Hepatol Res* 2002; **24**: 1

17 **Kato H**, Ruzibakiev R, Yuldasheva N, Hegay T, Kurbanov F, Achundjanov B, Tuichiev L, Usuda S, Ueda R, Mizokami M. Hepatitis B virus genotypes in Uzbekistan and validity of two different systems for genotyping. *J Med Virol* 2002; **67**: 477-483

18 **Chan HL**, Tsang SW, Wong ML, Tse CH, Leung NW, Chan FK, Sung JJ. Genotype B hepatitis B virus is associated with severe icteric flare-up of chronic hepatitis B virus infection in Hong Kong. *Am J Gastroenterol* 2002; **97**: 2629-2633

19 **Ding X**, Park YN, Taltavull TC, Thung SN, Jin X, Jin Y, Trung NS, Edamoto Y, Sata T, Abe K. Geographic characterization of hepatitis virus infections, genotyping of hepatitis B virus, and p53 mutation in hepatocellular carcinoma analyzed by in situ detection of viral genomes from carcinoma tissues: comparison among six different countries. *Jpn J Infect Dis* 2003; **56**: 12-18

20 **Lee CM**, Chen CH, Lu SN, Tung HD, Chou WJ, Wang JH, Chen TM, Hung CH, Huang CC, Chen WJ. Prevalence and clinical implications of hepatitis B virus genotypes in southern Taiwan. *Scand J Gastroenterol* 2003; **38**: 95-101

21 **Chan HL**, Hui AY, Wong ML, Tse AM, Hung LC, Wong VW, Sung JJ. Genotype C hepatitis B virus infection is associated with an increased risk of hepatocellular carcinoma. *Gut* 2004; **53**: 1494-1498

22 **Kao JH**, Wu NH, Chen PJ, Lai MY, Chen DS. Hepatitis B genotypes and the response to interferon therapy. *J Hepatol* 2000; **33**: 998-1002

23 **Wai CT**, Chu CJ, Hussain M, Lok AS. HBV genotype B is associated with better response to interferon therapy in HBeAg(+) chronic hepatitis than genotype C. *Hepatology* 2002; **36**: 1425-1430

24 **Janssen HL**, van Zonneveld M, Senturk H, Zeuzem S, Akarca US, Cakaloglu Y, Simon C, So TM, Gerken G, de Man RA, Niesters HG, Zondervan P, Hansen B, Schalm SW. Pegylated interferon alfa-2b alone or in combination with lamivudine for HBeAg-positive chronic hepatitis B: a randomised trial. *Lancet* 2005; **365**: 123-129

25 **Grandjacques C**, Pradat P, Stuyver L, Chevallier M, Chevallier P, Pichoud C, Maisonnas M, Trepo C, Zoulim F. Rapid detection of genotypes and mutations in the pre-core promoter and the pre-core region of hepatitis B virus genome: correlation with viral persistence and disease severity. *J Hepatol* 2000; **33**: 430-439

26 **Funk ML**, Rosenberg DM, Lok AS. World-wide epidemiology of HBeAg-negative chronic hepatitis B and associated precore and core promoter variants. *J Viral Hepat* 2002; **9**: 52-61

27 **Kondili LA**, Brunetto MR, Maina AM, Argentini C, Chionne P, La Sorsa V, Resuli B, Mele A, Rapicetta M. Clinical and molecular characterization of chronic hepatitis B in Albania: a country that is still highly endemic for HBV infection. *J Med Virol* 2005; **75**: 20-26

28 **Chan HL**, Hussain M, Lok AS. Different hepatitis B virus genotypes are associated with different mutations in the core promoter and precore regions during hepatitis B e antigen seroconversion. *Hepatology* 1999; **29**: 976-984

29 **Orito E**, Mizokami M, Sakugawa H, Michitaka K, Ishikawa K, Ichida T, Okanoue T, Yotsuyanagi H, Iino S. A case-control study for clinical and molecular biological differences between hepatitis B viruses of genotypes B and C. Japan HBV Genotype Research Group. *Hepatology* 2001; **33**: 218-223

30 **Alvarado-Esquivel C**, Wyseur A, Herrera-Ortiz FM, Ruiz-Maya L, Ruiz-Astorga R, Zarate-Aguilar A, Carrillo-Maravilla E, Herrera-Luna R, Morales-Macedo MC, Maertens G and Stuyver L. Therapies for Viral Hepatitis. London: International Medical Press, 1998: 35-41

31 **Sanchez LV**, Maldonado M, Bastidas-Ramirez BE, Norder H, Panduro A. Genotypes and S-gene variability of Mexican hepatitis B virus strains. *J Med Virol* 2002; **68**: 24-32

32 **Juarez-Figueroa LA**, Uribe-Salas FJ, Conde-Glez CJ, Hernandez-Avila M, Hernandez-Nevarez P, Uribe-Zuniga P, del Rio-Chiriboga C. Hepatitis B markers in men seeking human immunodeficiency virus antibody testing in Mexico City. *Sex Transm Dis* 1997; **24**: 211-217

33 **Stuyver L**, Van Geyt C, De Gendt S, Van Reybroeck G, Zoulim F, Leroux-Roels G, Rossau R. Line probe assay for monitoring drug resistance in hepatitis B virus-infected patients during antiviral therapy. *J Clin Microbiol* 2000; **38**: 702-707

34 **Hussain M**, Chu CJ, Sablon E, Lok AS. Rapid and sensitive assays for determination of hepatitis B virus (HBV) genotypes and detection of HBV precore and core promoter variants. *J Clin Microbiol* 2003; **41**: 3699-3705

35 **Kato H**, Gish RG, Bzowej N, Newsom M, Sugauchi F, Tanaka Y, Kato T, Orito E, Usuda S, Ueda R, Miyakawa Y, Mizokami M. Eight genotypes (A-H) of hepatitis B virus infecting patients from San Francisco and their demographic, clinical, and virological characteristics. *J Med Virol* 2004; **73:** 516-521

36 **Norder H**, Courouce AM, Coursaget P, Echevarria JM, Lee SD, Mushahwar IK, Robertson BH, Locarnini S, Magnius LO. Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. *Intervirology* 2004; **47**: 289-309

37 **Ohnuma H**, Yoshikawa A, Mizoguchi H, Okamoto H. Characterization of genotype H hepatitis B virus strain identified for the first time from a Japanese blood donor by nucleic acid amplification test. *J Gen Virol* 2005; **86**: 595-599

38 **Amini-Bavil-Olyaee S**, Sarrami-Forooshani R, Mahboudi F, Sabahi F, Adeli A, Noorinayer B, Azizi M, Reza Zali M. Genotype characterization and phylogenetic analysis of hepatitis B virus isolates from Iranian patients. *J Med Virol* 2005; **75**: 227-234

39 **Shibayama T**, Masuda G, Ajisawa A, Hiruma K, Tsuda F, Nishizawa T, Takahashi M, Okamoto H. Characterization of seven genotypes (A to E, G and H) of hepatitis B virus recovered from Japanese patients infected with human immunodeficiency virus type 1. *J Med Virol* 2005; **76**: 24-32

40 **Wai CT**, Fontana RJ, Polson J, Hussain M, Shakil AO, Han SH, Davern TJ, Lee WM, Lok AS. Clinical outcome and virological characteristics of hepatitis B-related acute liver failure in the United States. *J Viral Hepat* 2005; **12**: 192-198

41 **Schaefer S**. Hepatitis B virus: significance of genotypes. *J Viral Hepat* 2005; **12**: 111-124

42 **Bozdayi AM**, Aslan N, Bozdayi G, Turkyilmaz AR, Sengezer T, Wend U, Erkan O, Aydemir F, Zakirhodjaev S, Orucov S, Bozkaya H, Gerlich W, Karayalcin S, Yurdaydin C, Uzunalimoglu O. Molecular epidemiology of hepatitis B, C and D viruses in Turkish patients. *Arch Virol* 2004; **149**: 2115-2129

43 **Lusida MI**, Surayah H, Nagano-Fujii M, Soetjipto R, Boediwarsono PB, Nidom CA, Ohgimoto S, Hotta H. Genotype and subtype analyses of hepatitis B virus (HBV) and possible co-infection of HBV and hepatitis C virus (HCV) or hepatitis D virus (HDV) in blood donors, patients with chronic liver disease and patients on hemodialysis in Surabaya, Indonesia. *Microbiol Immunol* 2003; **47**: 969-975

44 **Teles SA**, Martins RM, Vanderborght B, Stuyver L, Gaspar

AM, Yoshida CF. Hepatitis B virus: genotypes and subtypes in Brazilian hemodialysis patients. *Artif Organs* 1999; **23**: 1074-1078

45 **Stuyver L**, De Gendt S, Van Geyt C, Zoulim F, Fried M, Schinazi RF, Rossau R. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. *J Gen Virol* 2000; **81**: 67-74

46 **Kato H**, Orito E, Gish RG, Sugauchi F, Suzuki S, Ueda R, Miyakawa Y, Mizokami M. Characteristics of hepatitis B virus isolates of genotype G and their phylogenetic differences from the other six genotypes (A through F). *J Virol* 2002; **76**: 6131-6137

47 **Kato H**, Orito E, Gish RG, Bzowej N, Newsom M, Sugauchi F, Suzuki S, Ueda R, Miyakawa Y, Mizokami M. Hepatitis B e antigen in sera from individuals infected with hepatitis B virus of genotype G. *Hepatology* 2002; **35**: 922-929

48 **Perez-Olmeda M**, Nunez M, Garcia-Samaniego J, Rios P, Gonzalez-Lahoz J, Soriano V. Distribution of hepatitis B virus genotypes in HIV-infected patients with chronic hepatitis B: therapeutic implications. *AIDS Res Hum Retroviruses* 2003; **19**: 657-659

49 **Chu CJ**, Keeffe EB, Han SH, Perrillo RP, Min AD, Soldevila-Pico C, Carey W, Brown RS Jr, Luketic VA, Terrault N, Lok AS. Prevalence of HBV precore/core promoter variants in the United States. *Hepatology* 2003; **38**: 619-628

50 **Sakurai M**, Sugauchi F, Tsai N, Suzuki S, Hasegawa I, Fujiwara K, Orito E, Ueda R, Mizokami M. Genotype and phylogenetic characterization of hepatitis B virus among multi-ethnic cohort in Hawaii. *World J Gastroenterol* 2004; **10**: 2218-2222

51 **Huy TT**, Ushijima H, Quang VX, Ngoc TT, Hayashi S, Sata T, Abe K. Characteristics of core promoter and precore stop codon mutants of hepatitis B virus in Vietnam. *J Med Virol* 2004; **74**: 228-236

52 **Locarnini SA**. Clinical relevance of viral dynamics and genotypes in hepatitis B virus. *J Gastroenterol Hepatol* 2002; **17** Suppl 3: S322-S328

53 **Wang Y**, Zhou G, Li X, Zhou Z, Zhou S, Ruan L, Chen M, Deng W. Genotyping of hepatitis B virus and clinical investigation. *Zhonghua Shiyan He Linchuang Bingduxue Zazhi* 2002; 16: 367-369

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RAPID COMMUNICATION

Prevalence of celiac disease in an urban area of Brazil with predominantly European ancestry

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Abstract

AIM: To determine the prevalence of celiac disease in a group of volunteer blood donors at a blood bank in the city of Curitiba, Brazil through detection of the serum marker immunoglobulin A (IgA) antitransglutaminase antibody.

METHODS: Blood samples collected from 2086 healthy subjects at the Paraná State Center for Hematology and Hemotherapy in Curitiba were submitted to ELISA testing for the IgA antitransglutaminase antibody. Positive samples received IgA antiendomysium antibody test through indirect immunofluorescence using human umbilical cord as substrate. Subsequently, patients who were positive on both tests underwent small bowel (distal duodenum) biopsy.

RESULTS: Six subjects, four males and two females, tested positive for the two serum markers. Five of the six were submitted to intestinal biopsy (one declined the procedure). Biopsy results revealed changes in the distal duodenum mucosa (three classified as Marsh Ⅲb lesions and two as Marsh Ⅱ lesions). Most donors diagnosed having celiac disease presented multiple symptoms (gastrointestinal tract complaints). One donor reported having a family history of celiac disease (in a niece).

CONCLUSION: Among apparently healthy blood donors, the prevalence of biopsy-confirmed celiac disease was approximately 1:417, similar to that seen in European countries.

Key words: Celiac disease; Epidemiology; Malabsorption; Prevalence; Small bowel disease

Pereira MAG, Ortiz-Agostinho CL, Nishitokukado I, Sato MN, Damião AOMC, Alencar ML, Abrantes-Lemos CP, Cançado ELR, de Brito T, Ioshii SO, Valarini SBM, Sipahi AM. Prevalence of celiac disease in an urban area of Brazil with predominantly European ancestry. *World J Gastroenterol* 2006; 12(40): 6546-6550

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INTRODUCTION

Celiac disease (CD) is a chronic disease that affects genetically susceptible individuals and is characterized by permanent intolerance to gluten and other related proteins, causing nonspecific, characteristic lesions in the small bowel mucosa[1]. These lesions prevent nutrient absorption in the affected area. Treatment involves a strict gluten-free diet (elimination of products containing wheat, barley, rye and probably oats). Clinical manifestations of CD are protean in nature and vary markedly with the age of the patient, the duration and extent of disease, and the presence of extraintestinal pathologic conditions. In addition to the classical gastrointestinal form, a variety of other clinical manifestations of the disease have been described, including atypical and asymptomatic forms[2].

Serologic tests developed over the past decades provide noninvasive tools for screening individuals at risk for the disease in both general and selected populations. However, for diagnosis of CD, it is necessary to perform histological evaluation in order to confirm intestinal damage in individuals presenting positive exam results.

Initially, the disease was considered a low-prevalence disorder and, consequently, diagnosis was based on clinical findings alone. Most prevalence studies of the disease have been carried out in European countries and have evaluated the prevalence based on symptomatic cases. Therefore,

atypical and asymptomatic cases were frequently misdiagnosed. Currently, prevalence is higher due to the availability of reliable, easily administered serologic tests of high sensitivity and specificity.

In CD patients, a wide variety of clinical manifestations are seen, and the disease may be seen in conjunction with other diseases. Therefore, as well as being important tools for prevalence studies, serologic tests (performed prior to the mandatory intestinal biopsy used for diagnosis confirmation) have become important markers for the disease[3].

Studies carried out in Europe, USA and Brazil in order to investigate the prevalence of CD have shown that the prevalence of the disease is considerably higher than previously presumed. Recent studies of serum markers in blood donors have shown a prevalence of 1 to 250 in Sweden[4], 1 to 524 in Denmark[5], 1 to 333 in Holland[6], 1 to 157 in Israel[7] and 1 to 250 in the USA[8]. The first Brazilian epidemiologic study using serum markers was published in 2000 and showed the prevalence of CD to be 1 to 681 among blood donors in the national capital of Brasília[9].

In order to get further insight into the prevalence of CD in Brazil and to evaluate the influence of genetic and environmental predisposition, we chose the city of Curitiba (in the state of Paraná). The city is located in the southern part of Brazil, and its population is of mostly European ancestry. Eating habits in Curitiba are similar to those seen in the countries of origin, and the diet features a significant wheat component. We decided to evaluate the epidemiology of CD in blood donors at a blood bank and to simultaneously analyze the ethnic profile of these donors. To that end, we used serum markers and conducted a genealogical study.

MATERIALS AND METHODS

Subjects

We analyzed 2086 serum samples collected from healthy donors at the Paraná State Center for Hematology and Hemotherapy in Curitiba from January to December 2001, regardless of gender. Donor ages ranged from 20 to 62 (mean, 33 years). The Ethics Committee of the University of São Paulo School of Medicine Hospital das Clínicas and the Ethics Committee of the Paraná State Center for Hematology and Hemotherapy approved the study. Donors gave written informed consent and answered a standardized questionnaire on demographics and health. No significant disease was diagnosed, and all serologic tests for human immunodeficiency virus (HIV), hepatitis B, hepatitis C, and alanine transaminase were normal. Serum samples were centrifuged and stored at -80℃ after serologic tests.

Methods

Serologic tests were performed in three University of São Paulo School of Medicine Medical Investigation Laboratories (LIM), LIM 06, LIM 07 and LIM 56. Samples positive for IgA antitissue transglutaminase (anti-tTG) by enzyme-linked immunosorbent assay (ELISA) were submitted to an immunofluorescence test for IgA antiendomysium antibody (EMA) using human umbilical cord as substrate. Each patient positive for both IgA anti-tTG and IgA EMA underwent small bowel (distal duodenum) endoscopic biopsy in which four samples were collected. This is the classical procedure accepted by the European Society of Pediatric Gastroenterology and Nutrition (ESPGAN)[10] for confirmation of a diagnosis of CD.

Anti-tissue transglutaminase antibody test

Technique carried out in accordance with the method described by Dieterich *et al*[11]: Microplates (96 wells; Corning, New York, NY, USA) were coated with 1 μg of guinea pig liver tTG (T 5398; Sigma, St. Louis, MO, USA) per well (activity: 1.5-3 KU/g of protein) in 100 μL Tris-HCl (50 mmol/L); NaCl (150 mmol/L) and $CaCl_2$ (5 mmol/L), pH 7.5, and incubated at 37℃ for 2 h. The wells were extensively rinsed in an Immunowash microplate washer (Bio-Rad, Hercules, CA, USA) in Tris-HCl (50 mmol/L), NaCl (150 mmol/L), EDTA (10 mmol/L) and Tween 20 (1 g/L), pH 7.4, and the microplates were incubated in washing buffer (100 μL/well) at 4℃ overnight. The microplate solution was aspirated; serum samples were diluted (1/100) in washing buffer, poured into the respective wells (100 μL/well) and incubated for one hour at room temperature. After rinsing the plates (3 times), peroxidase-conjugated IgA-α antibody (A 0295, Sigma), diluted (1/1000) in washing buffer (100 μL/well), was added to the wells, which were then incubated at room temperature for one hour. Extensive rinsing eliminated unbound antibodies. The tetramethylbenzidine substrate (100 μL/well) promoted the color reaction. Microplates were placed in the dark for approximately 10 min, and subsequently blocked with 0.5 mol/L H_2SO_4 (50 μL/well). Absorbance values were read at 450 nm using an ELISA reader (VERSAmax tunable microplate reader; Molecular Devices, Sunnyvale, CA, USA). After subtraction of background values < 0.100, absorbance (A) readings were multiplied by the serum dilution in order to calculate the ELISA titers. With the inclusion of two control serum samples in every test, intra-assay variations (n = 22) and inter-assay variations (n = 24) were 8.68% and 8.38%, respectively. Statistical evaluation was carried out through receiver operator characteristic (ROC) curve analysis[12] using SPSS® software.

Antiendomysium antibody test

Immunofluorescence tests for antiendomysium antibodies were carried out using 2 μm cryosections of human umbilical cord, which were incubated with patient serum prediluted (initial dilution = 1:5) in buffer (PBS and 1 g/L Tween 80, pH 7.2), in a humid chamber at 37℃ for 30 min. Slides were rinsed twice in PBS, pH 7.2, for 5 min. Samples were then incubated with fluorescein-conjugated anti-human IgA (Sigma) and diluted in dilution buffer (1:30). Subsequently, samples were rinsed twice with PBS and the slides were again incubated in humid chamber at 37℃ for 30 min. Later, samples were read under fluorescence microscopy. Samples were considered positive if there was a hexagonal pattern of fluorescence throughout the peritubular muscle layer of the human umbilical cord vessels, marking the extracellular connective tissue.

Table 1 Celiac disease diagnosed in apparently healthy blood donors

Age	Gender	Race	Ancestry	GI symptoms	Anti-tTG[1]	EMA[1]	Histology
26	F	W	Italian and Portuguese	Diarrhea, distension, abdominal pain and flatulence	1025	1/1280	Marsh IIIb
26	F	W	Italian, Portuguese and German	Pyrosis, obstipation, epigastric pain, flatulence, weakness and fatigue	295	1/640	Marsh IIIb
42	M	W	Portuguese, German and Spanish	Pyrosis, obstipation and flatulence	40	1/80	Marsh II
46	M	W	Italian	Pyrosis	2816	1/2560	Marsh II
57	M	W	Italian and Portuguese	Diarrhea	1540	1/640	Marsh IIIb
57	M	W	Italian	Asymptomatic	1840	1/640	No biopsy

F: Female; M: Male; W: White; GI: Gastrointestinal; Anti-tTG: Antitissue transglutaminase; EMA: Antiendomysium antibody; [1]Titers.

Histology

Samples were fixed with buffered formalin and stained with hematoxylin and eosin (H&E) for histological study. The following aspects were evaluated: (1) crypt/villus ratio; (2) crypt regeneration; (3) characteristics of the inflammatory infiltrate in the section itself; (4) type of atrophy. Two pathologists examined every slide for the standardization of the histological aspects, using the histological classification developed in 1992 by Marsh and modified in 1997 by Rostami *et al*[13-15]. This modified system establishes five lesion classes. In Marsh 0, there is normal architecture of the mucosa and less than 40 intraepithelial lymphocytes per 100 enterocytes in the villus epithelium. Marsh I is defined as normal architecture of the mucosa and more than 40 lymphocytes per 100 enterocytes in the villus epithelium. Marsh II involves crypt enlargement (hyperplasia), in which immature epithelial cells are produced in large numbers and there is an influx of lymphocytes and plasmocytes. Under this system Marsh III has been reclassified and divided into three separate classes. In Marsh IIIa, there is partial villus atrophy combined with slight lymphocyte infiltration in epithelial cells and crypt hyperplasia. Marsh IIIb is marked by near total atrophy of the villi (villi still recognizable), crypt hyperplasia in which immature epithelial cells are produced in greater proportions, and influx of inflammatory cells. The final designation, Marsh IIIc, indicates total villus atrophy, hyperplasic crypts and infiltrative lesions[13-15].

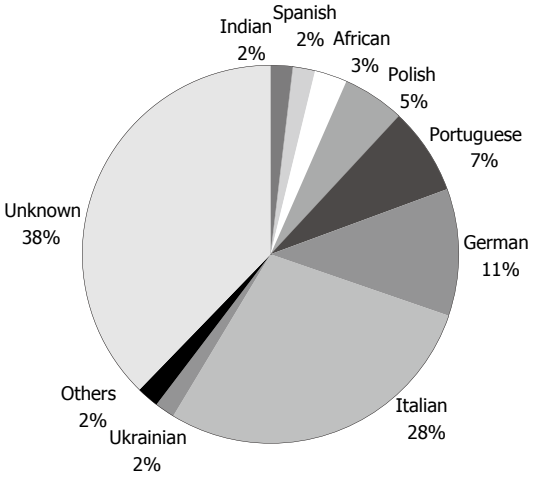

Figure 1 Blood donors by ancestry.

RESULTS

Of the 2086 blood donors, 1437 (68.88%) were males and 649 (31.12%) were females. Mean age was 33. There were 1977 Whites (94.77%), 82 Blacks (3.93%), and 27 Asians (1.30%). There were 1179 who claimed European ancestry (56.52%). Ethnic data were obtained through a genealogical study of the preceding three generations (Figure 1).

We identified six donors (four males and two females) who were positive for both anti-tTG and EMA. Five of these were submitted to intestinal biopsy and one declined the procedure. The procedure revealed that, in the mucosa of the small bowel (distal duodenum), three of the subjects presented Marsh IIIb lesions and two presented Marsh II lesions. Most subjects diagnosed with CD reported various gastrointestinal symptoms. One subject reported a family history of CD in a first-degree relative (a niece) (Table 1).

The prevalence of biopsy-confirmed CD was approximately 1:417 among apparently healthy blood donors. When the cases were positive for antitransglutaminase antibody were confirmed through the use of another marker, antiendomysium antibody, the prevalence was 1:347.

The sensitivity and specificity of the anti-tTG test were 100% and 96%, respectively. The OD cutoff value, established through analysis of the ROC curve, was 0.238. The area of the ROC curve was 0.999 ± 0.002.

DISCUSSION

In blood donors at a blood bank in Curitiba (Paraná), the prevalence of CD was 1:347 when samples positive for IgA anti-tTG antibodies were tested for a second marker (IgA antiendomysium antibodies). When subjects positive for both serum markers underwent distal duodenum biopsy, the prevalence was 1:417. This high prevalence is similar to that seen in European countries. This is unsurprising since most of the subjects in the study group were of European descent: 56.52% claimed European ancestry, and 37.78% were unaware of their ancestry (Figure 1). We can assume that most of the subjects of unknown ancestry were also of European descent since this ethnic group is predominant in Curitiba.

Factors that influence the high prevalence of CD are the genetic component (HLADQ_2 and DQ_8 haplotypes, which are strongly associated with CD and highly prevalent in the European population) and the consumption of grains containing gluten[2]. These two factors are present

in the city of Curitiba, where, in recent decades, there has been an increase in the consumption of wheat flour. This increase is related to the lifting of sanctions on the importation of special wheat grains. In addition, the consumption of foods containing wheat has increased as a consequence of economic plans that have created conditions which facilitate consumption among the population[17]. Currently, wheat consumption in the south of Brazil, where Curitiba is located, is 61 kg/person per year-the highest in the country[17]. This corresponds to 43.57% of the consumption in Italy, which, according to the World Health Organization, is 140 kg/person per year.

Another relevant and notable aspect is that, of the six positive donors, five were of Italian descent, most from the region of Veneto (northern Italy). A study carried out in that region using serum marker screening showed a CD prevalence of 1:200[18].

The CD prevalence observed in the present study was higher than the 1:681 prevalence found in a similar study involving blood donors at a blood bank in the city of Brasília[9]. This low prevalence could be attributed to low numbers of individuals of European descent, although the actual ethnic composition of the Brasília population is not known. However, it is known that most of the population of the city migrated from the Central-West region of Brazil, where individuals of European ancestry are in the minority and the consumption of wheat is lower (23 kg/person per year)[17].

In the literature, the prevalence of CD is higher in females when studies are based on cases with clinical evidence[19]. However, recent studies of blood donors showed that, when the diagnosis is made through the use of serum markers, this relationship is not maintained[7,8]. In our study, approximately one-third of the participants were female, and we found the prevalence of CD to be similar between males and females and proportionate to their respective representation in the study population (four males and two females tested positive).

A prevalence study of CD in a population composed of the descendants of European immigrants to New Zealand was conducted in the city of Wellington[20]. The study was based on clinical findings in children and adults and showed that prevalences of the disease in children and adults were lower and the same as those seen in the respective European cities of origin. The authors attributed the lower prevalence in children to underdiagnosis. Although the population under study presented the same ancestry and possibly the same eating habits, the authors evaluated only "the tip of the celiac iceberg". In the present study, which was conducted in a region of high wheat consumption and included a genealogical search and the use of serum markers, we evaluated "the rest of the iceberg". Our results show that, when ethnic and environmental factors are constant, the prevalence of the disease remains stable, even if members of the population migrate to other areas. A recent study[21], conducted in Argentina, using serum markers to evaluate a population of European descent in an area with 85 kg/person per year of wheat consumption, showed a CD prevalence of 1:167, reinforcing our hypothesis.

In the present study, we found a prevalence of CD of 1:417 among apparently healthy blood donors. Our results demonstrate that the high prevalence of CD in the city of Curitiba is comparable to that seen in European countries. This finding supports the hypothesis that the prevalence of the disease remains stable if predisposing genetic and environmental conditions are maintained.

REFERENCES

1 **Kagnoff MF**. Overview and pathogenesis of celiac disease. *Gastroenterology* 2005; **128**: S10-S18
2 **Dewar DH**, Ciclitira PJ. Clinical features and diagnosis of celiac disease. *Gastroenterology* 2005; **128**: S19-S24
3 **Romaldini CC**, Barbieri D. Serum antibodies in celiac disease. *Arq Gastroenterol* 1999; **36**: 258-264
4 **Grodzinsky E**, Franzen L, Hed J, Strom M. High prevalence of celiac disease in healthy adults revealed by antigliadin antibodies. *Ann Allergy* 1992; **69**: 66-70
5 **Weile B**, Grodzinsky E, Skogh T, Jordal R, Cavell B, Krasilnikoff PA. Screening Danish blood donors for antigliadin and antiendomysium antibodies. *Acta Paediatr Suppl* 1996; **412**: 46
6 **Rostami K**, Mulder CJ, Werre JM, van Beukelen FR, Kerchhaert J, Crusius JB, Pena AS, Willekens FL, Meijer JW. High prevalence of celiac disease in apparently healthy blood donors suggests a high prevalence of undiagnosed celiac disease in the Dutch population. *Scand J Gastroenterol* 1999; **34**: 276-279
7 **Shamir R**, Lerner A, Shinar E, Lahat N, Sobel E, Bar-or R, Kerner H, Eliakim R. The use of a single serological marker underestimates the prevalence of celiac disease in Israel: a study of blood donors. *Am J Gastroenterol* 2002; **97**: 2589-2594
8 **Not T**, Horvath K, Hill ID, Partanen J, Hammed A, Magazzu G, Fasano A. Celiac disease risk in the USA: high prevalence of antiendomysium antibodies in healthy blood donors. *Scand J Gastroenterol* 1998; **33**: 494-498
9 **Gandolfi L**, Pratesi R, Cordoba JC, Tauil PL, Gasparin M, Catassi C. Prevalence of celiac disease among blood donors in Brazil. *Am J Gastroenterol* 2000; **95**: 689-692
10 **Walker-Smith JA**, Guandalini S, Schmitz J, Shmerling DH, Visakorpi JK. Revised criteria for diagnosis of coeliac disease. Report of Working Group of European Society of Paediatric Gastroenterology and Nutrition. *Arch Dis Child* 1990; **65**: 909-911
11 **Dieterich W**, Laag E, Schopper H, Volta U, Ferguson A, Gillett H, Riecken EO, Schuppan D. Autoantibodies to tissue transglutaminase as predictors of celiac disease. *Gastroenterology* 1998; **115**: 1317-1321
12 **Fletcher R**, Fletcher SW, Wagner EH. Clinical epidemiology the essentials. 3th ed. Baltimore, Williams & Wilkins, 1984: 53-102
13 **Rostami K**, Kerckhaert J, Tiemessen R, von Blomberg BM, Meijer JW, Mulder CJ. Sensitivity of antiendomysium and antigliadin antibodies in untreated celiac disease: disappointing in clinical practice. *Am J Gastroenterol* 1999; **94**: 888-894
14 **Rostami K**, von Blomberg BM, Meijer JWR. Antiendomysium antibodies indicate severity of villous atrophy. *Eur J Gastroenterol Hepatol* 1997; **9**: A54
15 **Rostami K**, Kerckhaert J, von Blomberg BM, Meijer JW, Wahab P, Mulder CJ. SAT and serology in adult coeliacs, seronegative coeliac disease seems a reality. *Neth J Med* 1998; **53**: 15-19
16 **Marsh MN**. Gluten, major histocompatibility complex, and the small intestine. A molecular and immunobiologic approach to the spectrum of gluten sensitivity ('celiac sprue'). *Gastroenterology* 1992; **102**: 330-354
17 **Guth R**. Personal communication, 2002
18 **Volta U**, Bellentani S, Bianchi FB, Brandi G, De Franceschi L, Miglioli L, Granito A, Balli F, Tiribelli C. High prevalence of

celiac disease in Italian general population. *Dig Dis Sci* 2001; **46**: 1500-1505
19 **Marsh MN**. Coeliac disease. Oxford: Blackwell, 1992: 49-80
20 **Ussher R**, Yeong ML, Stace N. Coeliac disease: incidence and prevalence in Wellington 1985-92. *N Z Med J* 1994; **107**: 195-197
21 **Gomez JC**, Selvaggio GS, Viola M, Pizarro B, la Motta G, de Barrio S, Castelletto R, Echeverria R, Sugai E, Vazquez H, Maurino E, Bai JC. Prevalence of celiac disease in Argentina: screening of an adult population in the La Plata area. *Am J Gastroenterol* 2001; **96**: 2700-2704

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RAPID COMMUNICATION

Endoscopic management of acute cholangitis in elderly patients

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Abstract

AIM: To evaluate clinical presentation, etiology, complications and response to treatment in elderly patients with acute cholangitis.

METHODS: Demographics, etiology of biliary obstruction, clinical features, complications and associated systemic diseases of 175 patients with acute cholangitis were recorded. Endoscopic biliary drainage was performed using nasobiliary drain or stent. The complications related to ERCP, success of biliary drainage, morbidity, mortality and length of hospital stay were evaluated.

RESULTS: Of 175 patients, 52 aged ≥ 60 years (group Ⅰ, age < 60 years; group Ⅱ, age ≥ 60 years) and 105 were men. Fever was present in 38 of 52 patients of group Ⅱ compared to 120 of 123 in group Ⅰ. High fever (fever ≥ 38.0℃) was more common in group Ⅰ (118/120 *vs* 18/38). Hypotension (5/123 *vs* 13/52), altered sensorium (3/123 *vs* 19/52), peritonism (22/123 *vs* 14/52), renal failure (5/123 *vs* 14/52) and associated comorbid diseases (4/123 *vs* 21/52) were more common in group Ⅱ. Biliopancreatic malignancy was a common cause of biliary obstruction in group Ⅱ (*n* = 34) and benign diseases in group Ⅰ (*n* = 120). Indications for biliary drainage were any one of the following either singly or in combination: a fever of ≥ 38.0℃ (*n* = 136), hypotension (*n* = 18), peritonism (*n* = 36), altered sensorium (*n* = 22), and failure to improve within 72 h of conservative management (*n* = 22). High grade fever was more common indication of biliary drainage in group Ⅰ and hypotension, altered sensorium, peritonism and failure to improve within 72 h of conservative management were more common indications in group Ⅱ. Endoscopic biliary drainage was achieved in 172 patients (nasobiliary drain: 56 group Ⅰ, 24 group Ⅱ, stent: 64 group Ⅰ, 28 group Ⅱ) without any significant age related difference in the success rate. Abdominal pain, fever, jaundice, hypotension, altered sensorium, peritonism and renal failure improved after median time of 5 d in 120 patients in group Ⅰ (2-15 d) compared to 10 d in 47 patients of group Ⅱ (3-20 d). Normalization of leucocyte count was seen after a median time of 7 d (3-20 d) in 120 patients in group Ⅰ compared to 15 d (5-26 d) in 47 patients in group Ⅱ. There were no ERCP related complications in either group. Five patients (carcinoma gallbladder *n* = 3, CBD stones *n* = 2) died in group Ⅱ and they had undergone biliary drainage after failure of response to conservative management for 72 h. There was a higher mortality in patients in group Ⅱ despite successful biliary drainage (0/120 *vs* 5 /52). Length of hospital stay was longer in group Ⅱ patients (16.4 ± 5.6, 7-30 d) than in group Ⅰ patients (8.2 ± 2.4, 7-20 d).

CONCLUSION: Elderly patients with acute cholangitis have a high incidence of severe cholangitis, concomitant medical illnesses, hypotension, altered sensorium, peritonism, renal failure and higher mortality even after successful biliary drainage.

Key words: Acute cholangitis; Endoscopic biliary drainage; Endoscopic retrograde cholangio-pancreatography; Common bile duct stones; Carcinoma gall bladder

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INTRODUCTION

Acute cholangitis is a difficult diagnostic and therapeutic problem. Classically, Charcot's triad of jaundice, abdominal pain and fever have been the main basis of diagnosis however 30%-45% of the patients with acute cholangitis do not satisfy the criteria of Charcot's triad[1].

Altered sensorium, hypotension and renal failure can often be seen in patients with suppurative cholangitis[2]. In many cases, bile duct infection is latent and does not cause symptoms. Cholangitis varies in severity from a mild form which responds to parenteral antibiotics alone to severe or suppurative cholangitis which requires early drainage of biliary system to reduce the incidence of

systemic complications[3]. Endoscopic biliary drainage is an established mode of treatment for acute cholangitis with high success rate, low morbidity and mortality[1,3-20].

Old age is considered as a risk factor associated with significant mortality in acute cholangitis[21,22]. It is also a factor which adversely affects the outcome and survival in patients with acute cholangitis, which may be due to associated other systemic diseases and comorbid conditions, underlying etiology of biliary obstruction, poor immune status and poor general health in old age[21-23]. However, there is no study on clinical presentation, etiology, complications and survival after endoscopic biliary drainage in elderly patients with acute cholangitis. We prospectively compared the clinical presentation, etiology, associated systemic diseases, complications and response to treatment in elderly patients with acute cholangitis.

MATERIALS AND METHODS

Over past 5 years, 175 patients with acute cholangitis were recruited in this study. The diagnosis of acute cholangitis was based on presence of clinical evidence of infection (fever, leucocytosis and abdominal pain) in patients with biliary obstruction in the form of jaundice or hyperbilirubinemia[1,3]. Patients with biliary obstruction and associated hypotension, impaired level of consciousness and renal failure were specifically looked for associated features of acute cholangitis like fever and leucocytosis. They were labelled to be having acute cholangitis if they had any of features like hypotension, impaired level of consciousness and renal failure along with fever and /or leucocytosis in absence of infection at other sites or other causes for hypotension, impaired level of consciousness and renal failure. Details of demographic data, etiology of biliary obstruction, clinical features, biochemical parameters, microbiological spectrum, complications and associated other systemic diseases were recorded.

Immediate endoscopic biliary drainage was performed in patients with high fever, hypotension, peritonism, altered sensorium and associated renal failure. In patients without these features, endoscopic biliary drainage was performed only if they did not respond to conservative treatment. All patients were treated with intravenous antibiotics and metronidazole. Concomitant medical illnesses were also recorded and treatment was started to control these medical illnesses. Endoscopic retrograde cholangiopancreaticography (ERCP) was performed with a side viewing duodenoscope with a large accessory channel in a standard manner. Patients were subjected to either nasobiliary drain (NBD) placement or placement of biliary stent. After cannulation of the common bile duct, bile was aspirated to confirm the position of the cannula. Injection of contrast was avoided as far as possible to prevent any sudden increase in bile duct pressure which could lead to sudden cholangiovenous and cholangiolymphatic reflux. Endoscopic sphincterotomy was not performed. Either a 7-F nasobiliary drain or 7-F straight flap stent was placed in bile duct. After ERCP and biliary drainage, all the patients were kept under strict observation and treated in a critical care ward and the amount of bile drained from NBD was monitored daily.

Table 1 Demographic and clinical characteristics of patients with severe acute cholangitis

	Group Ⅰ	Group Ⅱ
n	123	52
Mean age ± SD (range) years	38.6 ± 12.4 (20-56)	68.4 ± 10.8[a] (60-90)
Gender (Male: Female)	72:51	33:19
Clinical presentation *n* (%)		
Right upper	116 (94.3)	27 (52)[a]
Quadrant pain	118 (96)	18 (34.6)[a]
Fever ⩾ 38.0℃	2 (1.6)	20 (38.4)[a]
< 38.0℃	120 (97.5)	48 (92.3)
Jaundice	3 (2.4)	19 (36.5)[a]
Altered sensorium	5 (4.0)	13 (25.0)[a]
Hypotension	22 (17.8)	14 (11.3)[a]
Peritonism	5 (4.0)	14 (11.3)[a]
Renal failure	4 (3.2)	21 (40.3)[a]
Other systemic illnesses		

[a]$P < 0.05$.

The patients were closely observed for evidence of ERCP-related complications. Clinical improvement was defined as normalization of fever, leucocytosis, hypotension, peritonism, altered sensorium and renal functions. After clinical improvement, a repeat ERCP or NBD cholangiogram was obtained in each patient. Clinical monitoring and biochemical tests were performed before ERCP and then on d 1, 3 and 7 after ERCP or earlier if indicated. After improvement of their cholangitis, patients underwent some form of definitive management. Primary outcome measures included complications related to ERCP and the treatment outcomes including success of biliary drainage, morbidity, mortality and length of hospital stay.

Statistical analysis

Quantitative data were expressed as the mean ± SD or as the median. The Mann-Whitney *U* test was used for comparing continuous variables and a Chi-square test with Yate's correction was used to analyze clinical variables and the two tailed Fisher's exact test was used when numbers were small. A P value < 0.05 was considered to be statistically significant.

RESULTS

Of 175 patients, 52 patients were aged ⩾ 60 years (group Ⅰ, age < 60 years; group Ⅱ, age ⩾ 60 years) and 105 were men. Most patients presented with right upper quadrant abdominal pain, jaundice and fever with chills. Of 175 patients, right upper quadrant abdominal pain and fever were more common in group Ⅰ compared to group Ⅱ (Table 1). Fever was present in 38 of 52 patients of group Ⅱ compared to 120 of 123 patients in group Ⅰ. However, high fever (fever ⩾ 38.0℃) was more common in patients of group Ⅰ (118/120 *vs* 18/38) (Table 1). Of 175 patients, hypotension (5/123 *vs* 13/52), altered sensorium (3/123 *vs* 19/52), peritonism (22/123 *vs* 14/52) and renal failure (5/123 *vs* 14/52) were more common in patients of group Ⅱ compared to patients with group Ⅰ.

Table 2 Etiology and site of biliary obstruction in common bile duct

Etiology of biliary obstruction	Site of biliary obstruction	Group Ⅰ *n* = 123(%)	Group Ⅱ *n* = 52(%)
Benign causes (*n* = 138)			
Common bile duct stone	Lower	104 (84.5)	18 (34.6)[a]
Chronic pancreatitis	Lower	2 (1.6)	0
Post operative biliary stricture	Upper	7 (5.7)	0
Hydatid cyst rupture with fistula	Upper	4 (3.2)	0
Rupture liver abscess with fistula	Upper	3 (2.4)	0
Malignant causes (*n* = 37)			
Gallbladder carcinoma	Upper	2 (1.6)	14 (27)[a]
Cholangiocacinoma	Upper + middle	0	5 (9.6)[a]
Periampullary carcinoma	Lower	1 (0.8)	15 (28.8)[a]

Table 3 Laboratory parameters before endoscopic biliary drainage (mean ± SD)

	Group Ⅰ (*n* = 123)	Group Ⅱ (*n* = 52)	*P*
Total leucocyte count, × 10^9/L	28.6 ± 6.4	20.4 ± 4.6	< 0.01
Total bilirubin, mg/dL	12.4 ± 8.2	20.6 ± 10.4	< 0.01
Aspartate aminotransferase, IU/L	62 ± 24	58 ± 20.6	NS
Alanine aminotransferase, IU/L	52 ± 18.4	56 ± 14.4	NS
Alkaline phosphatase, IU/L	402 ± 196	1126 ± 644	< 0.01
Serum albumin, g/dL	3.6 ± 0.8	3.8 ± 0.8	NS

Some patients in group Ⅱ presented with hypotension or altered sensorium despite no or mild abdominal pain and low grade fever.

Etiology and site of biliary obstruction in 175 patients are summarized in Table 2. The biliopancreatic malignancy was a common cause of biliary obstruction in patients of group Ⅱ (*n* = 34) compared to benign diseases in group Ⅰ (*n* = 120) (Table 2).

Of 175 patients, 25 patients had other associated comorbid systemic conditions (group Ⅰ, *n* = 4 and group Ⅱ, *n* = 21) like diabetes mellitus (*n* = 10), hypertension (*n* = 12), coronary artery disease (*n* = 6), cerebrovascular accidents (*n* = 4), chronic obstructive pulmonary disease (*n* = 4) and pulmonary tuberculosis (*n* = 3) in group Ⅱ patients compared to pulmonary tuberculosis (*n* = 2), rheumatic heart disease (*n* = 1), diabetes mellitus (*n* = 1), hypertension (*n* = 1) and thyrotoxicosis (*n* = 1). Laboratory investigation revealed higher elevation of leucocytes in group Ⅰ and higher elevations of bilirubin and alkaline phosphatase levels in group Ⅱ patients (Table 3). Blood cultures were positive in 70 cases (40%, group Ⅰ, *n* = 50; group Ⅱ, *n* = 20) with organisms such as *Escherichia Coli* (*n* = 50; group Ⅰ, *n* = 38; group Ⅱ, *n* = 12), Klebseilla (*n* = 12; group Ⅰ, *n* = 9; group Ⅱ, *n* = 3), Acinobacter (*n* = 13; group Ⅰ, *n* = 9; group Ⅱ, *n* = 4), Proteus (*n* = 9; group Ⅰ, *n* = 6; group Ⅱ, *n* = 3) and Pseudomonas (*n* = 9; group Ⅰ, *n* = 6; group Ⅱ, *n* = 3). Twenty-three patients had mixed infections. The biliary obstruction was most commonly found to be in the lower part of common bile duct in both group Ⅰ (*n* = 107) and group Ⅱ (*n* = 33) patients. Indications for biliary drainage were any one of the following either singly or in combination: a fever of ≥ 38.0℃ (*n* = 136), hypotension, with systolic blood pressure < 100 mm Hg (*n* = 18), right upper quadrant abdominal pain with guarding (peritonism) (*n* = 36), impaired level of consciousness (*n* = 22), and failure to improve within 72 h of conservative management (*n* = 22). High grade fever was more common indication of biliary drainage in group Ⅰ and hypotension, altered sensorium, peritonism and failure to improve within 72 h of conservative management were more common indications of biliary drainage in group Ⅱ patients (Table 4). None of the patients had undergone prior endoscopic or percutaneous transhepatic biliary drainage. After hospital admission, ERCP and endoscopic biliary drainage were performed in patients with severe acute cholangitis after a median interval of 20 h (range 3-42 h) in 153 patients and after a median interval of 96 h (range 72-106 h) in 22 patients who failed to respond to conservative management.

Table 4 Indications of biliary drainage

Indication	Group Ⅰ *n* = 123 (%)	Group Ⅱ *n* = 52 (%)	*P*
Fever ≥ 38.0℃	118 (96)	18 (34.6)	< 0.01
Hypotension	5 (4)	13 (25)	< 0.01
Peritonism	22 (18)	14 (26.9)	< 0.01
Altered sensorium	3 (2.6)	19 (36.5)	< 0.01
Failure to improve with conservative management	2 (1.6)	20 (38.4)	< 0.01

Endoscopic biliary drainage was achieved in 172 patients without any significant age related difference in the success rate. A nasobiliary drain was placed in 80 patients (56 group Ⅰ, 24 group Ⅱ) and a stent was placed in 92 patients (64 group Ⅰ, 28 group Ⅱ). ERCP with biliary drainage therefore could be achieved in 120 patients in group Ⅰ and 52 patients in group Ⅱ. Abdominal pain, fever, jaundice, hypotension, altered sensorium, peritonism and renal failure improved after median time of 5 d in all the 120 patients in group Ⅰ (range 2-15 d) and after median time of 10 d in 47 patients of group Ⅱ (range 3-20 d). Similar normalization of leucocyte count was seen after a median time of 7 d (range 3-20 d) in 120 patients in group Ⅰ and after a median time of 15 d (range 5-26 d) in 47 patients in group Ⅱ. There were no immediate ERCP related complications in either group of patients. There were no episodes of displacement, kinking or occlusion of NBD or of occlusion or migration of stent.

Five patients (carcinoma gallbladder *n* = 3, CBD stones *n* = 2) died in group Ⅱ after 3, 5, 6, 9 and 10 d of endoscopic biliary drainage. All the five patients were subjected to endoscopic biliary drainage (2 NBD, 3 stent) after they failed to respond to conservative management for 72 h. All the five patients died because of uncontrolled cholangitis and septicemia despite antibiotics and successful endoscopic biliary drainage.

There was a higher mortality in patients in group Ⅱ despite successful biliary drainage (0/120 *vs* 5 /52). There was no significant difference in mortality between group

Table 5 Clinical characteristics, indications and results of biliary drainage in patients with acute cholangitis due to stones in common bile duct (n = 122)

	Group Ⅰ n = 104 (%)	Group Ⅱ n = 18 (%)	P
Clinical parameters			
Fever	102 (98)	10 (55.5)	< 0.01
Jaundice	101 (97.1)	14 (77.7)	< 0.01
Altered sensorium	1 (0.96)	8 (44.4)	< 0.01
Hypotension	1 (0.96)	5 (27.8)	< 0.01
Peritonism	12 (11.5)	6 (33.3)	< 0.01
Renal failure	3 (2.8)	7 (38.9)	< 0.01
Indications of biliary drainage			
Fever ⩾ 38.0℃	102 (98)	6 (33.3)	< 0.01
Hypotension	1 (0.96)	5 (27.8)	< 0.01
Peritonism	12 (11.5)	6 (33.3)	< 0.01
Altered sensorium	1 (0.96)	8 (44.4)	< 0.01
Failure to improve with conservative treatment	1 (0.96)	7 (38.9)	< 0.01
Results of biliary drainage			
Time taken for improvement			
Median (range) days	5 (2-15)	10 (3-20)	< 0.01
Mortality	0	2	< 0.01
Length of hospital stay			
Mean (range) days	8.2 (7-20)	16.4 (7-30)	< 0.01

Ⅱ patients with (2/21) and without (3/31) concurrent diseases. Also there was no difference in mortality between group Ⅱ patients with benign (2/18) and malignant causes (3/34) of biliary obstruction. Length of hospital stay was significantly longer in group Ⅱ patients (16.4 ± 5.6, range 7-30 d) than in group Ⅰ patients (8.2 ± 2.4, range 7-20 d). Altered sensorium, hypotension, peritonism, mortality and length of hospital stay were more in group Ⅱ patients with CBD stones (n = 18) than in group Ⅰ patients with CBD stones (n = 104) (Table 5).

DISCUSSION

Older age has been considered a risk factor for increased morbidity and mortality rates in the treatment of acute cholangitis[21,22]. However there is little specific information available on clinical features and treatment of acute cholangitis in the elderly. In present study elderly patients with acute cholangitis were found to have relatively higher incidence of severe cholangitis, hypotension, altered sensorium, peritonism and renal failure. Symptoms in elderly patients did not correlate with the severity of acute cholangitis. Many elderly patients with severe cholangitis presented with deceptively mild symptoms. Presence of abdominal pain and high grade fever was less common in elderly patients compared to young patients. Almost 40% of elderly patients had low grade fever and nearly ¼ did not have fever. Therefore early diagnosis of acute cholangitis based on symptoms alone was difficult as reported earlier[21]. However on examination many elderly patients had features suggestive of severe cholangitis in form of jaundice, hypotension, altered sensorium, peritonism and associated renal failure. All the elderly patients showed abnormal results of laboratory tests. However rise in leucocyte count was less compared to leucocytosis in younger patients and bilirubin and alkaline phosphatase levels were higher in elderly patients than in younger patients. This could be due to biliopancreatic malignancy, a common cause of biliary obstruction in elderly patients compared to benign diseases in younger patients. Elderly patients also had a higher incidence of coexisting medical problems like cardiovascular, pulmonary, neurological and other systemic diseases. Due to the high incidence of severe cholangitis, features like hypotension and altered sensorium and associated other concurrent systemic diseases, the management of acute cholangitis in the elderly becomes difficult[21].

Urgent endoscopic biliary drainage has been recommended for severe acute cholangitis or cholangitis that does not respond to conservative treatment[1,3-20]. Endoscopic biliary drainage including endoscopic sphincterotomy, nasobiliary drainage and stenting has been advocated as safe and effective measure for the treatment of acute cholangitis with a mortality ranging from 2% to 8%. In present series, urgent endoscopic biliary drainage yielded favorable outcome for all the young patients. However endoscopic biliary drainage for elderly patients was associated with significant mortality. Earlier studies have shown that the success rates of therapeutic ERCP are similar between elderly and young patients[24,25]. However the morbidity and mortality associated with therapeutic ERCP are significantly greater in the elderly than in young patients[23-25]. There were no serious complications of endoscopic biliary drainage procedures in both elderly and younger patients. In elderly patients higher morbidity and mortality even after biliary drainage are considered due to advanced acute cholangitis, exacerbation of concurrent medical illnesses, unsuccessful biliary drainage procedures, underlying biliopancreatic malignancy as a cause of biliary obstruction, complications characteristic of elderly such as delirium, pneumonia or atelectasis and need for prolonged ventilatory support after the endoscopic drainage procedures[21-23]. Elderly patients are often unable to tolerate endoscopic procedures because they are critically ill or uncooperative. In this situation, the endoscopic procedures can be performed safely and successfully under general anesthesia and endotracheal intubation[21,25]. However none of the elderly patients in present series required general anesthesia, endotracheal intubation and ventilatory support after endoscopic procedures for biliary drainage.

In present study, the clinical profile and response to endoscopic biliary drainage in elderly patients was similar in patients having biliary obstruction due to benign and malignant causes. Elderly patients with CBD stones had different clinical profile, poorer response to biliary drainage and higher mortality as compared to young patients with CBD stones. In present series, all the five elderly patients were subjected to endoscopic biliary drainage after they failed to respond to conservative management for 72 h. All the five patients died because of uncontrolled cholangitis and septicemia despite antibiotics and successful endoscopic biliary drainage. None of the patients who underwent immediate endoscopic biliary drainage because of high grade fever or associated features like hypotension, altered sensorium and peritonism had died. It is possible that patients with low grade fever and

without features like hypotension, altered sensorium and peritonism also had severe acute cholangitis which was not clinically evident. Therefore they did not fit into indications of immediate endoscopic biliary drainage without waiting for assessment of response to conservative management for 72 h and accounted for 25% (5/20) of mortality in subgroup of elderly patients not responding to conservative treatment for 72 h. Some of the elderly patients presented with hypotension or altered sensorium despite absent or mild abdominal pain and without or with low grade fever suggesting thereby that elderly patients may have severe acute cholangitis without fever or with low grade fever and should be subjected to immediate endoscopic biliary drainage without waiting for 72 h to assess the response to conservative treatment for 72 h. Higher mortality has earlier been reported in patients with acute cholangitis who fail to respond to antibiotics and therapeutic intervention is performed after 72 h of hospital admission[23,26]. It has been found that many times it is not always clinically apparent which patients will respond to medical treatment alone and which will require urgent biliary drainage. The mortality rate is high for patients who undergo delayed biliary drainage after failure of medical therapy[23,26]. In elderly patients, the levels of bilirubin and alkaline phosphatase were significantly higher than in young patients. It has been reported that higher levels of bilirubin and alkaline phosphatase are associated with higher mortality in patients with acute cholangitis[22]. The differences in clinical profile, laboratory parameters and response to biliary drainage in elderly patients can not be attributed to differences in type of bacteria causing cholangitis because the bacteriological spectrum was similar in elderly and young patients in present study.

Elderly patients with acute cholangitis have a high incidence of severe cholangitis, concomitant medical illnesses, hypotension, altered sensorium, peritonism, renal failure and higher mortality even after successful biliary drainage.

REFERENCES

1 **Sharma BC**, Kumar R, Agarwal N, Sarin SK. Endoscopic biliary drainage by nasobiliary drain or by stent placement in patients with acute cholangitis. *Endoscopy* 2005; **37**: 439-443
2 **Boey JH**, Way LW. Acute cholangitis. *Ann Surg* 1980; **191**: 264-270
3 **van Lent AU**, Bartelsman JF, Tytgat GN, Speelman P, Prins JM. Duration of antibiotic therapy for cholangitis after successful endoscopic drainage of the biliary tract. *Gastrointest Endosc* 2002; **55**: 518-522
4 **Lai EC**, Paterson IA, Tam PC, Choi TK, Fan ST, Wong J. Severe acute cholangitis: the role of emergency nasobiliary drainage. *Surgery* 1990; **107**: 268-272
5 **Leung JW**, Chung SC, Sung JJ, Banez VP, Li AK. Urgent endoscopic drainage for acute suppurative cholangitis. *Lancet* 1989; **1**: 1307-1309
6 **Chawla YK**, Sharma BC, Dilawari JB. Endoscopic nasobiliary drainage in acute suppurative cholangitis. *Indian J Gastroenterol* 1994; **13**: 83-85
7 **Leese T**, Neoptolemos JP, Carr-Locke DL. Successes, failures, early complications and their management following endoscopic sphincterotomy: results in 394 consecutive patients from a single centre. *Br J Surg* 1985; **72**: 215-219
8 **Leese T**, Neoptolemos JP, Baker AR, Carr-Locke DL. Management of acute cholangitis and the impact of endoscopic sphincterotomy. *Br J Surg* 1986; **73**: 988-992
9 **Chawla YK**, Sharma BC, Singh R, Sharma TR, Dilawari JB. Emergency endoscopic nasobiliary drainage without the aid of fluoroscopy. *Indian J Gastroenterol* 1993; **12**: 97-98
10 **Lai EC**, Mok FP, Tan ES, Lo CM, Fan ST, You KT, Wong J. Endoscopic biliary drainage for severe acute cholangitis. *N Engl J Med* 1992; **326**: 1582-1586
11 **Lin XZ**, Chang KK, Shin JS, Lin CY, Lin PW, Yu CY, Chou TC. Emergency endoscopic nasobiliary drainage for acute calculous suppurative cholangitis and its potential use in chemical dissolution. *J Gastroenterol Hepatol* 1993; **8**: 35-38
12 **Cotton PB**. Endoscopic drainage for suppurative cholangitis. *Lancet* 1989; **2**: 213
13 **Sharma BC**, Agarwal DK, Baijal SS, Saraswat VA, Choudhuri G, Naik SR. Endoscopic management of acute calculous cholangitis. *J Gastroenterol Hepatol* 1997; **12**: 874-876
14 **Gogel HK**, Runyon BA, Volpicelli NA, Palmer RC. Acute suppurative obstructive cholangitis due to stones: treatment by urgent endoscopic sphincterotomy. *Gastrointest Endosc* 1987; **33**: 210-213
15 **Hui CK**, Lai KC, Yuen MF, Ng M, Chan CK, Hu W, Wong WM, Lai CL, Wong BC. Does the addition of endoscopic sphincterotomy to stent insertion improve drainage of the bile duct in acute suppurative cholangitis? *Gastrointest Endosc* 2003; **58**: 500-504
16 **Leung JW**. Does the addition of endoscopic sphincterotomy to stent insertion improve drainage of the bile duct in acute suppurative cholangitis? *Gastrointest Endosc* 2003; **58**: 570-572
17 **Lee DW**, Chan AC, Lam YH, Ng EK, Lau JY, Law BK, Lai CW, Sung JJ, Chung SC. Biliary decompression by nasobiliary catheter or biliary stent in acute suppurative cholangitis: a prospective randomized trial. *Gastrointest Endosc* 2002; **56**: 361-365
18 **Lau JY**, Chung SC, Leung JW, Ling TK, Yung MY, Li AK. Endoscopic drainage aborts endotoxaemia in acute cholangitis. *Br J Surg* 1996; **83**: 181-184
19 **Sugiyama M**, Atomi Y. The benefits of endoscopic nasobiliary drainage without sphincterotomy for acute cholangitis. *Am J Gastroenterol* 1998; **93**: 2065-2068
20 **Kumar R**, Sharma BC, Singh J, Sarin SK. Endoscopic biliary drainage for severe acute cholangitis in biliary obstruction as a result of malignant and benign diseases. *J Gastroenterol Hepatol* 2004; **19**: 994-997
21 **Sugiyama M**, Atomi Y. Treatment of acute cholangitis due to choledocholithiasis in elderly and younger patients. *Arch Surg* 1997; **132**: 1129-1133
22 **Pitt HA**, Cameron JL, Postier RG, Gadacz TR. Factors affecting mortality in biliary tract surgery. *Am J Surg* 1981; **141**: 66-72
23 **Gigot JF**, Leese T, Dereme T, Coutinho J, Castaing D, Bismuth H. Acute cholangitis. Multivariate analysis of risk factors. *Ann Surg* 1989; **209**: 435-438
24 **Deenitchin GP**, Konomi H, Kimura H, Ogawa Y, Naritomi G, Chijiiwa K, Tanaka M, Ikeda S. Reappraisal of safety of endoscopic sphincterotomy for common bile duct stones in the elderly. *Am J Surg* 1995; **170**: 51-54
25 **Sugiyama M**, Atomi Y. Endoscopic sphincterotomy for bile duct stones in patients 90 years of age and older. *Gastrointest Endosc* 2000; **52**: 187-191
26 **Thompson JE Jr**, Tompkins RK, Longmire WP Jr. Factors in management of acute cholangitis. *Ann Surg* 1982; **195**: 137-145

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CASE REPORT

Portal thrombosis and steatosis after preoperative chemotherapy with FOLFIRI-bevacizumab for colorectal liver metastases

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Abstract

In order to discuss the role of preoperative chemotherapy for colorectal liver metastases, which is used frequently before hepatic resection, even in patients with resectable disease at presentation, we herein report the development of two complications, partial portal vein thrombosis and hepatic steatosis with lobular inflammation, during the course of preoperative chemotherapy with FOLFIRI plus bevacizumab for colorectal liver metastases, which recognition led to timely discontinuation of chemotherapy as well as a change in the surgical strategy to resect the tumors and the damaged liver through advanced techniques. We conclude that duration of treatment and drug doses and combinations may impact the development of chemotherapy-induced liver injury. Surgeons and medical oncologists must work together to devise safe, rational, and oncologically appropriate treatments for patients with multiple colorectal liver metastases, and to improve the understanding of the pathogenesis of chemotherapy-induced liver injury.

Key words: Chemotherapy; Colorectal liver metastases; Resection

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INTRODUCTION

Surgical resection of colorectal liver metastases is nowadays a standard of care for resectable disease with 5-year survival rate approaching 60%[1-3]. Because of several theoretical benefits, preoperative systemic chemotherapy has been frequently used to downsize the disease.

We report here the development of two complications, partial portal vein thrombosis and hepatic steatosis with lobular inflammation, during the course of preoperative chemotherapy with FOLFIRI plus bevacizumab for colorectal liver metastases, and discuss the surgical management and implications.

CASE REPORT

A 61-year-old woman was referred for treatment of synchronous multiple bilobar colorectal liver metastases. Six months after a laparoscopic right hemicolectomy for a pT3N2 colon cancer, a multiphase spiral computed tomography (CT) performed without and with intravenous (Ⅳ) contrast revealed bilateral disease with a total of five lesions distributed in segments Ⅰ, Ⅱ, ⅣA, Ⅵ and Ⅶ amenable to hepatic resection (Figure 1). Because of the known high risk of disease recurrence after liver resection for multiple bilobar and synchronous metastases in patients with N2 classification of primary tumor, the patient was treated with six courses of preoperative chemotherapy with FOLFIRI (irinotecan, 5-fluorouracil, and high-dose leucovorin) plus bevacizumab.

Repeat CT performed without and with Ⅳ contrast 3 wk after the completion of chemotherapy with bevacizumab showed downsizing of the lesions in segments Ⅰ, ⅣA, Ⅵ-Ⅶ, and stable disease in the segment Ⅱ. Non-contrast images also showed the development of diffuse fatty infiltration of the liver with sparing of segments Ⅴ and Ⅷ while the images acquired after Ⅳ contrast administration revealed absent portal perfusion of the right anterior sector associated with right anterior portal branch occlusion. The lack of portal flow to segments Ⅴ and Ⅷ explained sparing from steatosis and increased arterial flow to these segments. Some compensatory hypertrophy of the left liver was evident (Figure 2). The patient had no evidence of hypercoagulation and no other known risk factors for thrombosis. Even though this patient experienced downsizing of bilobar liver metastases, the new finding

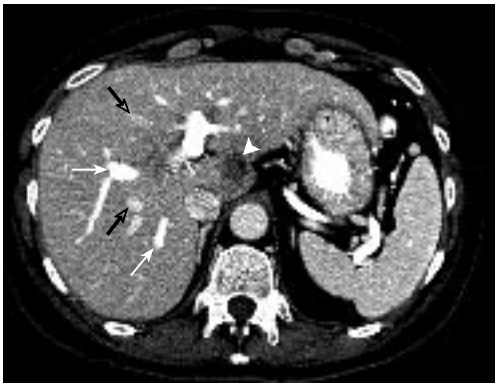

Figure 1 Contrast enhanced CT at the level of the portal bifurcation before treatment shows metastasis in segment I (arrowhead), opacified right anterior and posterior segmental portal veins (white arrows), and opacified middle and right hepatic veins (open arrows).

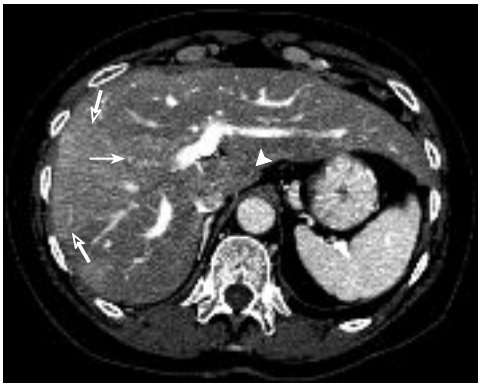

Figure 2 Contrast enhanced CT at the level of the portal bifurcation after treatment shows improved hepatic metastasis in segment I (arrowhead), non-visualization of the thrombosed anterior right segmental portal vein (arrow), opacified posterior segmental portal veins, middle and right hepatic veins, and wedge shaped increased enhancement (open arrows) of the anterior sector secondary to portal vein occlusion.

of portal vein thrombosis led to discontinuation of chemotherapy and reassessment of the treatment plan. Prior to occurrence of portal vein thrombosis, a partial right hepatectomy plus resection of the metastases in segments IVA, Ⅱ and Ⅰ, was planned. The finding of right portal vein thrombosis led to a change in operative plan to include resection of the segments involved both by tumor and by portal vein thrombosis in a 2-stage fashion. At the first stage, segmental resection of the lesions in segments Ⅰ and Ⅱ was performed, preserving the majority of the parenchyma of the lateral bisegment. Three-dimensional liver volumetry revealed the planned remnant liver (bisegment Ⅱ/Ⅲ) was of inadequate volume to permit resection of the right liver plus segment Ⅳ. Therefore, in order to increase volume and function of the liver remnant prior to the 2-stage resection, percutaneous right portal vein embolization extended to segment Ⅳ was performed. Repeat CT volumetry after 4 wk revealed adequate liver remnant hypertrophy, and the remaining diseased liver was resected by extended right hepatectomy (resection of Couinaud segments Ⅳ through Ⅷ). The postoperative course after each stage was uneventful. The pathological review of the specimen revealed 50% necrosis in the tumors and moderate-severe steatosis with lobular inflammation in the resected liver.

DISCUSSION

We report the development of partial portal vein thrombosis in combination with hepatic steatosis and lobular inflammation (steatohepatitis) during the course of preoperative chemotherapy with FOLFIRI plus bevacizumab for colorectal liver metastases. This thrombotic complication, which occurred during chemotherapy, was possibly not because of the presence or treatment effect of a metastatic lesion since no tumor was associated with the thrombosed portal vessel. Absence of previous data on vascular events in association with 5-fluorouracil alone, a drug which has been in use for more than 50 years, raises the possibility of an event associated with bevacizumab and/or irinotecan treatment.

Bevacizumab is a humanized recombinant murine monoclonal antibody to vascular endothelial growth factor A (VEGF-A) that competitively blocks binding of VEGF-A to its receptors, resulting in inhibition of angiogenesis. Bevacizumab has been reported to be associated with delayed wound healing, venous and arterial thromboembolism; however, to our knowledge bevacizumab has not been reported to cause portal venous thrombosis[4-8]. The proposed mechanism of bevacizumab-related thrombosis is complex-both hemorrhagic and thrombotic events may be involved. Briefly, bevacizumab by antagonizing VEGF's functions might decrease the renewal capacity of endothelial cells in response to trauma, leading simultaneously to a tendency to bleeding and thrombosis[9,10].

Preliminary evidence that irinotecan may be associated with arterial and venous thrombotic events has been reported as well, but portal vein thrombosis has not been reported previously and the pathophysiology of irinotecan-related thrombotic events remains unclear[6,11].

The baseline incidence of thrombosis in cancer patients is significant, and the development of portal vein thrombosis in our patient cannot be definitively attributed to chemotherapy[12]. However, the pattern of thrombosis, the occurrence during chemotherapy treatment, and the absence of any vascular complications after colectomy, portal vein embolization or 2 sequential hepatectomy procedures, suggest that combination of FOLFIRI and bevacizumab contributed to this complication.

Histopathologic review of the resected liver confirmed not only moderate-severe steatosis consistent with the CT findings, but also revealed lobular inflammation[13]. Steatosis plus lobular inflammation can progress to steatohepatitis, which has been associated with preoperative chemotherapy with irinotecan, and may raise the risk of death after liver surgery[14-17]. Adverse outcomes related to chemotherapy-induced injury likely relate to a decreased regenerative capacity of injured hepatocytes in response to major hepatectomy, possibly through alterations of nuclear factors such as nuclear factor-kappa B, which is crucial for the priming phase of liver regeneration[18].

In the patient reported, who had resectable disease at presentation, development of both portal vein thrombosis

and hepatic steatosis suggests that chemotherapy treatment contributed to these events and raised the possibility that curative surgery would not be possible. Fortunately, the recognition of these complications led to timely discontinuation of chemotherapy as well as a change in the surgical strategy to resect the tumors and the damaged liver through advanced techniques (portal vein embolization, extended hepatectomy, 2-stage approach) in an effort to minimize morbidity.

Chemotherapy will be used with more frequency before hepatic resection. Duration of treatment and drug doses and combinations may impact the development of chemotherapy-induced liver injury. Surgeons and medical oncologists must work together to devise safe, rational, and oncologically appropriate treatments for patients with multiple colorectal liver metastases, and to improve the understanding of the pathogenesis of chemotherapy-induced liver injury.

REFERENCES

1 **Choti MA**, Sitzmann JV, Tiburi MF, Sumetchotimetha W, Rangsin R, Schulick RD, Lillemoe KD, Yeo CJ, Cameron JL. Trends in long-term survival following liver resection for hepatic colorectal metastases. *Ann Surg* 2002; **235**: 759-766

2 **Abdalla EK**, Vauthey JN, Ellis LM, Ellis V, Pollock R, Broglio KR, Hess K, Curley SA. Recurrence and outcomes following hepatic resection, radiofrequency ablation, and combined resection/ablation for colorectal liver metastases. *Ann Surg* 2004; **239**: 818-825; discussion 825-827

3 **Pawlik TM**, Scoggins CR, Zorzi D, Abdalla EK, Andres A, Eng C, Curley SA, Loyer EM, Muratore A, Mentha G, Capussotti L, Vauthey JN. Effect of surgical margin status on survival and site of recurrence after hepatic resection for colorectal metastases. *Ann Surg* 2005; **241**: 715-722, discussion 722-724

4 **Gordon MS**, Cunningham D. Managing patients treated with bevacizumab combination therapy. *Oncology* 2005; **69** Suppl 3: 25-33

5 **Shah MA**, Ilson D, Kelsen DP. Thromboembolic events in gastric cancer: high incidence in patients receiving irinotecan- and bevacizumab-based therapy. *J Clin Oncol* 2005; **23**: 2574-2576

6 **Ellis LM**, Curley SA, Grothey A. Surgical resection after downsizing of colorectal liver metastasis in the era of bevacizumab. *J Clin Oncol* 2005; **23**: 4853-4855

7 **Scappaticci FA**, Fehrenbacher L, Cartwright T, Hainsworth JD, Heim W, Berlin J, Kabbinavar F, Novotny W, Sarkar S, Hurwitz H. Surgical wound healing complications in metastatic colorectal cancer patients treated with bevacizumab. *J Surg Oncol* 2005; **91**: 173-180

8 **Skillings JR**, Johnson DH, Miller K, Kabbinavar E, Bergsland E, Holmgren SN Holden SN, Hurwitz H, Scappaticci F. Arterial thromboembolic events (ATEs) in a pooled analysis of 5 randomized, controlled trials (RCTs) of bevacizumab (BV) with chemotherapy. *Proc Am Soc Clin Oncol* 2005; 3019

9 **Kilickap S**, Abali H, Celik I. Bevacizumab, bleeding, thrombosis, and warfarin. *J Clin Oncol* 2003; **21**: 3542; author reply 3543

10 **Zakarija A**, Soff G. Update on angiogenesis inhibitors. *Curr Opin Oncol* 2005; **17**: 578-583

11 **Rothenberg ML**, Meropol NJ, Poplin EA, Van Cutsem E, Wadler S. Mortality associated with irinotecan plus bolus fluorouracil/leucovorin: summary findings of an independent panel. *J Clin Oncol* 2001; **19**: 3801-3807

12 **Lee AY**. Epidemiology and management of venous thromboembolism in patients with cancer. *Thromb Res* 2003; **110**: 167-172

13 **Kleiner DE**, Brunt EM, Van Natta M, Behling C, Con tos MJ, Cummings OW, Ferrell LD, Liu YC, Torbenson MS, Unalp-Arida A, Yeh M, McCullough AJ, Sanyal AJ. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 2005; **41**: 1313-1321

14 **Vauthey JN**, Pawlik TM, Ribero D, Wu TT, Zorzi D, Hoff PM, Xiong HQ, Eng C, Lauwers GY, Mino-Kenudson M, Risio M, Muratore A, Capussotti L, Curley SA, Abdalla EK. Chemotherapy regimen predicts steatohepatitis and an increase in 90-day mortality after surgery for hepatic colorectal metastases. *J Clin Oncol* 2006; **24**: 2065-2072

15 **Kooby DA**, Fong Y, Suriawinata A, Gonen M, Allen PJ, Klimstra DS, DeMatteo RP, D'Angelica M, Blumgart LH, Jarnagin WR. Impact of steatosis on perioperative outcome following hepatic resection. *J Gastrointest Surg* 2003; **7**: 1034-1044

16 **Parikh AA**, Gentner B, Wu TT, Curley SA, Ellis LM, Vauthey JN. Perioperative complications in patients undergoing major liver resection with or without neoadjuvant chemotherapy. *J Gastrointest Surg* 2003; **7**: 1082-1088

17 **Fernandez FG**, Ritter J, Goodwin JW, Linehan DC, Hawkins WG, Strasberg SM. Effect of steatohepatitis associated with irinotecan or oxaliplatin pretreatment on resectability of hepatic colorectal metastases. *J Am Coll Surg* 2005; **200**: 845-853

18 **DeAngelis RA**, Markiewski MM, Taub R, Lambris JD. A high-fat diet impairs liver regeneration in C57BL/6 mice through overexpression of the NF-kappaB inhibitor, IkappaBalpha. *Hepatology* 2005; **42**: 1148-1157

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CASE REPORT

Gallbladder tuberculosis: False-positive PET diagnosis of gallbladder cancer

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Abstract

Gallbladder tuberculosis (GT) is an extremely rare disease, and very few cases have been reported in the literature. The first case of GT was described in 1870 by Gaucher. A correct preoperative diagnosis of GT is unusual, and it is frequently confused with various gallbladder diseases. We present a new case of a patient who underwent surgery with the preoperative diagnosis of gallbladder cancer after a false positive positron emission tomography scan in the diagnostic work-up.

Key words: Positron emission tomography; Gallbladder; Tuberculosis; Cancer; Review

Ramia JM, Muffak K, Fernández A, Villar J, Garrote D, Ferron JA. Gallbladder tuberculosis: False-positive pet diagnosis of gallbladder cancer. *World J Gastroenterol* 2006; 12(40): 6559-6560

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INTRODUCTION

Abdominal tuberculosis is common in developing countries but gallbladder tuberculosis (GT) is an extremely rare disease[1,2], and very few cases have been reported in the literature[1]. The first case of GT was described in 1870 by Gaucher.

A correct preoperative diagnosis of GT is unusual, and it is frequently confused with various gallbladder diseases. We present a new case of a patient who underwent surgery with the preoperative diagnosis of gallbladder cancer after a false positive positron emission tomography (PET) scan in the diagnostic work-up.

CASE REPORT

A 64-year-old man was admitted to our department for abdominal pain and jaundice. Abdominal examination revealed a palpable mass in right hypochondrium. Laboratory tests were normal. A gallbladder mass was observed on ultrasound. A computed tomography (CT) scan showed a thickened gallbladder wall with possible infiltration of liver parenchyma and duodenum, and we proposed a diagnosis of advanced neoplasm of gallbladder (Figure 1). A PET scan showed a hypermetabolic area in the right liver lobe in the usual location of the gallbladder, infiltrating the liver parenchyma (Figure 2) and reaffirming our suspected diagnosis of advanced neoplasm of gallbladder.

We performed a subcostal laparotomy and observed a very large tumor that involved gallbladder and duodenum. A biopsy of the gallbladder wall was taken, and the histologic perioperative study reported gallbladder tuberculosis with no signs of malignancy. We then performed a cholecystectomy and observed the presence of cholecistoduodenal fistulae, so that we closed the duodenum. An exhaustive examination of the abdominal cavity revealed no other foci of tubercular infection. The definitive histologic study showed granulomatous reaction with the presence of multinucleated giant cells and caseum necrosis compatible with GT.

The postoperative course was uneventful. A Mantoux test was positive. Other techniques (chest X-ray, bronchoscopy) showed no tubercular infection in other organs. Antitubercular drugs were prescribed for six months.

DISCUSSION

The gallbladder is highly resistant to tubercular infection, and the presence of cholelitiasis and cystic duct obstruction is essential for the development of GT[1,2]. About 70% of GT cases are accompanied by gallstones[2]. Four types of GT have been described, according to whether only the gallbladder is involved or there is generalized tuberculosis and whether the gallbladder mucosa is histologically involved[1]. GT often occurs together with other intra-abdominal tuberculosis, usually in women over 30 years of age[2]. However, in the present case it was restricted to the gallbladder, because the duodenum was only locally involved. The route of infection may be peritoneal, hematogenous or lymphatic[2]. Histologically, the lesion may be in the form of a localized

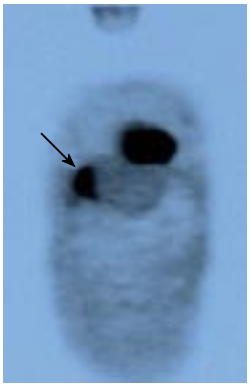

Figure 1 CT scan suggestive of image of gallbladder neoplasm.

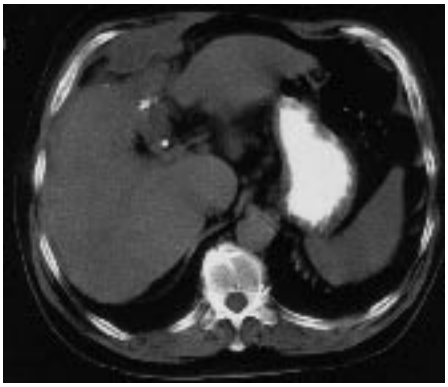

Figure 2 High uptake in gallbladder fossa (false positive for gallbladder cancer).

ulceration or there may be typical tuberculosis nodules in the wall of varied size and numbers.

A wide spectrum of symptoms have been described in patients with GT, including abdominal pain in right hypochondrium, weight loss, fever, anorexia, diarrhea, nausea, vomiting and a palpable abdominal mass[2]. Anemia, elevated ESR and positive tuberculin test are usually found in laboratory examinations.

The correct diagnosis of GT is difficult, and it is usually made after a cholecystectomy[1]. Ultrasound and CT may show an enlarged gallbladder, a thickened gallbladder wall, soft tissue masses, or nodular lesions, although neither ultrasound nor CT lesions are specific[2]. The diagnosis of tuberculosis in other abdominal organs (liver, spleen, lymph nodes or peritoneum) is very helpful for diagnosing GT.

The differential diagnosis of GT includes acute and chronic cholecystitis, polypoid lesions and gallbladder carcinoma[2]. The presence of a mass that fills the gallbladder associated with cholelitiasis is indistinguishable from carcinoma of the gallbladder[1]. Moreover, both GT and carcinoma can give rise to regional lymph nodes. The presence of liver metastasis or liver infiltration suggests the presence of a gallbladder carcinoma. On the other hand, lung lesions or mesenteric thickening is frequent in patients with tubercular infection.

PET is a very accurate diagnostic method for cancer. PET can image the metabolic differences between normal and malignant cells using tumor-seeking tracers[3]. According to some authors, it is difficult to distinguish patients with lung cancer from those with pulmonary tuberculosis due to the high uptake rate of patients with active tuberculosis[3,4]. This is because an increased glycolysis of macrophages, cells present in tubercular infection, results in a high FDG uptake value[3]. 11C-choline tracer is more accurate than 18F-fluorodeoxyglucose for making the differential diagnosis between tubercular infection and cancer[3].

There has been little experience with PET as a diagnostic tool in gallbladder cancer but the first published reports are fairly encouraging, showing a sensitivity of 75%[5,6]. At our department, a prospective trial is in progress to establish the sensitivity of PET scan in patients with suspicion of gallbladder cancer based on CT scan. The present patient was included in this trial and the results obtained appeared to confirm the suspected diagnosis of gallbladder cancer. To our knowledge, this is the first case of a false positive PET scan in a gallbladder cancer due to GT. There is one previous report of pancreatic tuberculosis that was misdiagnosed as pancreatic cancer after a positive PET scan[7].

Tuberculosis was common in Spain in the past but is now an uncommon finding in immunocompetent patients, and the GT diagnosis was therefore unexpected. In the countries with a high rate of tuberculosis infection, the possibility of a tuberculosis infection should be considered in patients suspected of a hepatobiliary cancer (liver, pancreas or gallbladder) with a positive PET if the patient has a positive Mantoux test or a history of tuberculosis.

REFERENCES

1 **Jain R**, Sawhney S, Bhargava D, Berry M. Gallbladder tuberculosis: sonographic appearance. *J Clin Ultrasound* 1995; **23**: 327-329

2 **Yu R**, Liu Y. Gallbladder tuberculosis: case report. *Chin Med J* (Engl) 2002; **115**: 1259-1261

3 **Hara T**, Kosaka N, Suzuki T, Kudo K, Niino H. Uptake rates of 18F-fluorodeoxyglucose and 11C-choline in lung cancer and pulmonary tuberculosis: a positron emission tomography study. *Chest* 2003; **124**: 893-901

4 **Goo JM**, Im JG, Do KH, Yeo JS, Seo JB, Kim HY, Chung JK. Pulmonary tuberculoma evaluated by means of FDG PET: findings in 10 cases. *Radiology* 2000; **216**: 117-121

5 **Koh T**, Taniguchi H, Yamaguchi A, Kunishima S, Yamagishi H. Differential diagnosis of gallbladder cancer using positron emission tomography with fluorine-18-labeled fluorodeoxyglucose (FDG-PET). *J Surg Oncol* 2003; **84**: 74-81

6 **Rodriguez-Fernandez A**, Gomez-Rio M, Llamas-Elvira JM, Ortega-Lozano S, Ferron-Orihuela JA, Ramia-Angel JM, Mansilla-Rosello A, Martinez-del-Valle MD, Ramos-Font C. Positron-emission tomography with fluorine-18-fluoro-2-deoxy-D-glucose for gallbladder cancer diagnosis. *Am J Surg* 2004; **188**: 171-175

7 **Sanabe N**, Ikematsu Y, Nishiwaki Y, Kida H, Murohisa G, Ozawa T, Hasegawa S, Okawada T, Toritsuka T, Waki S. Pancreatic tuberculosis. *J Hepatobiliary Pancreat Surg* 2002; **9**: 515-518

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CASE REPORT

Isolated splenic vein thrombosis secondary to splenic metastasis: A case report

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Abstract

A 49-year-old, previously healthy woman sought treatment for abdominal pain. Colonoscopy revealed ascending colon cancer. Computed tomography and angiography showed splenic metastasis and thrombosis extending from the splenic vein to the portal vein. She underwent right hemicolectomy, splenectomy, and distal pancreatomy. Histological findings showed no malignant cell in the splenic vein which was filled with organizing thrombus. We postulate the mechanism of splenic vein thrombosis in our case to be secondary to the extrinsic compression of the splenic vein by the splenic metastasis or by the inflammatory process produced by the splenic metastasis. In conclusion, we suggest that splenic metastasis should be added to the list of differential diagnosis which causes splenic vein thrombosis. In the absence of other sites of neoplastic disease, splenectomy seems to be the preferred therapy because it can be performed with low morbidity and harbors the potential for long-term survival.

Key words: Colon cancer; Splenic metastasis; Splenic vein occlusion; Splenic vein thrombosis

Hiraiwa K, Morozumi K, Miyazaki H, Sotome K, Furukawa A, Nakamaru M, Tanaka Y, Iri H. Isolated splenic vein thrombosis secondary to splenic metastasis: A case report. *World J Gastroenterol* 2006; 12(40): 6561-6563

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INTRODUCTION

Isolated splenic vein thrombosis is a rare clinical syndrome that may lead to left-sided portal hypertension. Metastasis to the spleen from solid tumors is also considered rare. When identified it usually occurs in the setting of widely disseminated diseases. We report a case of isolated splenic vein thrombosis secondary to splenic metastasis of an adenocarcinoma of the colon. The splenic lesion was detected before the resection of the primary colon lesion during a complete metastatic work-up.

CASE REPORT

A 49-year-old woman presented complaining of abdominal pain in May 2003. There was no relevant medical history. Her performance status was not impaired. Physical examination revealed upper abdominal tenderness with mild guarding. Laboratory findings revealed anemia with hemoglobin of 84 g/L and hematocrit of 29.9%. Esophagogastroduodenoscopy revealed no remarkable finding. Colonoscopy revealed ascending colon cancer of 7 cm in diameter. The level of tumor markers was elevated with carcinoembryonic antigen of 36.7 μg/L and CA19-9 of 1870 kU/L. Computed tomography of the abdomen showed splenic metastasis (Figure 1A), but no metastasis in the liver. Contrast-enhanced computed tomography and angiography showed occlusion of the splenic vein, a shadow defect at the portal vein, and development of collaterals, which suggested tumor thrombosis extending from the splenic vein to the portal vein (Figures 1B, 2A, B).

We diagnosed her as having an ascending colon cancer with splenic metastasis and splenic vein tumor thrombosis (T3NXM1, Stage Ⅳ). She underwent right hemicolectomy, splenectomy, and distal pancreatomy. The tumor of the spleen was adherent to the left hemidiaphragm, so a small segment of the diaphragm was removed en bloc. The portal vein was resected at its junction with the splenic vein and reanastomosed.

Histological findings showed a moderately differentiated adenocarcinoma of the ascending colon (Figure 3A) that invaded into serosa, with one of the five lymph nodes being positive for metastatic adenocarcinoma.

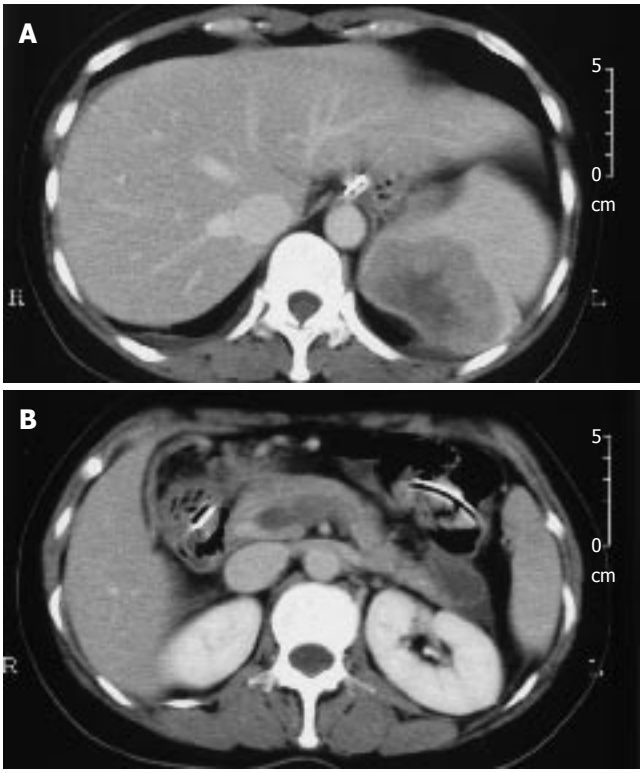

Figure 1 Contrast-enhanced computed tomography of the abdomen. **A**: Enlarged spleen with hypodense lesion in the superior spleen which was considered to be splenic metastasis; **B**: Hypoattenuating thrombus in the splenic vein and the portal vein.

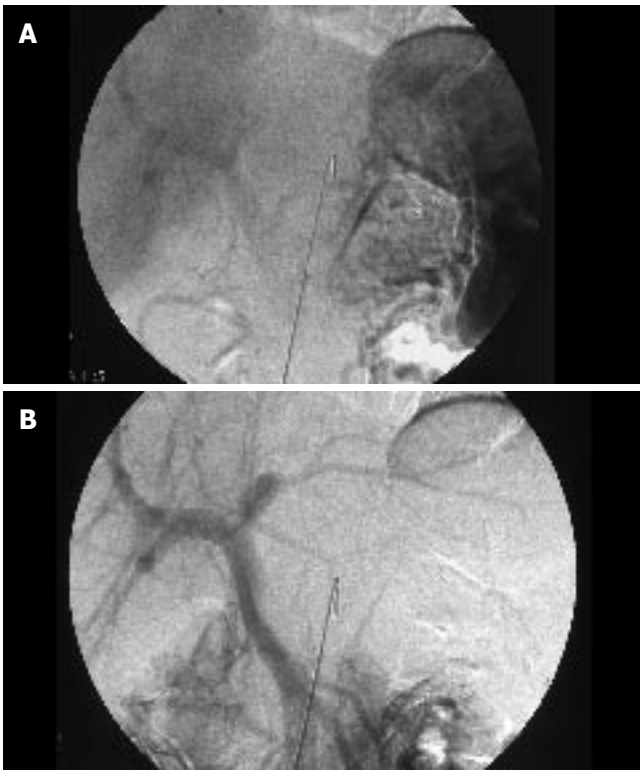

Figure 2 Digital substraction angiography. **A**: The venous phase of the celiac artery angiogram reveals occlusion of the splenic vein and development of collaterals; **B**: The venous phase of the superior mesenteric artery angiogram reveals a defect shadow in the portal vein at its junction with the splenic vein.

The tumor of the spleen consisted of a moderately

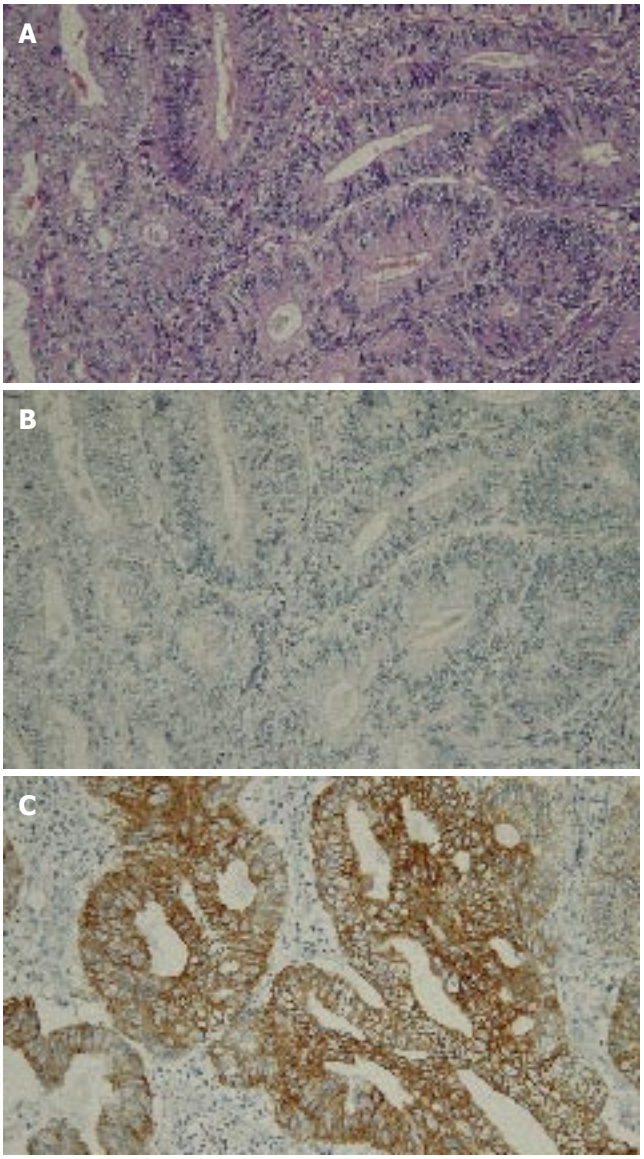

Figure 3 Histological findings of the tumor of the ascending colon. **A**: Moderately differentiated adenocarcinoma (HE, x 20); **B**: Immunostaining for cytokeratin 7 showing a negative reaction (x 20); **C**: Immunostaining for cytokeratin 20 showing a positive reaction (x 20).

differentiated adenocarcinoma similar in histology to the tumor of the ascending colon (Figure 4A). These sections were stained with immunohistochemical reactions of antibodies to cytokeratins 7 and 20 (Figures 3B, C, 4B, C). Negative staining for cytokeratin 7 and positive staining for cytokeratin 20 was consistent with splenic metastasis of an adenocarcinoma of the colon[1].

On the contrary to the preoperative diagnosis, no malignant cell was recognized in the splenic vein which was filled with organizing thrombus (Figure 5).

The postoperative course was uneventful. Two years and three months later, the patient is alive and well except for a nodule in the Douglas fossa which seems to be metastasis.

DISCUSSION

Isolated splenic vein thrombosis is a rare clinical syndrome that causes left-sided portal hypertension. It may lead to

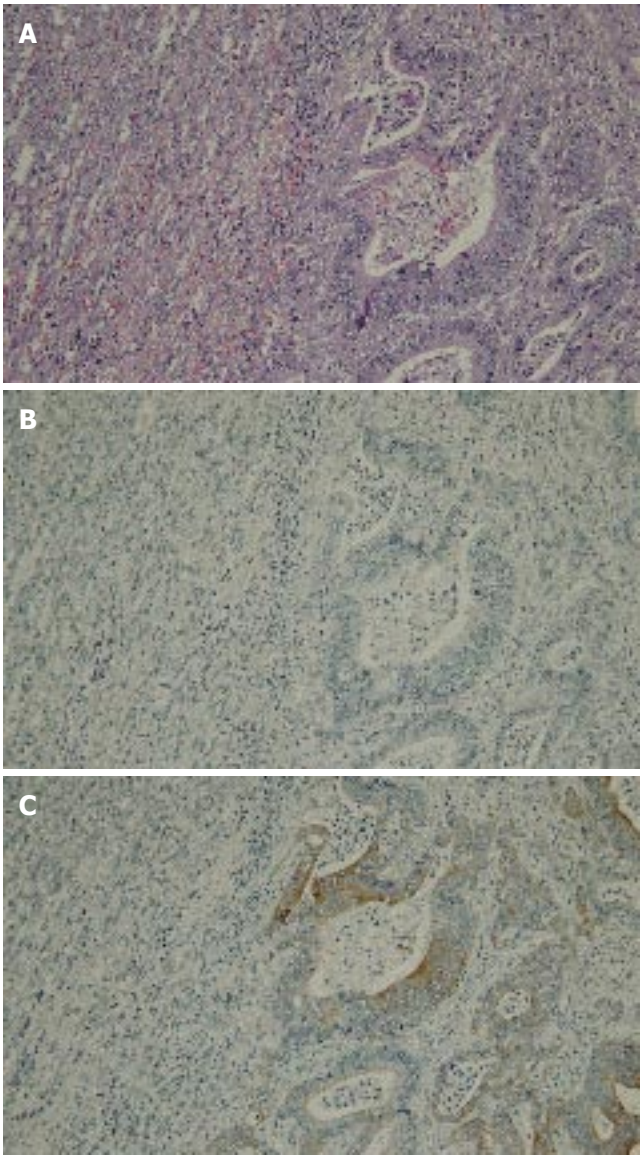

Figure 4 Histological findings of the tumor of the spleen. **A**: Moderately differentiated adenocarcinoma (HE x 20); **B**: Immunostaining for cytokeratin 7 showing a negative reaction (x 20); **C**: Immunostaining for cytokeratin 20 showing a positive reaction (x 20).

bleeding from gastric varices. The majority of splenic vein thrombosis are the result of pancreatic pathologies, including acute and chronic pancreatitis, pancreatic pseudocyst, pancreatic tumor and abscesses[2]. Other reported causes are traumas, umbilical vein catheterizations, lymphomas and sarcomas, retroperitoneal fibrosis, gastric surgeries, splenic artery aneurysms, myeloproliferative diseases, hereditary thrombocythemia[3], renal diseases including renal cell carcinomas[4] and benign renal cysts[5], and tuberculous lymphadenitis[6]. Our case did not have these diseases, and so we postulate the mechanism of splenic vein thrombosis in our case to be secondary to the extrinsic compression of the splenic vein by the splenic metastasis or by the inflammatory process produced by the splenic metastasis. To our knowledge, there has been no report of isolated splenic vein thrombosis secondary to splenic metastasis.

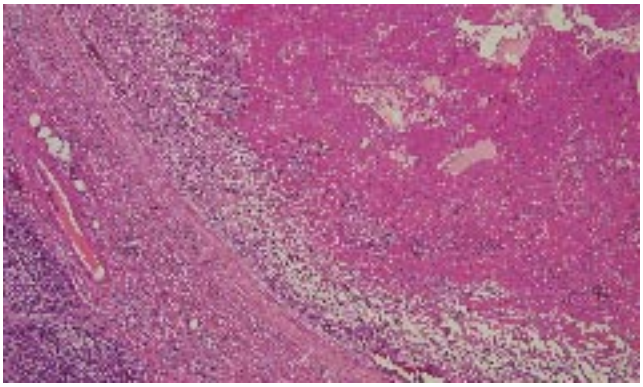

Figure 5 Histological findings of the splenic vein filled with not tumor thrombus, but organizing thrombus. The elastic tissue ring is intact (HE, x 4).

Carcinoma of the colon frequently metastasizes to the liver but only occasionally involves the spleen. The spleen is considered an unfavourable site for the development of metastases but the reason for this is not fully understood. Metastatic carcinoma that involves the spleen is usually a manifestation of widely disseminated disease involving multiple organs. Berge conducted an autopsy study on 7246 patients with various types of malignant tumors[7]. Splenic metastases were found in 21 out of the 1019 (2%) patients with colorectal carcinoma, but all were associated with involvement of other organs as well.

There are no long-term follow-up data of splenectomy for metastasis from colon cancer. Our patient underwent splenectomy, as it would deem to provide the only chance for long-term survival.

In conclusion, we suggest that metastatic tumor of the spleen should be added to the list of differential diagnosis which causes splenic vein thrombosis. Splenic metastasis from adenocarcinoma of the colon is a rare finding. In the absence of other sites of neoplastic disease, splenectomy seems to be the preferred therapy because it can be performed with low morbidity and harbors the potential for long-term survival.

REFERENCES

1 **Tot T**. Cytokeratins 20 and 7 as biomarkers: usefulness in discriminating primary from metastatic adenocarcinoma. *Eur J Cancer* 2002; **38**: 758-763
2 **Smith TA**, Brand EJ. Pancreatic cancer presenting as bleeding gastric varices. *J Clin Gastroenterol* 2001; **32**: 444-447
3 **Glynn MJ**. Isolated splenic vein thrombosis. *Arch Surg* 1986; **121**: 723-725
4 **Hasan A**, Ahmed M. Isolated splenic vein occlusion: a report of two cases. *J Pak Med Assoc* 1982; **32**: 79-80
5 **Koehler RE**. Case: splenic vein obstruction due to metastatic hypernephroma. *Gastrointest Radiol* 1981; **6**: 365-370
6 **Takeuchi H**, Suzuki M, Unno M, Kakita T, Matsuno S, Nakura H. Splenic vein occlusion secondary to tuberculous lymphadenitis at the splenic hilum: report of a case. *Surg Today* 2000; **30**: 383-385
7 **Berge T**. Splenic metastases. Frequencies and patterns. *Acta Pathol Microbiol Scand* 1974; **82**: 499-506

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CASE REPORT

Budd-Chiari like syndrome in decompensated alcoholic steatohepatitis and liver cirrhosis

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Abstract

A rare case of pseudo-Budd-Chiari Syndrome in a patient with decompensated alcoholic liver disease is reported. Although clinical and radiological findings suggested Budd-Chiari Syndrome, the liver biopsy revealed micronodular cirrhosis and absence of histological signs of hepatic outflow obstruction.

Key words: Pseudo Budd-Chiari; Budd-Chiari syndrome; Liver cirrhosis; Alcoholic steatohepatitis

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INTRODUCTION

Budd-Chiari Syndrome (BCS) is a rare, heterogeneous and potentially lethal condition caused by obstruction of the hepatic venous outflow tract[1], situated anywhere between the small hepatic venules until the right atrium[2]. In Western countries, thrombosis from multiples causes is the predominant factor in the etiology of BCS[3]. The correct diagnosis is very important for the therapeutic approach. Doppler ultrasound, computed tomography (CT) scan, and magnetic resonance imaging (MRI) provide evidence of hepatic vein (HV) thrombosis, with different specificity and sensitivity[4-7].

Pseudo-BCS is a condition which until now has not been well known. Only four reports were found in the literature and all of these concerned patients with a mechanical compression of the hepatic outflow due to an enlarged liver and liver cirrhosis.

The diagnosis of pseudo-BCS is difficult, because clinical and radiological findings are extremely similar to BCS, making invasive methods necessary.

We report a case of presumed BCS in a patient with alcoholic liver disease and emphasize the importance of the differential diagnosis for the correct management of these patients.

CASE REPORT

A 49-year-old female was admitted to our hospital with a 6-mo history of abdominal pain, progressive ascites, weight loss, anorexia and fatigue. There was no previous history of GI bleeding, surgery, hemotransfusions or spontaneous abortion. Also, she denied the use of oral contraceptives. Right up until admission day, she had consumed excessive amounts of alcohol for over 8 years. On physical examination we found mild jaundice, anemia, spider nevi, palmar erythema, hepatomegaly of 16 cm below the lower edge of the ribs, moderate ascites and edema. A systolic ejection murmur at the abdominal right upper quadrant was also found. Cardiac and pulmonary auscultation was normal. There was no jugular venous distention or pulsatile liver. Laboratory results are listed in Table 1.

Spontaneous bacterial peritonitis was diagnosed and treated. During endoscopy, three small esophageal varices were found. Doppler ultrasound revealed gross hepatomegaly 20 cm, with only a narrowed middle HV visible and a tributary branch. Absence of flow in the HV was reported. The CT-scan showed the massive hepatomegaly with irregular distribution of contrast, enlarged caudate lobe, and contrast hypercaptation at the left hepatic lobe.

The possibility of hepatocarcinoma plus BCS was considered. Serum viral markers were all negative and α-fetoprotein level was normal (4.61 U/mL).

MRI examination suggested the diagnosis of BCS due to patchy enhancement, absence of left and right HV and a narrowed middle HV with two narrowed tributary branches. An arterio-portal shunt was seen and hepatocarcinoma was excluded (Figure 1).

The diagnosis of BCS was made with a high degree of certainty by radiological exams. The search for hypercoagulable states was all negative.

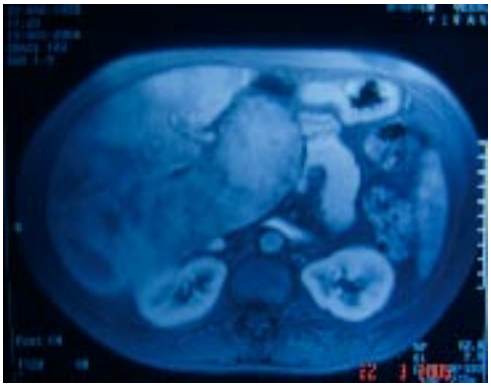

Figure 1 MRI in arterial phase showing a massive hepatomegaly with enlarged caudate lobe and patchy enhancement of contrast. A branch of portal vein is shown (arterio-portal shunt).

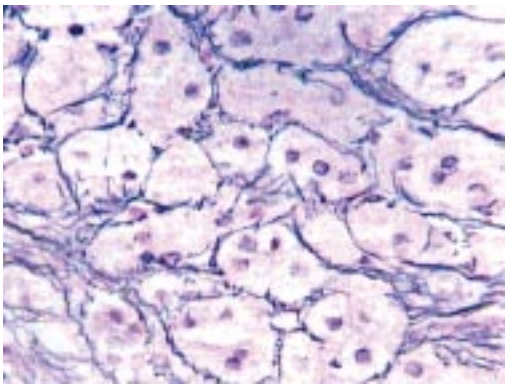

Figure 2 Liver biopsy reveals perisinusoidal and pericellular fibrosis (Gomori¡'s reticulin stain, original magnification x 100).

Due to the patient's previous history and clinical condition, a liver biopsy was performed in order to clarify the extent of liver damage due to alcohol abuse as well as venous occlusion.

The liver biopsy showed micronodular cirrhosis, perisinusoidal and pericellular fibrosis (Figure 2). There was neither pericentral congestion nor other histological signs of hepatic outflow congestion and the use of anticoagulant therapy was not indicated. The venography was not performed due to the results of the liver biopsy.

The patient was discharged four weeks after admission with improvement of her clinical condition and laboratory results (Table 1).

Table 1 Laboratory results

	Admission day	Discharge day	Normal values
Hemoglobin (mg/dL)	9.7	11.0	12.0-16.0
Mean corpuscular volume (fL)	108.1	103	80.0-96.0
Leukocytes (/mm^3)	21800	10100	4000-11000
Platelets (mil/mm^3)	306	250	140-360
Prothrombin time (s)	17.6	15.2	13
INR	1.52	1.2	1.0-1.4
Aspartate aminotransferase (U/L)	135	44	15-37
Alanin aminotransferase (U/L)	32.7	40	30-65
Gamma-GT (U/L)	907	312	5-85
Bilirubin (mg/dL)	2.73	1.4	0-1.0
Albumin (g/L)	24	36	34-50
Creatinine (mg/dL)	0.5	0.6	0.6-1.3
Alkaline fosfatase (U/L)	217	118	50-136

DISCUSSION

BCS is a rare disease, varied in cause, presentation and progression, with different diagnostic and therapeutic approaches. The radiological methods are part of the diagnostic work-up for BCS[1,8].

The first-line test is Doppler ultrasound of the liver[1] that has a sensitivity of 87.5% in the diagnosis of BCS[4]. The presence of a caudate vein equal or larger than 3 mm in diameter[9] or abnormalities in the flow of the HV outflow tract are suggestive of BCS[5,10].

MRI is the second-line test for BCS[1]. It permits differentiation between all forms of BCS[11]. Due to the high cost and low availability of this test, MRI is only recommended when the diagnosis is not clear after Doppler ultrasound[1,8,12].

A CT-scan may show an enlargement of the liver (caudate lobe), with patchy enhancement after contrast; but with indeterminate results due to false-positive in 50% of the cases[5,7]. Combination of the three techniques in the appropriate clinical setting increases sensitivity to 80%-90%[13].

In accordance with international guidelines, in BCS, liver biopsy and venography of hepatic veins are indicated when the diagnosis remains uncertain[1,14]. The third-line investigation is liver biopsy, which provides information for important differential diagnoses[14]. In current practice venography is rarely considered necessary for establishing a diagnosis and is carried out only when treatment is being planned[1].

Clinical exams and radiological testing strongly indicated that our patient was suffering from BCS. Due to the radiological results during the clinical evolution, it was no necessary to perform the venography. However, when the possible causes of BCS in our patient were extensively sought, by other means, all of them turned out to be negative.

The hypothesis of hepatocarcinoma was made due to the findings in the CT-scan. But this, too, was excluded after the MRI showed an arterio-portal shunt. It is well known that arterio-portal shunts can be present in a cirrhotic liver and confuse the diagnosis with hepatocarcinoma[15], as was the case in our patient.

Hypercoagulable states and thrombosis of other causes represent more than 75% of the etiology in patients with BCS[8]. Other authors suggest that more than 85% of idiopathic causes of BCS are due to myeloproliferative disorders[16,17].

One doubt in this case was the possibility that alcoholic intake could have been the cause of this BCS or in some way be associated with it. Two reports in the literature show this rare association[13,18].

Shankel *et al* in 1987 described a patient with BCS and alcoholic liver disease. The patient presented both inferior vena cava (IVC) and nephrotic syndromes. In spite of the BCS diagnosis, absence of thrombus and only a mechanical compression of the IVC was found at

autopsy[18].

The second report was made by Janssen *et al*, fifteen years later. It described three patients with alcoholic liver disease and BCS, without any thrombotic occlusion. These cases were denominated pseudo-BCS[13].

Another two cases of pseudo-BCS were reported in association with liver cirrhosis of unknown causes, without HV occlusion findings[19,20].

Dhawan *et al* in 1978, described a patient with clinical and radiological findings of BCS and concomitant liver cirrhosis. A complete constriction of the right HV and a narrowed left HV were shown to be present due to the hypertrophy of the left lobe of the liver and a regenerative nodule. No thrombotic occlusions were seen at the autopsy[19]. This was the first paper to introduce the term pseudo-BCS.

Rector *et al* two years later, also reported a patient with liver cirrhosis, probably by non-alcoholic steatohepatitis and BCS. A distortion of the IVC caused by cirrhosis and increased abdominal pressure suggested BCS due to membranous obstruction, but no thrombus or membranous occlusion was found[20].

All the cases reported had similar clinical, laboratory and radiological features and diagnostics.

Some of the described patients had a good evolution after alcohol withdrawal[13,18], including our patient. The other patients had fatal outcomes because of severe hepatic damage due to excessive alcohol intake or advanced liver disease[18,19]. One patient died after unnecessary anticoagulant therapy[13], which worsened the coagulation profile. No patients had pericentral congestion nor histological signs of hepatic outflow obstruction characteristic of BCS. A mechanical compression of the hepatic outflow due to an enlarged liver was seen in all cases. Histological changes in the architecture of the small hepatic veins were also observed in our patient.

Structural lesions of the hepatic venules, such as veno-occlusive changes, perivenular fibrosis and lymphocytic phlebitis have been well documented in patients with alcoholic liver disease[21].

Probably the structural lesions at the hepatic venules in association with mechanical compression due to anatomic abnormalities caused by an enlarged liver contributed to the misleading picture of BCS in these patients.

In conclusion the alcoholic steatohepatitis with liver cirrhosis can show a BC-like syndrome. Patients with this rare association denominated pseudo-BCS should be extensively investigated because management will depend on the correct diagnosis. The treatment of pseudo-BCS caused by alcoholic liver disease consists basically in discontinuing alcohol ingestion and generally is associated with a good prognosis.

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REFERENCES

1 **Valla DC**. The diagnosis and management of the Budd-Chiari syndrome: consensus and controversies. *Hepatology* 2003; **38**: 793-803
2 **Ludwig J**, Hashimoto E, McGill DB, van Heerden JA. Classification of hepatic venous outflow obstruction: ambiguous terminology of the Budd-Chiari syndrome. *Mayo Clin Proc* 1990; **65**: 51-55
3 **Okuda K**, Kage M, Shrestha SM. Proposal of a new nomenclature for Budd-Chiari syndrome: hepatic vein thrombosis versus thrombosis of the inferior vena cava at its hepatic portion. *Hepatology* 1998; **28**: 1191-1198
4 **Bolondi L**, Gaiani S, Li Bassi S, Zironi G, Bonino F, Brunetto M, Barbara L. Diagnosis of Budd-Chiari syndrome by pulsed Doppler ultrasound. *Gastroenterology* 1991; **100**: 1324-1331
5 **Miller WJ**, Federle MP, Straub WH, Davis PL. Budd-Chiari syndrome: imaging with pathologic correlation. *Abdom Imaging* 1993; **18**: 329-335
6 **Soyer P**, Rabenandrasana A, Barge J, Laissy JP, Zeitoun G, Hay JM, Levesque M. MRI of Budd-Chiari syndrome. *Abdom Imaging* 1994; **19**: 325-329
7 **Mori H**, Maeda H, Fukuda T, Miyake H, Aikawa H, Maeda T, Nakashima A, Isomoto I, Hayashi K. Acute thrombosis of the inferior vena cava and hepatic veins in patients with Budd-Chiari syndrome: CT demonstration. *AJR Am J Roentgenol* 1989; **153**: 987-991
8 **Menon KV**, Shah V, Kamath PS. The Budd-Chiari syndrome. *N Engl J Med* 2004; **350**: 578-585
9 **Bargallo X**, Gilabert R, Nicolau C, Garcia-Pagan JC, Bosch J, Bru C. Sonography of the caudate vein: value in diagnosing Budd-Chiari syndrome. *AJR Am J Roentgenol* 2003; **181**: 1641-1645
10 **Ohta M**, Hashizume M, Tomikawa M, Ueno K, Tanoue K, Sugimachi K. Analysis of hepatic vein waveform by Doppler ultrasonography in 100 patients with portal hypertension. *Am J Gastroenterol* 1994; **89**: 170-175
11 **Noone TC**, Semelka RC, Siegelman ES, Balci NC, Hussain SM, Kim PN, Mitchell DG. Budd-Chiari syndrome: spectrum of appearances of acute, subacute, and chronic disease with magnetic resonance imaging. *J Magn Reson Imaging* 2000; **11**: 44-50
12 **Kane R**, Eustace S. Diagnosis of Budd-Chiari syndrome: comparison between sonography and MR angiography. *Radiology* 1995; **195**: 117-121
13 **Janssen HL**, Tan AC, Tilanus HW, Metselaar HJ, Zondervan PE, Schalm SW. Pseudo-Budd-Chiari Syndrome: decompensated alcoholic liver disease mimicking hepatic venous outflow obstruction. *Hepatogastroenterology* 2002; **49**: 810-812
14 **Janssen HL**, Garcia-Pagan JC, Elias E, Mentha G, Hadengue A, Valla DC. Budd-Chiari syndrome: a review by an expert panel. *J Hepatol* 2003; **38**: 364-371
15 **Tamura S**, Kihara Y, Yuki Y, Sugimura H, Shimizu T, Adjei ON, Watanabe K. Pseudo lesions on CTAP secondary to arterio-portal shunts. *Clin Imaging* 1997; **21**: 359-365
16 **Valla D**, Casadevall N, Lacombe C, Varet B, Goldwasser E, Franco D, Maillard JN, Pariente EA, Leporrier M, Rueff B. Primary myeloproliferative disorder and hepatic vein thrombosis. A prospective study of erythroid colony formation in vitro in 20 patients with Budd-Chiari syndrome. *Ann Intern Med* 1985; **103**: 329-334
17 **Pagliuca A**, Mufti GJ, Janossa-Tahernia M, Eridani S, Westwood NB, Thumpston J, Sawyer B, Sturgess R, Williams R. In vitro colony culture and chromosomal studies in hepatic and portal vein thrombosis--possible evidence of an occult myeloproliferative state. *Q J Med* 1990; **76**: 981-989
18 **Dhawan VM**, Sziklas JJ, Spencer RP. Pseudo-Budd-Chiari syndrome. *Clin Nucl Med* 1978; **3**: 30-31
19 **Shankel S**. Budd-Chiari and the nephrotic syndromes secondary to massive hepatomegaly. *Am J Gastroenterol* 1987; **82**: 155-158
20 **Rector WG Jr**. Pseudo-Budd-Chiari syndrome: extrinsic deformity of the intrahepatic inferior vena cava mimicking membranous obstruction. *J Clin Gastroenterol* 1989; **11**: 88-91
21 **Goodman ZD**, Ishak KG. Occlusive venous lesions in alcoholic liver disease. A study of 200 cases. *Gastroenterology* 1982; **83**: 786-796

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CASE REPORT

A case of hepatocellular carcinoma arising within large focal nodular hyperplasia with review of the literature

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Abstract

Focal nodular hyperplasia (FNH) is a relatively rare benign hepatic tumor, usually presenting as a solitary lesion; however, multiple localizations have also been described. The association of FNH with other hepatic lesions, such as adenomas and haemangiomas has been reported by various authors. We herein report a case of a hepatocellular carcinoma arising within a large focal nodular hyperplasia, in a young female patient.

Key words: Focal nodular hyperplasia; Hepatocellular carcinoma; Liver tumors

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INTRODUCTION

Focal nodular hyperplasia (FNH) is a relatively rare benign liver tumor, often asymptomatic and discovered incidentally[1,2]. It occurs in both men and women, but shows a predilection for young women. FNH presents as a solitary lesion in 70% of the cases, while in 30% of patients two to five lesions are present[3]. Multiple lesions occur rarely[4-7].

Although an association with the use of oral contraceptives has been shown[6,7], its pathogenesis is still unclear. The potential for malignant transformation of FNH into hepatocellular carcinoma (HCC) has not been demonstrated[8]. However, cases of anatomical adjacency of fibrolamellar carcinoma (FL-HCC) and FNH in the same patient have been described, and some authors have suggested a direct link between the two tumors[9,10].

The simultaneous presence of HCC in its typical form and FNH is exceptional. To the best of our knowledge, only few cases regarding simultaneous occurrence of FNH and HCC of the liver have been reported in literature[8,11]. In this case report we describe a case of co-existent FNH and HCC and discuss the clinical management and therapeutic implications.

CASE REPORT

A 23-year-old woman was referred to the Outpatient Department of our hospital with a present history of fatigue and a past medical history of atresia of oesophagus which was reconstructed during her neonatal age and underwent oesophageal dilatations in her adolescence. She has never used oral contraceptives and had no history of hepatitis or alcohol abuse. Physical examination did not reveal any abnormal findings. Laboratory findings including serum α-fetoprotein and carcinoembryogenic antigen levels were within normal ranges. Contrast-enhanced abdominal CT scan showed five lesions in the liver (both left and right lobes), with the largest of the lesions located in the left lobe, an angiomyolipoma in the right kidney and multiple small angiomyolipomas of the left kidney. All liver lesions enhanced greatly during the early arterial phase (Figure 1A and B).

Three months later, a new contrast-enhanced abdominal CT scan was repeated and revealed apart from the known masses an increase in the size of the tumor in the left lobe of the liver (diameter from 4 cm to 7 cm). On unenhanced scans all masses appeared well-defined and homogeneously hypodense. Following an i.v. bolus of contrast, the masses showed early arterial contrast enhancement, with the exception of a centrally located area in the largest lesion, which remained hypodense (Figure 2A and B). On portal venous phase scans all lesions became isodense. The central hypodense area of the largest lesion remained unchanged during the portal venous phase scans, while became hyperdense on delayed scans. This area was attributed to a central scar, a finding consistent with a typical FNH. The remaining three masses were also con-

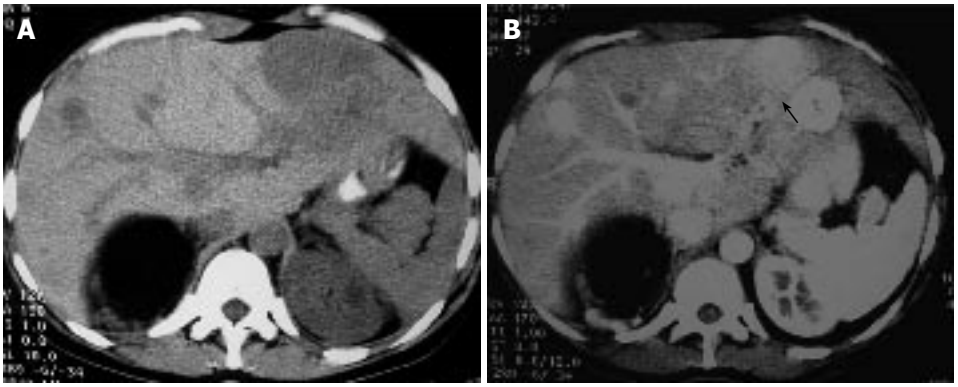

Figure 1 **A**: Unenhanced transverse CT scan demonstrates multiple hypoattenuating masses located on both liver lobes. Fatty tissue (known angiomyolipoma) replaces the most of the upper pole of the right kidney; **B**: Post-contrast CT scan depicts multiple round liver lesions, with a smooth margin, which demonstrate intense homogeneous enhancement. The lesion located in segment three, has a small central area of hypodensity, consistent with a central scar.

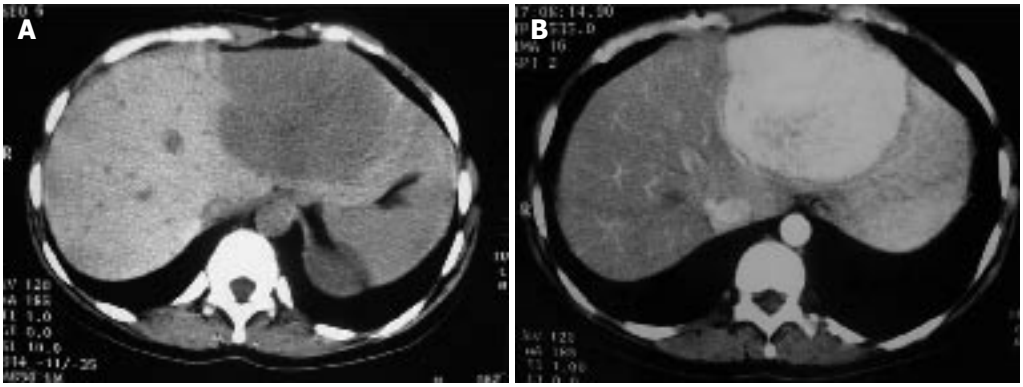

Figure 2 **A**: Pre-contrast CT scan 3 mo later, revealing the significant increase in the size of the lesion located in the left liver lobe; **B**: Post-contrast image at the same level showing the increased contrast uptake of the lesion.

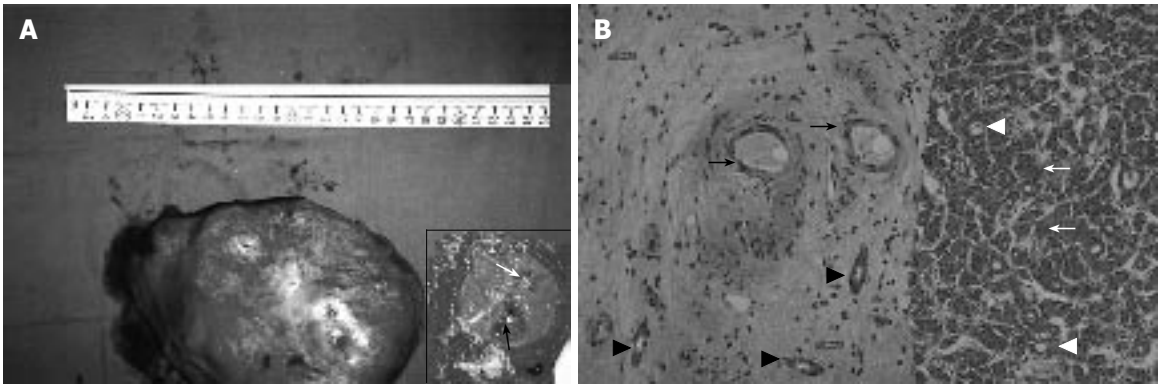

Figure 3 **A**: Photograph of the surgical specimen. The inset shows the cut surface: White arrow points at the scar of FNH and black arrow shows the hepatocellular carcinoma; **B**: Photomicrograph from the lesion. Left: Connective tissue from the core of FNH containing thin wall vessels (black arrows) and cholangioles (black arrowheads). Right: hepatocellular carcinoma. The tumor shows a trabecular growth pattern (white arrows) and focal pseudoglandular transformation (white arrowheads) (HE x 100).

sidered as FNHs. MR imaging was carried out at the same time and the findings were consistent with multiple FNHs.

Due to the great increase in the size of the mass located in the left lobe, during such a short period (3 mo), a CT guided core liver biopsy was performed in the largest lesion. Pathologic examination of the biopsy revealed that the specimen composed of fibrous tissue surrounded a nodule of hyperplastic hepatocytes, contained numerous thin-walled vessels, as well as numerous proliferated bile ductules. No evidence of malignancy was observed. Based on the radiological and histopathological data, the provisional diagnosis of FNH was made. In order to exclude any coexistent brain pathology, the patient underwent brain MR imaging, which was normal.

The surgical procedure involved the resection of the Ⅱ and Ⅲ liver segments. During the operation a frozen section was performed, revealing FNH. The surgical specimen measured 17.3 cm × 15.0 cm × 10.2 cm (Figure 3A). It consisted of a portion of liver which contained two masses, the larger measuring 9 cm × 6 cm × 5 cm and the smaller 4.5 cm × 3.5 cm × 2.0 cm. Macroscopically, both masses had a yellow-white cut surface and a central scar. In addition, the smaller mass contained another smaller tumor measuring 2.1 cm × 1.8 cm × 1.0 cm, which was located in the periphery and showed a brown-green cut surface (Figure 3A-inset). Microscopic examination of the specimen revealed the presence of FNH (two discrete tumors), whereas the smaller tumor included within the smaller mass represented a well-differentiated hepatocellular carcinoma (Figure 3B). Diagnosis of HCC was based

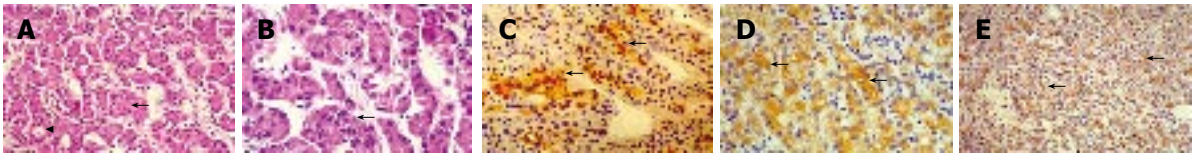

Figure 4 Microphotograph showing details of the tumor. **A**, **B**: The tumor displays a trabecular growth pattern (**A**-arrow, **B**-arrow) with focal pseudoglandular transformation (A-arrowhead) (HE, **A** × 250, **B** × 400); **C**: Tumor cells show positive immunohistochemical stain for hepatocyte antigen (arrows-streptavidin biotin peroxidase × 250); **D**: Tumor cells show positive immunohistochemical stain for cytokeratin 18 (arrows-streptavidin biotin peroxidase × 400); **E**: Immunohistochemical stain for polyclonal carcinoembryonic antigen displays a canalicular pattern (arrows-streptavidin biotin peroxidase × 100).

on morphological (HE stain) and immunohistochemical grounds (Figure 4): The neoplastic cells exhibited positive stain for hepatocyte paraffin antigen and cytokeratin 18 and negative stain for cytokeratin CK19, chromogranin, synaptophysin, common leukocyte antigen negative, CD34 antigen. In addition, polyclonal carcinoembryonic antigen displayed a canalicular pattern.

The postoperative period was uneventful. During the follow-up period, the patient underwent liver transplantation in another center abroad. Pathologic examination at that time revealed that the two small tumors in the right liver lobe represented liver angiomyolipomas (personal communication). At present time, eight years after the initial diagnosis, the patient is alive.

DISCUSSION

Benign liver tumors are uncommon in surgical practice (3%-5% of all liver tumors); haemangioma is the most common type (55%-60%), whereas adenomas (8%-19%) and FNH (21%-27%) are less frequent. FNH, also called mixed tumor represents a small percentage (1%-5%); its definition is derived from the coexistence of two or more tumor phenotypes[1,12,13]. FNH is generally considered to be a hyperplastic response to an abnormal blood supply[14].

The potential of benign liver tumors for malignant degeneration has been extensively discussed in the literature; in particular, a considerable risk of malignant transformation has been reported for hepatic adenoma[15], which was supported by findings of HCC foci within the benign tumor mass. This is why surgical resection of hepatic adenomas is recommended by most authors (apart from considerations about possible rupture of larger adenomas and subsequent intra-abdominal bleeding).

Conversely, there is no agreement on the malignant potential of FNH. To the best of our knowledge only a few cases of co-existent FNH and HCC have been reported in the literature[8,11] and the pathological correlation is not always clear. Saul *et al* reported one case in 1987 in which FNH and HCC were concomitant, although the HCC was a fibrolamellar variant (FL-HCC)[9]; Chen *et al* recently reported one case of HCC partially surrounded by FNH[8], while even more recently Cucchetti *et al* reported a case of simultaneous presence of FNH and HCC[11].

Simultaneous appearance of FNH with adenoma[16,17] and haemangioma[18] has been reported. The simultaneous occurrence of adenoma, focal nodular hyperplasia and haemangioma has also been described and the authors have concluded that adenoma, FNH and haemangiomas might have a common origin that should be attributed to vascular changes primarily caused by a congenital abnormality of the angioarchitecture and blood circulation of the liver[19]. Various authors have described the co-existence of FNH with vascular cranial malformations[20], cystic dysplasia of the kidneys[21], Klippel-Trenaunay syndrome[22]. In all cases described so far, the coexistence of the above findings were attributed to a so called syndrome or were described as coincidental[23]. A useful diagnostic tool for the distinction between liver cell adenoma and FNH, when the routine histopathologic features are not very clear, is clonality analysis[24].

The histopathological features of FL-HCC suggest a direct link between this tumor and FNH and some authors hypothesize a direct evolution from FNH to FL-HCC[9,10]. Also, the simultaneous presence of adjacent adenoma does not exclude the development of HCC from malignant degeneration of the adenoma. Therefore co-existence of FNH and HCC in the same patient is an exceptional event, to the best of our knowledge reported only in two cases.

Recent insight into the molecular characteristics of the clonal growth of FNH failed to support further a possible derivation of HCC from FNH; two different studies, conducted by Gaffey[12] and Paradis[13], were undertaken to clarify the monoclonal or polyclonal nature of these tumors by a method that scans the molecular pattern of inactivation of chromosome X. The authors eventually came to opposite conclusions. In another study[8], clonal analysis was applied to macroscopically different portions having different histological features within the same tumor, with concomitant FNH and HCC; the results showed that FNH was of monoclonal origin, but the FNH clone was similar to that of HCC and therefore the authors' conclusions did not support the hypothesis that HCC was the product of malignant transformation from FNH. The issue of an identical clonal origin of FNH and HCC therefore remains a matter of debate, although we feel it is rather imprudent at present to completely exclude any malignant potential of FNH.

The potential of FNH for malignant evolution would appear unlikely on the basis of the follow-up of patients with non-surgically excised FNH. Weimann *et al* observed an increase in the size of FNH lesions only in 9.5% of 53 cases, with no malignant transformation during a mean follow-up of three years[25]. Likewise, in other studies no increase in lesion size was observed among 11 patients in a two-year follow-up, and in some cases the FNH even completely disappeared over time[26]. This evidence

recently led Charny *et al*, to conclude that, if the diagnosis is unequivocal, surgical resection is not indicated for asymptomatic FNH[27].

A clear and precise diagnosis of a benign liver tumor is difficult to obtain in every patient, particularly in the case of FNH. In the series collected by Terkivatan, difficulty in differentiating FNH from adenoma or HCC represented the indication for surgery in 32% of cases[28]. FNH was rarely encountered by radiologists prior to the current practice of helical multiphasic CT or MR imaging. It is also believed that as the imaging methods improve, FNH will be encountered more frequently. Many authors have reported the CT features of FNH. Imaging characteristics, typical of FNH, include a homogeneous well-defined hypo-, or iso-dense lesion on unenhanced scans, which shows bright enhancement during early arterial phase and becomes isodense on portal venous phase. Central scars are hypodense on early arterial and portal venous phases and become hyperdense on delayed scans[29-32]. Magnetic resonance imaging is another modality also used for the confident diagnosis of FNH. The reported sensitivity and specificity values for contrast enhanced-MRI diagnosis of FNH was 70% and 98% respectively in an article by Cherqui *et al* in a series of 41 patients with clinical radiological and pathological correlations. The central scar was detected in 78% of the cases[33]. An alternative to angiography could be hepatic cholescintigraphy, which according to the study of Weimann, best reflected the vascular pattern and the typical biliary ductule proliferation of FNH (sensitivity $\geqslant$ 82%, specificity $\geqslant$ 97%)[25].

Finally, the diagnostic value of liver biopsy in suspected FNH is rather limited; the lesion itself may not be reached or the specimen may not be sufficient for an accurate diagnosis, and false negative results are deleterious for the final outcome as hepatic adenomas or HCC may bleed or seed along the needle track.

In conclusion, at present there is no clear-cut evidence supporting the potential for malignant degeneration of FNH into HCC; the indication for surgery, particularly in small lesions ($\leqslant$ 4 cm) and asymptomatic patients, is therefore rather controversial. In view of this uncertainty, a correct diagnosis which differentiates between FNH and HCC must be achieved for all cases by means of a multidisciplinary approach. Due to the rarity of the association between FNH and HCC it is difficult to draw solid conclusions for both the pathology of this entity and the appropriate management of these patients.

REFERENCES

1 **Reddy KR**, Kligerman S, Levi J, Livingstone A, Molina E, Franceschi D, Badalamenti S, Jeffers L, Tzakis A, Schiff ER. Benign and solid tumors of the liver: relationship to sex, age, size of tumors, and outcome. *Am Surg* 2001; **67**: 173-178

2 **Finch MD**, Crosbie JL, Currie E, Garden OJ. An 8-year experience of hepatic resection: indications and outcome. *Br J Surg* 1998; **85**: 315-319

3 **Benhamou JP**, Erlinger S. Maladies du foie et des voies biliaires. 3rd ed. Paris: Medicines-Sciences: Flammarion, 1995: 71-90

4 **Colle I**, de Beeck BO, Hoorens A, Hautekeete M. Multiple focal nodular hyperplasia. *J Gastroenterol* 1998; **33**: 904-908

5 **Vilgrain V**, Flejou JF, Arrive L, Belghiti J, Najmark D, Menu Y, Zins M, Vullierme MP, Nahum H. Focal nodular hyperplasia of the liver: MR imaging and pathologic correlation in 37 patients. *Radiology* 1992; **184**: 699-703

6 **Scott LD**, Katz AR, Duke JH, Cowan DF, Maklad NF. Oral contraceptives, pregnancy, and focal nodular hyperplasia of the liver. *JAMA* 1984; **251**: 1461-1463

7 **Knowles DM**, Wolff M. Focal nodular hyperplasia of the liver: a clinicopathologic study and review of the literature. *Hum Pathol* 1976; **7**: 533-545

8 **Chen TC**, Chou TB, Ng KF, Hsieh LL, Chou YH. Hepatocellular carcinoma associated with focal nodular hyperplasia. Report of a case with clonal analysis. *Virchows Arch* 2001; **438**: 408-411

9 **Saul SH**, Titelbaum DS, Gansler TS, Varello M, Burke DR, Atkinson BF, Rosato EF. The fibrolamellar variant of hepatocellular carcinoma. Its association with focal nodular hyperplasia. *Cancer* 1987; **60**: 3049-3055

10 **Vecchio FM**, Fabiano A, Ghirlanda G, Manna R, Massi G. Fibrolamellar carcinoma of the liver: the malignant counterpart of focal nodular hyperplasia with oncocytic change. *Am J Clin Pathol* 1984; **81**: 521-526

11 **Cucchetti A**, Vivarelli M, De Ruvo N, Bellusci R, Cavallari A. Simultaneous presence of focal nodular hyperplasia and hepatocellular carcinoma: case report and review of the literature. *Tumori* 2003; **89**: 434-436

12 **Gaffey MJ**, Iezzoni JC, Weiss LM. Clonal analysis of focal nodular hyperplasia of the liver. *Am J Pathol* 1996; **148**: 1089-1096

13 **Paradis V**, Laurent A, Flejou JF, Vidaud M, Bedossa P. Evidence for the polyclonal nature of focal nodular hyperplasia of the liver by the study of X-chromosome inactivation. *Hepatology* 1997; **26**: 891-895

14 **Wanless IR**, Mawdsley C, Adams R. On the pathogenesis of focal nodular hyperplasia of the liver. *Hepatology* 1985; **5**: 1194-1200

15 **Foster JH**, Berman MM. The malignant transformation of liver cell adenomas. *Arch Surg* 1994; **129**: 712-717

16 **Friedman LS**, Gang DL, Hedberg SE, Isselbacher KJ. Simultaneous occurrence of hepatic adenoma and focal nodular hyperplasia: report of a case and review of the literature. *Hepatology* 1984; **4**: 536-540

17 **Grange JD**, Guechot J, Legendre C, Giboudeau J, Darnis F, Poupon R. Liver adenoma and focal nodular hyperplasia in a man with high endogenous sex steroids. *Gastroenterology* 1987; **93**: 1409-1413

18 **Mathieu D**, Zafrani ES, Anglade MC, Dhumeaux D. Association of focal nodular hyperplasia and hepatic hemangioma. *Gastroenterology* 1989; **97**: 154-157

19 **Di Carlo I**, Urrico GS, Ursino V, Russello D, Puleo S, Latteri F. Simultaneous occurrence of adenoma, focal nodular hyperplasia, and hemangioma of the liver: are they derived from a common origin? *J Gastroenterol Hepatol* 2003; **18**: 227-230

20 **Goldin RD**, Rose DS. Focal nodular hyperplasia of the liver associated with intracranial vascular malformations. *Gut* 1990; **31**: 554-555

21 **Kinjo T**, Aoki H, Sunagawa H, Kinjo S, Muto Y. Congenital absence of the portal vein associated with focal nodular hyperplasia of the liver and congenital choledochal cyst: a case report. *J Pediatr Surg* 2001; **36**: 622-625

22 **Bathgate A**, MacGilchrist A, Piris J, Garden J. Multiple focal nodular hyperplasia in Klippel-Trenaunay syndrome. *Gastroenterology* 1999; **117**: 284-285

23 **Wanless IR**, Albrecht S, Bilbao J, Frei JV, Heathcote EJ, Roberts EA, Chiasson D. Multiple focal nodular hyperplasia of the liver associated with vascular malformations of various organs and neoplasia of the brain: a new syndrome. *Mod Pathol* 1989; **2**: 456-462

24 **Gong L**, Su Q, Zhang W, Li AN, Zhu SJ, Feng YM. Liver cell adenoma: a case report with clonal analysis and literature review. *World J Gastroenterol* 2006; **12**: 2125-2129

25 **Weimann A**, Ringe B, Klempnauer J, Lamesch P, Gratz KF, Prokop M, Maschek H, Tusch G, Pichlmayr R. Benign liver tumors: differential diagnosis and indications for surgery. *World J Surg* 1997; **21**: 983-990; discussion 990-991

26 **Di Stasi M**, Caturelli E, De Sio I, Salmi A, Buscarini E, Buscarini L. Natural history of focal nodular hyperplasia of the liver: an ultrasound study. *J Clin Ultrasound* 1996; **24**: 345-350
27 **Charny CK**, Jarnagin WR, Schwartz LH, Frommeyer HS, DeMatteo RP, Fong Y, Blumgart LH. Management of 155 patients with benign liver tumours. *Br J Surg* 2001; **88**: 808-813
28 **Terkivatan T**, de Wilt JH, de Man RA, van Rijn RR, Zondervan PE, Tilanus HW, IJzermans JN. Indications and long-term outcome of treatment for benign hepatic tumors: a critical appraisal. *Arch Surg* 2001; **136**: 1033-1038
29 **Procacci C**, Fugazzola C, Cinquino M, Mangiante G, Zonta L, Andreis IA, Nicoli N, Pistolesi GF. Contribution of CT to characterization of focal nodular hyperplasia of the liver. *Gastrointest Radiol* 1992; **17**: 63-73
30 **Choi CS**, Freeny PC. Triphasic helical CT of hepatic focal nodular hyperplasia: incidence of atypical findings. *AJR Am J Roentgenol* 1998; **170**: 391-395
31 **Kehagias D**, Moulopoulos L, Antoniou A, Hatziioannou A, Smyrniotis V, Trakadas S, Lahanis S, Vlahos L. Focal nodular hyperplasia: imaging findings. *Eur Radiol* 2001; **11**: 202-212
32 **Brancatelli G**, Federle MP, Grazioli L, Blachar A, Peterson MS, Thaete L. Focal nodular hyperplasia: CT findings with emphasis on multiphasic helical CT in 78 patients. *Radiology* 2001; **219**: 61-68
33 **Cherqui D**, Rahmouni A, Charlotte F, Boulahdour H, Metreau JM, Meignan M, Fagniez PL, Zafrani ES, Mathieu D, Dhumeaux D. Management of focal nodular hyperplasia and hepatocellular adenoma in young women: a series of 41 patients with clinical, radiological, and pathological correlations. *Hepatology* 1995; **22**: 1674-1681

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Meetings

MAJOR MEETINGS COMING UP

First Biennial Congress of the Asian-Pacific Hepato-Pancreato-Biliary Association
March, 2007
Fukuoka, Japan
http://www.congre.co.jp/1st-aphpba

American College of Gastroenterology Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3-8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26-30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
14-16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1-5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

ILTS 12th Annual International Congress
3-6 May 2006
Milan
ILTS
www.ilts.org

Internal Medicine: Gastroenterology
22 July 2006-1 August 2006
Amsterdam
Continuing Education Inc
jbarnhart@continuingeducation.net

6th Annual Gastroenterology And Hepatology
15-18 March 2006
Rio Grande
Office of Continuing Medical Education
cmenet@jhmi.edu
www.hopkinscme.net

World Congress on Gastrointestinal Cancer
28 June 2006-1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6-8 September 2006
Stockholm
European Society of Clinical Microbiology and Infectious Diseases
icsi2006@stocon.se
www.icsi2006.se/9/23312.asp

7th World Congress of the International Hepato-Pancreato-Biliary Association
3-7 September 2006
Edinburgh
Edinburgh Convention Bureau
convention@edinburgh.org
www.edinburgh.org/conference

Society of American Gastrointestinal Endoscopic Surgeons
26-29 April 2006
Dallas - TX
www.sages.org

Digestive Disease Week 2006
20-25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25-26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
3-7 June 2006
Seatlle - Washington
www.fascrs.org

EVENTS AND MEETINGS IN 2006

10th World Congress of the International Society for Diseases of the Esophagus
22-25 February 2006
Adelaide
isde@sapmea.asn.au
www.isde.net

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
Sydney - NSW
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Easl 2006 - the 41st annual
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Digestive Disease Week Administration
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www.cag-acg.org

Prague Hepatology 2006
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12th International Symposium on Viral Hepatitis and Liver Disease
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www.isvhld2006.com/

Falk Seminar: XI Gastroenterology Seminar Week
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European Multidisciplinary Colorectal Cancer Congress 2006
12-14 February 2006
Berlin
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www.colorectal2006.org

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
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Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
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2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci U S A* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900]

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9 Outreach: bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ Cell Tumour Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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