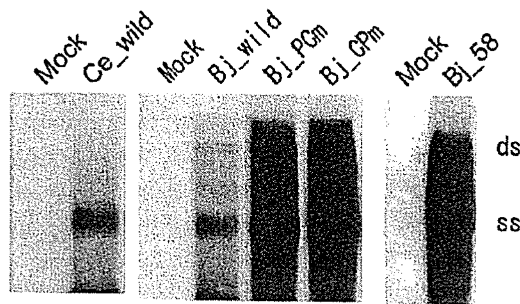

Fig. 3. Southern blot analysis for replicative activity of the wild-type HBV clones (HBV/Ce_wild and Bj_wild), as well as mutants with precore (Bj_PCm) or core-promoter (Bj_CPm) mutation, and Bj_58 with precore stop-codon mutation obtained from a patient with fulminant hepatitis.

densities of migration patterns of the wild-type, precore, and core-promoter mutants in Southern blotting analysis. The wild-type HBV/Bj displayed a band for single-stranded (ss) HBV DNA and an additional band for double-stranded (ds) HBV DNA. Of note, the densities of these bands were far greater for HBV/Bj mutants incorporated with precore or core-promoter mutation, as well as Bj_58 with the precore mutation, thereby indicating much enhanced replicative activity of precore or core-promoter mutant *in vitro*. Although the intracellular HBV DNA level for the wild-type HBV/Bj was comparable with that for the wild-type Ce (Fig. 3), the extracellular HBV DNA level in culture media was approximately threefold higher for Bj than Ce ($P < .01$) (Sugiyama M et al., manuscript in submission).

Discussion

A nationwide survey of genotypes/subgenotypes in patients with acute HBV infection from Japan during the past 2 decades has examined their influence on fulminant and chronic outcomes. The study was feasible in a country where mass vaccination has not been performed because of an extremely high efficacy of immunoprophylaxis on babies born to carrier mothers; it has decreased the persistent HBV carrier rate from 1.4% to 0.3%.[26] Acute HBV infection keeps increasing, however, predominantly through promiscuous sexual contacts in Japan.

Fulminant hepatitis developed rather frequently in 40 of the 301 (13%) patients. This is likely due to selection bias because the study included only patients who were hospitalized for acute hepatitis B. Exclusion of subclinical cases of acute HBV infection would have overestimated the incidence of fulminant hepatitis. Regardless of such a selection bias, influence of HBV genotypes/subgenotypes was evident in comparison with the 40 patients with fulminant and the 261 with acute self-limited hepatitis. Remarkably, none of the 33 patients infected with HBV/Ae developed fulminant hepatitis. In sharp contrast, 12 of the 22 (55%) patients infected with HBV/Bj developed it. Furthermore, both precore (G1896A) and core-promoter (A1762T/G1764A) mutations were detected significantly more frequently in patients with fulminant than acute self-limited hepatitis. In infection with HBV/Bj, in particular, the frequency of core-promoter mutation was much higher in the patients with fulminant (67%) than that reported in those with chronic hepatitis (16%).[27] Precore and core-promoter mutations are very frequent in patients with fulminant hepatitis from Asia[28-30] and the Middle East.[31] The failure in detecting these mutations in Western countries[32-35] could be attributed to frequent HBV/Ae and rare Bj there. In multivariate analysis, HBeAg-negative, HBV/Bj, and the precore stop-codon mutation for G1896A were independent risk factors for the development of fulminant hepatitis (Table 4). Various mutations at nt 1753 for enhanced HBV replication,[36] as well as those adjacent at nt 1754 prevailing in patients with fulminant hepatitis,[37] occurred more frequently in patients with fulminant than acute self-limited hepatitis. Host factors, such as age and total bilirubin, contributed to the development of fulminant hepatitis as well (Table 4).

In vitro replication analysis demonstrated the intracellular HBV DNA level of the wild-type HBV/Bj comparable with that of the wild-type Ce (Fig. 3). The extracellular HBV DNA level of HBV/Bj-clone, however, was much higher than those of the other genotypes, indicating its strong inclination to be secreted from cells (Sugiyama et al., manuscript in submission). Such a high concentration of HBV/Bj in the circulation of patients would rapidly and extensively promote infection of hepatocytes.

Enhanced replication capacities of precore (G1896A) and core-promoter (A1762T/G1764A) mutants for HBeAg-minus and -reduced phenotypes, respectively, were demonstrated in a replication model *in vitro* (Fig. 3). These observations were concordant with those in previous reports[38,39]; however no data are available on the replication of HBV/Bj *in vitro*, either of the wild-type or variants with these mutations. Extremely high intracellular and extracellular expressions of viral DNA were observed for the HBV/Bj clone with precore stop-codon mutation from a patient with fulminant hepatitis. These results might implicate high replication due to mutations of precore region and core-promoter in the induction of fulminant hepatitis. In support of this view, Bocharov et al.[40] have proposed that enhanced HBV replication would efficiently stimulate immune reactions, represented by the cytotoxic T lymphocyte response, suggesting that enhanced replication by HBV/Bj or precore/

core-promoter mutation might lead to fulminant hepatitis.

That HBV DNA levels were lower in patients with fulminant than acute hepatitis, despite a high replication capacity of HBV/Bj incriminated in the development of fulminant hepatic failure, may seem surprising. Because destruction of hepatocytes proceeds swiftly in patients with fulminant hepatitis, hepatic mass for HBV to thrive would have been extremely reduced in them at presentation. As a consequence, some patients with fulminant hepatitis B are without serum HBsAg; they are diagnosed by high-titered IgM anti-HBc.[41] On the contrary, HBV DNA levels were higher in the patients with HBV/Ae than Bj (Table 1); those with Ae tend to delay reducing HBV DNA, some of whom have chronic outcome. Combined, correlating HBV DNA levels with the clinical outcome in acute HBV infection would be difficult.

A wide variation has been seen in the rate of persistence after acute HBV infection in adulthood. No chronic outcomes of acute hepatitis B were seen in female recipients of red blood cells contaminated with HBV (0/28)[42] or patients in an acupuncture-associated outbreak (0/35).[43] In marked contrast, they ranged from 0.2% (14/715) in Greece[44] through 2.7% (1/37) in university students in Taiwan[45] to 10.4% (5/8) in Alaskan Eskimos[46] and 12.1% (7/58) in Germany.[47] HBV genotypes are implicated in a high rate of persistence in European countries where HBV/A is predominant.[48] In Japan, also, adulthood infection tends to persist longer with HBV/A than B or C (23% 3/13 vs. 13% 1/8 or 12% 3/25).[49] In the current series on 256 patients with acute hepatitis B in Japan who were followed rigorously, HBV infection persisted in only three (1%), representing 2 of the 32 (6%) with HBV/Ae and 1 of the 21 (5%) with Ba. Hence, 99% of patients lost their HBsAg by 6 months. Persistence of HBV observed in the patients with HBV/Ae (6%) is less frequent than that in 4 of the 31 (13%) patients with Ae from a hospital in metropolitan Tokyo.[49] The difference would be ascribable, at least in part, to lamivudine given to some patients in this study (18%). All patients treated with lamivudine recovered from acute hepatitis, whereas none of the three patients with chronic outcome had received antiviral treatment during their acute phase of illness, indicating that lamivudine might be able to prevent the chronic outcome. Likewise, some patients from metropolitan Tokyo, in whom HBV persisted,[49,50] had received immunosuppressants in the acute phase of infection before referral to their hospital.

Using cell culture and chimeric mice models for the replication system of different genotype/subgenotype clones, we have observed that the replication of HBV is the highest for HBV/Bj or C and the lowest for Aa/Ae (Sugiyama M et al., manuscript in submission). It is probable that the propensity of HBV/A infection to chronicity would be due to less intensive immune response against its slow viral dynamics. Taken together, the infection with HBV/A appears to persist longer than those with the other genotypes; this needs to be confirmed by further investigation in patients from various countries.

In conclusion, persistence of HBV after acute infection is rare and occurs more often in patients infected with HBV/Ae than others. Fulminant outcome is frequent in hospitalized patients and associated with HBV/Bj accompanied by the lack of serum HBeAg as well as high replication due to precore stop-codon mutation (G1896A), a finding supported by an *in vitro* replication model.

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Liver International 2006: 26: 90–96

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Liver International
DOI: 10.1111/j.1478-3231.2005.01200.x

Clinical Studies

Measurement of hepatitis B virus core-related antigen is valuable for identifying patients who are at low risk of lamivudine resistance

Tanaka E, Matsumoto A, Suzuki F, Kobayashi M, Mizokami M, Tanaka Y, Okanoue T, Minami M, Chayama K, Imamura M, Yatsuhashi H, Nagaoka S, Yotsuyanagi H, Kawata S, Kimura T, Maki N, Iino S, Kiyosawa K, HBV Core-Related Antigen Study Group. Measurement of hepatitis B virus core-related antigen is valuable for identifying patients who are at low risk of lamivudine resistance.
Liver International 2006: 26: 90–96. © Blackwell Munksgaard 2005

Abstract: *Objective:* The clinical usefulness of hepatitis B virus core-related antigen (HBVcrAg) assay was compared with that of HBV DNA assay in predicting the occurrence of lamivudine resistance in patients with chronic hepatitis B. *Patients:* Of a total of 81 patients who were treated with lamivudine, 25 (31%) developed lamivudine resistance during a median follow-up period of 19.3 months. *Results:* The pretreatment positive rate of HBe antigen, or pretreatment levels of HBVcrAg or HBV DNA did not differ between patients with and without lamivudine resistance. Levels of both HBVcrAg and HBV DNA decreased after the initiation of lamivudine administration; however, the level of HBVcrAg decreased significantly more slowly than that of HBV DNA. The occurrence of lamivudine resistance was significantly less frequent in the 56 patients whose HBV DNA level was less than 2.6 log copy/ml at 6 months of treatment than in the remaining 25 patients. The cumulative rate of lamivudine resistance was as high as 70% within 2 years in the latter group, while it was only 28% in the former group. Lamivudine resistance did not occur during the follow-up period in the 19 patients whose HBVcrAg level was less than 4.6 log U/ml at 6 months of treatment, while it did occur in 50% of the remaining patients within 2 years. *Conclusion:* These results suggest that measurement of HBV DNA is valuable for identifying patients who are at high risk of developing lamivudine resistance, and that, conversely, measurement of HBVcrAg is valuable for identifying those who are at low risk of lamivudine resistance.

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Key words: chronic hepatitis B – HBV core-related antigen – HBV DNA – lamivudine resistance

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Received 19 June 2005,
accepted 8 August 2005

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Lamivudine, a nucleoside analogue that inhibits reverse transcriptases, was first developed as an anti-viral agent against human immunodeficiency virus (HIV). It was later also found to be effective against hepatitis B virus (HBV) because HBV is a member of the Hepadnaviridae family of viruses, which use reverse transcriptases in their replication process (1, 2). Lamivudine was found to inhibit the replication of HBV, reduce hepatitis, and improve histological findings of the liver in long-term treatment (3–5). Furthermore, it has been shown that lamivudine treatment improves the long-term outcome of patients with chronic hepatitis B (6, 7). However, there are a number of problems with lamivudine therapy, such as relapse of hepatitis because of the appearance of YMDD mutant viruses and the reactivation of hepatitis after discontinuation of the treatment (8–11).

The concentration of HBV DNA in serum decreases and usually becomes undetectable during lamivudine administration, but it rapidly increases when HBV becomes resistant to lamivudine. Thus, the measurement of HBV DNA is useful for monitoring the anti-viral effects of lamivudine. However, a negative result of HBV DNA in serum does not necessarily indicate a good outcome of lamivudine therapy, because lamivudine resistance may occur even if HBV DNA levels remain undetectable during therapy (11–13). Recently, a chemiluminescence enzyme immunoassay (CLEIA) was developed in our laboratory for the detection of hepatitis B virus core-related antigen (HBVcrAg) (14, 15). The assay reflects the viral load of HBV in a similar manner to that used in assays, which detect HBV DNA. HBVcrAg consists of HBV core and e antigens; both proteins are transcribed from the precore/core gene and their first 149 amino acids are identical (16–18). The HBVcrAg CLEIA simultaneously measures the serum levels of hepatitis B core (HBc) and e (HBe) antigens, using monoclonal antibodies, which recognize common epitopes of these two denatured antigens. In the present study, we analyzed the clinical significance of the HBVcrAg assay in monitoring the anti-viral effects of lamivudine treatment.

Patients and methods

Patients

A total of 81 patients with chronic hepatitis B, who received lamivudine therapy, were enrolled in the present study. These were 58 men and 23 women with a median age of 49 years (range 24–79 years). The 81 patients were selected retrospectively from six medical institutions in Japan (Shinshu University Hospital, Toranomon Hospital, Nagoya City University Hospital, Kyoto Prefectural University Hospital, Hiroshima University Hospital, National Nagasaki Medical Center). Eight to 25 patients who met the following three criteria were selected consecutively in each institution: the first, a daily dose of 100 mg lamivudine was administered for at least 6 months in a period from 1999 to 2004; the second, histologically confirmed for chronic hepatitis without liver cirrhosis; and the third, serum samples at several time points available for testing. All patients were naive for lamivudine therapy. Chronic hepatitis B was defined as positive hepatitis B surface (HBs) antigen for more than 6 months with elevated levels of serum transaminases. The HBV genotype was A in two patients, B in three and C in 76. Serum HBV DNA was detectable in all patients, and HBe antigen was positive in 51 (63%) of the 81 patients just before lamivudine administration. The median follow-up period was 19 months with a range from 6 to 50 months. Follow-up of patients ended when lamivudine administration was discontinued. Written informed consent was obtained from each patient.

The occurrence of lamivudine resistance was defined as a rapid increase in serum HBV DNA levels with the appearance of the YMDD mutations during lamivudine administration. Using this criteria, resistance appeared in 27 (33%) of the 81 patients. The median period from the start of lamivudine administration to the occurrence of resistance was 12 months with a range from 4 to 37 months.

Serological markers for HBV

HBs antigen, HBe antigen and anti-HBe antibody were tested using commercially available enzyme immunoassay kits (Abbott Japan Co., Ltd., Tokyo, Japan). Six major genotypes (A–F) of HBV can be detected using the method reported by Mizokami et al. (19), in which the surface gene sequence amplified by polymerase chain reaction (PCR) is analyzed by restriction fragment length polymorphism. The YMDD motif, that is, lamivudine resistant mutations in the active site of HBV polymerase, was detected with an enzyme-linked mini-sequence assay kit (HBV YMDD Mutation Detection Kit, Genome Science Laboratories Co., Ltd., Tokyo, Japan) (20).

Serum concentration of HBV DNA was determined using Amplicor HBV monitor kit (Roche, Tokyo, Japan), which had quantitative range from 2.6 to 7.6 log copy/ml. Sera containing

over 7.0 log copy/ml HBV DNA were diluted 10- or 100-fold with normal human serum and re-tested to obtain the end titer.

Serum concentrations of HBVcrAg were measured using the CLEIA method reported previously (10, 11). Briefly, 100 µL serum was mixed with 50 µL pretreatment solution containing 15% sodium dodecylsulfate and 2% Tween 60. After incubation at 70 °C for 30 min, 50 µL pretreated serum was added to a well coated with monoclonal antibodies against denatured HBc and HBe antigens (HB44, HB61 and HB114) and filled with 100 µL assay buffer. The mixture was incubated for 2 h at room temperature and the wells were then washed with buffer. Alkaline phosphatase-labeled monoclonal antibodies against denatured HBc and HBe antigens (HB91 and HB110) were added to the well, and the mixture was incubated for 1 h at room temperature. After washing, CDP-Star with Emerald II (Applied Biosystems, Bedford, MA) was added and the plate was incubated for 20 min at room temperature. The relative chemiluminescence intensity was measured, and the HBVcrAg concentration was determined by comparison with a standard curve generated using recombinant pro-HBe antigen (amino acids, 10–183 of the precore/core gene product). The HBVcrAg concentration was expressed as units/ml (U/ml) and the immunoreactivity of recombinant pro-HBe antigen at 10 fg/ml was defined as 1 U/ml. In the present study, the cutoff value was tentatively set at 3.0 log U/ml. Sera containing over 7.0 log U/ml HBVcrAg were diluted 10- or 100-fold in normal human serum and re-tested to obtain the end titer.

Statistical analysis

The Mann–Whitney *U*-test and Wilcoxon signed-ranks test were utilized to analyze quantitative data, and Fisher's exact test was used for qualitative data. A log-rank test was used to compare the occurrence of lamivudine resistance. Statistical analyses were performed using the SPSS 5.0 statistical software package (SPSS, Inc., Chicago, IL). A *P*-value of less than 0.05 was considered to be statistically significant.

Results

Table 1 shows a comparison of the clinical and virological backgrounds of the 27 patients who showed lamivudine resistance and the 54 patients who did not. Median age, gender distribution and median follow-up period did not differ between the two groups, and the positive rate of HBe antigen was similar. Both HBV DNA and HBVcrAg levels at the beginning of lamivudine administration were similar between the two groups; however, both HBV DNA and HBVcrAg levels at 6 months after the start of lamivudine administration were significantly lower in the lamivudine resistance negative group than in the positive group. ALT level was normal at the beginning in eight (15%) of the 54 patients without lamivudine resistance and in two (7%) of the 27 patients with it ($P>0.2$).

Table 1. Comparison of the clinical and virological backgrounds of patients who showed lamivudine resistance and those who did not

Characteristics	Appearance of lamivudine resistance: Negative ($n=54$)	Positive ($n=27$)	P
Age (years)*	47.0 (24–79)	50.6 (34–67)	0.140†
Gender (male %)	74%	67%	>0.2‡
Follow-up period (months)*	16 (6–50)	21 (9–43)	>0.2†
HBV genotype (A/B/C)	2/2/50	0/1/26	>0.2‡
HBe antigen (positive %)	59%	70%	>0.2‡
ALT (IU/ml)*			
Initial	85 (22–713)	95 (20–1140)	>0.2†
At 6 months	27 (11–115)	30 (15–92)	>0.2†
HBV DNA (log copy/ml)*			
Initial	7.0 (3.5–9.1)	7.3 (4.2–9.2)	>0.2†
At 6 months	<2.6 (<2.6–4.8)	3.3 (<2.6–6.6)	<0.001†
HBVcrAg (log U/ml)*			
Initial	6.2 (<3.0–8.8)	7.3 (4.4–9.1)	0.073†
At 6 months	5.2 (<3.0–6.7)	5.8 (4.7–8.4)	<0.001†

HBe antigen, hepatitis B e antigen; HBV, hepatitis B virus; ALT, alanine aminotransferase; HBVcrAg, HBV core-related antigen. *Data are expressed as median (range). †Mann–Whitney *U* test. ‡χ^2-test.

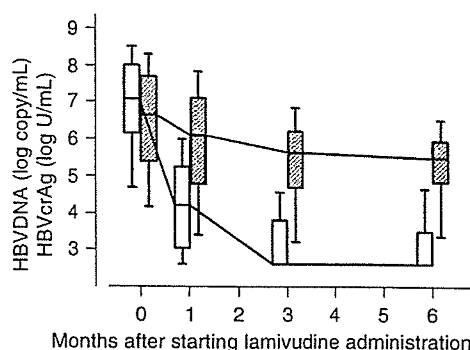

Fig. 1. Changes in the median levels of hepatitis B virus core-related antigen (HBVcrAg) and hepatitis B virus (HBV) DNA during lamivudine administration. The box plots show the 10th, 25th, 50th, 75th and 90th percentiles, with the open boxes indicating HBV DNA and shaded boxes indicating HBVcrAg. The median amount of decrease from the baseline in HBVcrAg levels was significantly smaller (Wilcoxon signed-ranks test) than that in HBV DNA level at 1 (2.80 log copy/ml vs. 0.27 log U/ml, $P<0.001$), 3 (3.60 log copy/ml vs. 0.83 log U/ml, $P<0.001$) and 6 months (3.90 log copy/ml vs. 1.15 log U/ml, $P<0.001$) after the initiation of lamivudine administration.

Figure 1 shows changes in HBV DNA and HBVcrAg levels during lamivudine treatment in all patients. The level of HBV DNA decreased rapidly and became undetectable at 3 months after treatment was initiated. On the other hand, although HBVcrAg levels decreased continuously, the median amount of decrease from the base-line was significantly lower than that in HBV DNA levels at 1, 3 and 6 months after starting lamivudine administration (Wilcoxon signed-ranks test, $P<0.001$ at all analyzed points in time).

Changes in HBV DNA and HBVcrAg levels during lamivudine administration are compared in Fig. 2 between the 27 patients who showed lamivudine resistance and the 54 patients who did not. Serum HBV DNA levels were found to decrease rapidly and become undetectable within 6 months in 45 (83%) of the 54 patients without lamivudine resistance. On the other hand, only 11 (41%) of the 27 patients with lamivudine resistance showed a similar rapid decrease, and the HBV DNA levels of the remaining patients stayed above the detection limit during the follow-up period. HBVcrAg levels decreased but did not reach levels lower than 4.7 log U/ml (5000 U/ml) in the 27 patients with lamivudine resistance. In 19 (35%) of the 54 patients without lamivudine resistance, on the other hand, the levels decreased to levels below 4.7 log U/ml within 6 months after the start of lamivudine administration. The level of HBVcrAg increased rapidly as did the level of HBV DNA when lamivudine resistance occurred.

The occurrence of lamivudine resistance was significantly less frequent in the 56 patients whose HBV DNA level was less than 2.6 log copy/ml at 6 months after the initiation of treatment than in the remaining 25 patients (Fig. 3). The cumulative occurrence of lamivudine resistance was as high as 70% within 2 years in the latter group, while it was only 28% in the former group. There was no occurrence of lamivudine resistance during the follow-up period in the 19 patients whose HBVcrAg levels were less than 4.6 log U/ml at 6 months after the initiation of lamivudine therapy (Fig. 3). On the other hand, lamivudine resistance occurred in 50% of the remaining patients within 2 years.

Discussion

The HBVcrAg assay is a unique assay, which measures the amounts of e and core antigens

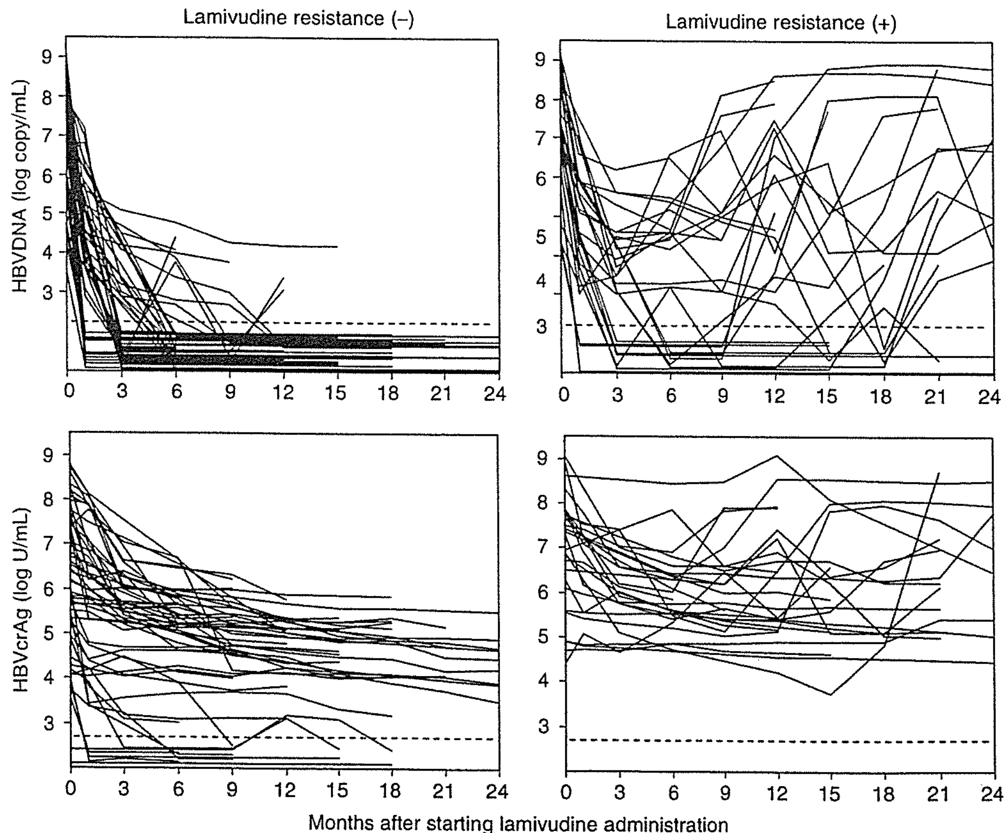

Fig. 2. Comparison of changes in serum hepatitis B virus (HBV) DNA and serum HBV core-related antigen (HBVcrAg) levels between patients who showed lamivudine resistance and those who did not. The broken lines indicate the detection limit of each assay.

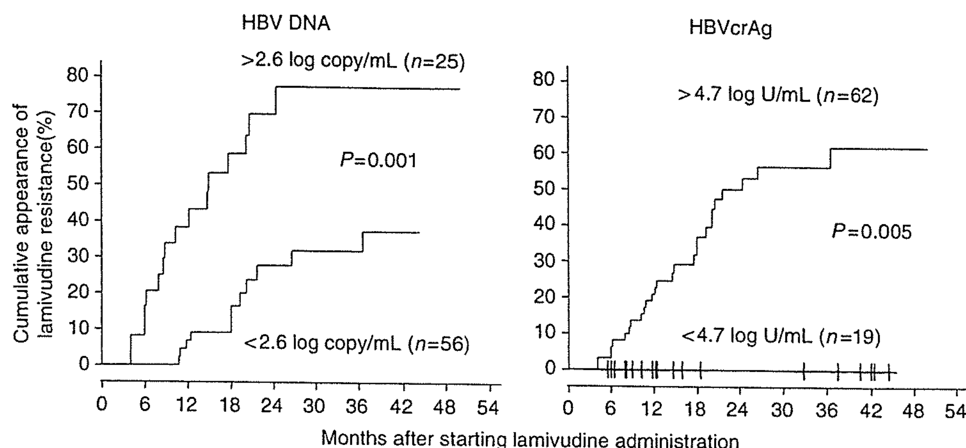

Fig. 3. Comparison of the cumulative occurrence of lamivudine resistance between patients who showed hepatitis B virus (HBV) DNA levels of less than the detection limit (2.6 log copy/ml) at 6 months after starting lamivudine administration and those who did not (left figure), and similarly between patients who showed HBV core-related antigen (HBVcrAg) levels of less than 4.7 log U/ml and those who did not (right figure).

coded by the core gene of the HBV genome with high sensitivity and a wide quantitative range. Serum HBVcrAg levels reflect the viral load in the natural course because these levels correlate linearly with those of HBV DNA (14, 15). On the other hand, the character of HBVcrAg is somewhat different from that of HBV DNA in patients undergoing anti-viral therapies such as lamivudine. That is, HBVcrAg levels decrease significantly more slowly than those of HBV DNA after the initiation of lamivudine administration.

HBV is an enveloped DNA virus containing a relaxed circular DNA genome, which is converted into a covalently closed circular DNA (cccDNA) episome in the nucleus of infected cells (18, 21–23). The cccDNA molecules serve as the transcriptional template for the production of viral RNAs that encode viral structural and nonstructural proteins. Reverse transcription of the viral pregenomic RNA and second-strand DNA synthesis occur in the cytoplasm within viral capsids formed by the HBV core protein. Because lamivudine, a nucleoside analogue, inhibits reverse transcription of the pregenomic RNA, it directly suppresses the production of HBV virion. Thus, serum HBV DNA levels decrease rapidly after the initiation of lamivudine administration. On the other hand, the production of viral proteins is not suppressed by lamivudine because the production process does not include reverse transcription. Furthermore, it has been reported that the amount of cccDNA, which serves as a template for mRNA, decreases quite slowly after starting the administration of nucleoside analogues (24–26). Thus, it is reasonable that serum HBVcrAg levels decrease much more slowly than HBV DNA levels after the initiation of lamivudine therapy.

Significant markers that can predict the presence or absence of lamivudine resistance are clinically valuable because the emergence of this resistance and the subsequent recurrence of hepatitis are fundamental problems in lamivudine therapy. Serum markers that reflect the activity of HBV replication have been reported to be associated with the occurrence of lamivudine resistance (11, 12, 27, 28). However, neither the pretreatment existence of HBe antigen nor pretreatment levels of HBV DNA or HBVcrAg were found to be significant markers in the present study. These results may reflect a weak association between the pretreatment activity of HBV replication and the occurrence of lamivudine resistance (13, 29). Changes in HBV DNA and HBVcrAg levels after starting lamivudine administration clearly differed between patients with and without lamivudine resistance. Thus, HBV DNA and HBVcrAg levels at 6 months after starting lamivudine administration were analyzed to determine whether these levels might serve as predictive markers; both were found to be significantly lower in patients without lamivudine resistance at the tested point in time. Furthermore, patients who showed higher levels of HBV DNA and HBVcrAg at 6 months after the initiation of treatment were significantly more likely to develop lamivudine resistance than those who showed lower levels.

We believe that the measurement of HBV DNA levels is useful to identify patients who are at high risk for lamivudine resistance because as many as 70% of patients who were positive for HBV DNA at 6 months after starting lamivudine

administration developed lamivudine resistance within 2 years. However, a negative result of HBV DNA at 6 months does not necessarily guarantee the absence of lamivudine resistance because nearly 30% of such patients developed resistance within 2 years. On the other hand, HBVcrAg levels of less than 4.7 log U/ml at 6 months are a useful indicator of patients who are unlikely to develop lamivudine resistance, because no such patients developed resistance during the follow-up period in the present study. Lower serum HBVcrAg levels may reflect lower levels of cccDNA in hepatocytes because the mRNAs of HBVcrAg are transcribed from the cccDNA (18, 22, 23). This possibility may explain our finding that patients whose HBVcrAg levels decreased sufficiently were unlikely to develop lamivudine resistance, because cccDNA provides the templates for viral and pregenomic messenger RNA (18, 22, 23), which may be a source of lamivudine-resistant strains.

In conclusion, our results suggest that measurement not only of HBV DNA but also of HBVcrAg is useful for predicting the occurrence of lamivudine resistance. HBV DNA measurement is valuable for identifying patients who are at high risk of developing this resistance and HBcrAg measurement is valuable for identifying those who are at low risk.

Acknowledgements

This research was supported in part by a research grant on hepatitis from the Ministry of Health, Labour and Welfare of Japan.

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Increasing hepatitis C virus-associated hepatocellular carcinoma mortality and aging: Long term trends in Japan

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Received 13 June 2005; received in revised form 10 November 2005; accepted 28 November 2005

Abstract

Background: The incidence of hepatocellular carcinoma (HCC) in Japan has been increasing. The aim of the study was to determine the epidemiological trends in HCC mortality in Japan.

Methods: We reviewed the medical records of all patients whose death was caused by liver disease between 1981 and 2000 at two hospitals. The courses of death were separated based on presence or absence of HCC when death ensued. Additionally, cohorts of patients with HCC were analyzed in 5-year time periods.

Results: The number of deaths from hepatitis C virus (HCV)-associated HCC steadily increased 2.6 times from 49 to 128 during observation period. The mean age at death from HCV-associated HCC from 1996 to 2000 was significantly higher than that in the period from 1981 to 1985 ($p < 0.0001$).

Interpretation: Deaths from HCV-associated HCC increased from 1981 to 2000, consistent with the aging of the population in Japan.

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Keywords: Hepatitis C virus; Hepatocellular carcinoma; Epidemiological

1. Introduction

Hepatocellular carcinoma (HCC) affects approximately half a million people each year worldwide, making it the fifth most common malignancy in men and the ninth most common in women [1–7]. Recently, a trend of increasing rates of HCC has been reported from several developed countries in North America, Europe and Asia [1–9], and the incidence of primary liver cancer in Japan has been increasing over the past four decades [10,11]. HCC often develops in patients with liver cirrhosis caused by hepatitis C virus (HCV), hepatitis B virus (HBV) or excessive alcohol consumption.

Of the hepatitis viruses that cause HCC, HCV is more common than HBV in Japan [12–15]. Although the age-adjusted incidence rates of HCC have been increasing during the period of rising HCC mortality, the temporal and demographic features of survival for HCC patients in Japan are unknown. Hence, we have analyzed these trends over time, using information from two independent databases that deal with HCC in Japan.

2. Patients and methods

We reviewed the medical records of all patients who died from liver disease and received medical care between 1981 and 2000 at the Liver Disease Center, National Nagasaki Medical Center and at The First Department of Internal

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doi:10.1016/j.hepres.2005.11.007

Medicine, Nagasaki University School of Medicine. A total of 1001 patients were studied. All the patients were followed-up after diagnosis until death in one of the two hospitals and we were able to confirm their date of death and that death had occurred after severe liver disease.

All patients were entered into this study because sera were stored at −80 °C. These sera were used to assay HBV or HCV infection. A diagnosis of chronic HCV infection was based on the presence of anti-HCV antibody and HCV-RNA detected by polymerase chain reaction (PCR), whereas diagnosis of chronic HBV infection was based on the presence of hepatitis B surface antigen (HBsAg) or anti-hepatitis B core antigen (anti-HBc) reactivity. Diagnosis of HCC was based on histological findings or on characteristic images in dynamic computed tomography, dynamic magnetic resonance imaging and hepatic angiography. Demographic information, including age at death, sex and year of death, was collected from the patients' chart. Excessive alcohol consumers (an alcohol consumption of >50 g/day for 5 years) were not including in this study.

The courses of death were separated into those occurring with or without HCC when death ensued. Additionally, the patients with HCC were analyzed in 5 yearly intervals (1981–1985, 1986–1990, 1991–1995 and 1996–2000). Patients were classified according to 5-year age groups, and by HBV or HCV infection, and the number of patients in each age group with HBV- or HCV-associated HCC was calculated in each time period.

The SAS computer program for Windows was used to perform statistical analysis of the data, using analysis of variance (ANOVA).

3. Results

A total of 1001 patients died at the Liver Disease Center, National Nagasaki Medical Center and at The First Department of Internal Medicine, Nagasaki University School of Medicine from 1981 to 2000. The patients with HBV-associated HCC were 73.7% (210 of 285) in HBV-related disease and the patients with HCV-associated HCC were 84.5% (381 of 451) in HCV-related disease. There were 653 patients with HCC died. The mean time during followed-up were 2.5 years. The proportion of patients diagnosed with HBV-associated HCC was 32% (210 of 653), whereas 58% (381 of 653) had HCV-associated HCC, and an additional 2% (12 of 653) had HCC associated with both viruses (Table 1).

From 1981 to 2000, 210 patients died of HBV-associated HCC, whereas 381 died of HCV-associated HCC. Table 2 shows the number and the mean age at death from HBV- or HCV-associated HCC during the 5-year periods 1981–1985, 1986–1990, 1991–1995 and 1996–2000. The number of deaths from HBV-associated HCC was not changed within the range from 49 to 58 during the four 5-year periods: 54 (1981–1986), 49 (1986–1990), 49 (1991–1995) and 58 (1996–2000), and the mean age at death was not also statistically significantly different among the periods: 55.4 ± 9.9 (1981–1985), 55.6 ± 10.3 (1986–1990), 55.5 ± 10.6 (1991–1995) and 59.3 ± 10.2 (1996–2000). In contrast, the number of deaths from HCV-associated HCC steadily increased 2.6 times from 49 to 128 during same observation period: 49 (1981–1986), 90 (1986–1990), 114

Table 1
Course of death from 1981 to 2000

	HBV	HCV	Overlap	Others	Total
HCC (%)	210 (32)	381 (58)	12 (2)	50 (8)	653 (100)
Chronic liver failure	47	35	1	36	119
GI bleeding	8	17	1	13	39
Other disease	3	5	0	16	24
Acute liver failure	10	1	3	19	33
Other cancer	7	12	0	114	133
Total (%)	285 (28)	451 (45)	17 (2)	248 (25)	1001 (100)

HCC, hepatocellular carcinoma; GI bleeding, gastrointestinal bleeding; HBV, hepatitis B virus; HCV, hepatitis C virus; overlap, both HBV and HCV positive; other, both HBV and HCV negative.

Table 2
Mean age of KBV associated HCC deaths

Year	1981 - 1985	1986 - 1990	1991 - 1995	1996 - 2000	total
Number	54	49	49	58	210
Mean age (y.o.)	55.4	55.6	55.5	59.3	56.8
SD	9.9	10.3	10.6	10.2	10.3

NS NS NS
NS
NS
NS

Mean age of HCV-associated HCC deaths

Year	1981 - 1985	1986 - 1990	1991 - 1995	1996 - 2000	total
Number	49	90	114	128	381
Mean age (y.o.)	60.0	63.0	64.1	67.0	64.3
SD	8.1	7.0	7.2	7.9	7.8

NS NS 0.0267
0.0176
0.0016
< 0.0001

HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; S.D., standard deviation; NS, not significant.

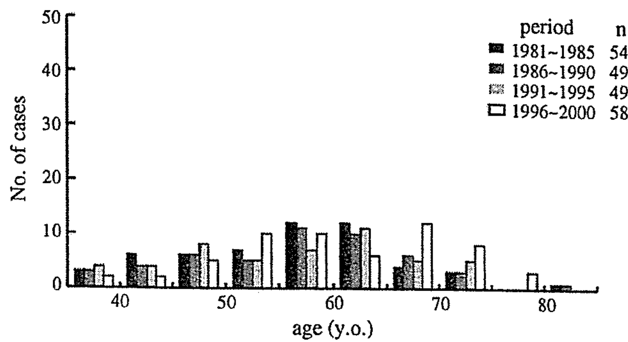

Fig. 1. Age distribution of the total number of deaths from hepatitis B virus-associated hepatocellular carcinoma from 1981 to 2000. There was no change of number of patients and age distribution of patients who died from hepatitis B virus-associated hepatocellular carcinoma during the four time periods.

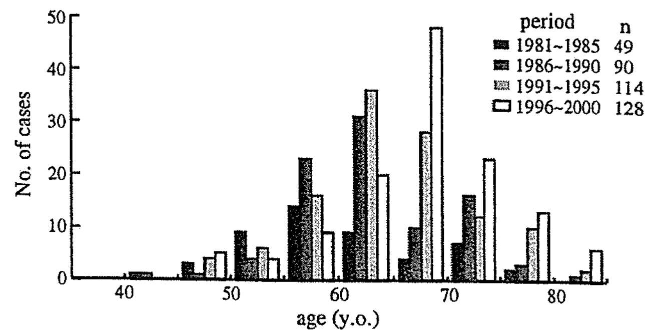

Fig. 2. Age distribution of the total number of deaths from hepatitis C virus-associated hepatocellular carcinoma from 1981 to 2000. The number of death from HCV associated HCC has increased 2.6 times during recent 20 years and this increase was provided by a close association with older shift of age distribution.

(1991–1995) and 128 (1996–2000). In addition, the mean age at death from HCV-associated HCC also increased over time. The mean age at death from 1996 to 2000 (67.0 ± 7.9 years old) was significantly higher than that from 1981 to 1985 (60.0 ± 8.1) ($p < 0.0001$), 1986 to 1990 (63.0 ± 7.0) ($p = 0.0016$) and 1991 to 1995 (64.1 ± 7.2) ($p = 0.0267$), respectively.

Fig. 1 shows the age distribution for deaths from HBV-associated HCC during the four 5-year periods. There was no change of number of patients and age distribution for deaths from HBV-associated HCC during these periods. In contrast, Fig. 2 shows the age distribution for deaths from HCV-associated HCC during the four 5-year periods. The number of patients with HCV-associated HCC aged more than 60 years in 1981–1985, 1986–1990, 1991–1995 and 1996–2000 were 22, 61, 88 and 110 patients, respectively. Fig. 2 indicated that the number of death from HCV associated HCC has increased during recent 20 years and this increase was provided by a close association with older shift of age distribution.

Table 3 shows the age distribution of HCC deaths in 5-year period (1981–1985, 1986–1990, 1991–1995 and 1996–2000). The number of patients with HCV-associated HCC obviously had an increase in the ratio of patients aged more than 60 years ($p < 0.0001$): 18.6% (1981–1985), 37.9% (1986–1990), 51.2% (1991–1995) and 54.4% (1996–2000). There was a significant difference of age distribution in the patients with HCV-associated HCC between aged more than and less than 60 years old in each 5-year period ($p < 0.0001$). In contrast, there was no difference in the age distribution of patients with other types of during these periods.

Fig. 3 shows the ratio between HCV-associated deaths and HBV-associated HCC deaths in 5-year period (1981–1985, 1986–1990, 1991–1995 and 1996–2000). The ratio between HCV-associated HCC and HBV-associated HCC increased and reached a plateau during the observation period: 0.9 (1981–1985), 1.8 (1986–1990), 2.3 (1991–1995) and 2.2 (1996–2000) (1981–1985 versus 1991–1995, $p = 0.0030$; 1981–1985 versus 1996–2000, $p = 0.0042$). Above all, the ratio of patients aged more than 60 years old increased during the observation period: 1.1 (1981–1985), 3.0 (1986–1990), 4.2 (1991–1995) and 3.8 (1996–2000) (1981–1985 versus

Table 3
Age distribution of HCC deaths in 5-year period

Age (y.o.)	1981–1985, no. (%)	1986–1990, no. (%)	1991–1995, no. (%)	1996–2000, no. (%)	*p*-Value
HBV					
<60	34 (28.8)	29 (18.0)	28 (16.3)	29 (14.4)	NS
>60	20 (17.0)	20 (12.5)	21 (12.2)	29 (14.4)	
HCV					
<60	27 (22.9)	29 (18.0)	26 (15.1)	18 (8.9)	<0.0001
>60	22 (18.6)	61 (37.9)	88 (51.2)	110 (54.4)	
Overlap					
<60	1 (0.9)	3 (1.9)	2 (1.2)	1 (0.5)	NS
>60	0	2 (1.2)	0	3 (1.5)	
Other					
<60	5 (4.2)	2 (1.2)	4 (2.3)	2 (1.0)	NS
>60	9 (7.6)	15 (9.3)	3 (1.7)	10 (4.9)	
Total	118 (100)	161 (100)	172 (100)	202 (100)	

HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; overlap, both HBV and HCV positive; other, both HBV and HCV negative.

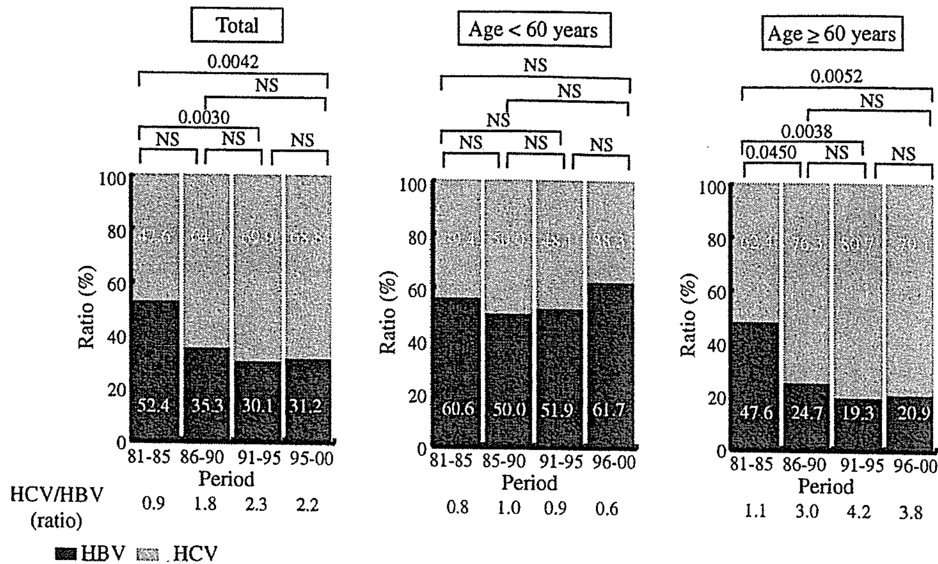

Fig. 3. Ratio between hepatitis C virus-associated hepatocellular carcinoma deaths and hepatitis B virus-associated hepatocellular carcinoma from 1981 to 2000. The ratio between HCV-associated HCC and HBV-associated HCC increased and reached a plateau during the observation period.

1986–1990, $p=0.0450$; 1981–1985 versus 1991–1995, $p=0.0038$; 1981–1985 versus 1996–2000, $p=0.0052$). In contrast, there was no difference in the ratio of patients aged more than 60 years old of during these periods.

4. Discussion

HCC accounts for approximately 6% of all human cancers. It is estimated that half a million cases occur annually worldwide, making HCC the fifth most common malignancy in men and the ninth in women [1–7,9]. The age-adjusted mortality rate from HCC has increased over the past decades in Japan [16], and in the current study more than 90% of deaths from HCC were HBV- and/or HCV-related and the number of deaths from HCV-associated HCC apparently increased 2.6 times from 1981 to 2000, and the mean age of deaths from HCV-associated HCC also significantly rose. During the same period, the number and the age distribution of deaths from HBV-associated HCC remained unchanged. The increase in the number of deaths from HCV-associated HCC seemed to be closely associated with the shift of age distribution of HCV infected population between 1981 and 2000. Although our data had the limitations of applying the findings from two hospitals to a general population, Kiyosawa described that deaths due to HCC in Japan have continued to increase in males, particularly in those older than 60 years of age between 1982 and 2003. This also suggests that the average age of diagnosis of HBV-related HCC was similar in all three time periods. In contrast, the average age of patients with HCV-related HCC rose from 61.6 years in 1982 to 63.1 years in 1990 and 67.8 years in 2003 [11]. The research group for population-based cancer registration in Japan described that incidence of HCC in Japan have continued to increase and reached a plateau in males and female from 1975 to 1999. Above all, the age distribution incidence and incident rate of HCC reached a peak older than 65 years old in males and female [17]. And, this study suggested that the ratio between HCV-associated HCC and HBV-associated HCC increased and reached a plateau from 1981 to 2000, especially more than 60 years old. Where did these findings and difference of HCC development between HCV and HBV, which were considered to be both oncogenic virus after long-term persistent infection with inflammation and fibrotic change in the liver but popular hepatitis virus infections in Japan, come from?

The simple reason may be explained as follows. From 1981 to 2000, mortality from a variceal hemorrhage in cirrhotic patients has declined [9,18]. Long term nutritional supplementation with oral branched-chain amino acids has been useful in the prevention of progressive hepatic failure, and improvement of surrogate markers and perceived health status in advanced cirrhosis has occurred [19,20]. Additionally, many new treatments and techniques have been introduced for HCC, including transcatheter arterial embolization, percutaneous ethanol injection therapy, microwave coagulation therapy, radiofrequency ablation, systemic chemotherapy and advance surgical techniques. However, these advances of medical treatment cannot explain the difference between HBV-associated HCC and HCV-associated HCC.

Alternatively, well considered reasons of the recent rapid increase of the number of patients who died from HCV-associated HCC in Japan, were shown in the current two studies. First, Hamada et al. recently reported that the majority of HCC patients develop HCC when they are aged over 60 years old, regardless of the timing of HCV infection. This result was obtained by the long-term observation of the patients infected by post-transfused HCV infection [21]. This also suggests that HCC has increased among patients over 60 years old with HCV infection and such phenomenon has never been observed nor reported till now in patients with HBV infection.

Second, the chronically HCV-infected population is aging in Japan. Yoshizawa et al. reported that age-specific prevalence rates for the presence of anti-HCV antibody among ~300,000 voluntary blood donors from Hiroshima in 1999 clearly increased with the age, reaching the highest rate of 7% in individuals who were more than 70 years old [11,22]. In a word, HCV infected people become older with years in Japan and they were regarded as a high risk for HCC. Then, the number of deaths from HCV-associated HCC has been increased recent 20 years in Japan.

El-Serag et al. reported that an increase in the number of cases of HCC affecting mainly younger age groups has occurred in the United States (U.S.) over the past two decades [23,24]. HCV infection accounts for most of the increase in the number of cases of primary liver cancer [4,6,7,9,25], while the rates of primary liver cancer associated with alcoholic cirrhosis and HBV infection have remained unchanged [4,6,9]. Tanaka et al. reported that HCV was introduced into the U.S. population around 100 years ago and was widely disseminated between 1954 and 1978 [26]. Most HCV-infected patients in the U.S. were born between 1940 and 1965 [27,28], and are therefore younger than HCV-infected Japanese patients. Hence, the burden of disease associated with HCV infection will probably increase in the U.S. during the next 10–20 years, as has occurred in Japan, as this cohort reaches an age at which complications of chronic liver disease typically occur [1–7,26]. The current study suggests that increased HCV-associated HCC will occur in the U.S. over the next two to three decades.

In conclusion, we found that the number of patients with HCV-associated HCC in Japan has increased, consistent with aging of the population, but the number of patients with HBV-associated HCC has remained unchanged over the last 20 years.

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ELSEVIER

Serum Levels of Interleukin-6 and Its Soluble Receptors in Patients with Hepatitis C Virus Infection

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ABSTRACT: Interleukin-6 (IL-6) is an important cytokine in liver regeneration, and elevated levels of IL-6 have been demonstrated in patients with chronic liver diseases (CLD). Many biological effects of IL-6 depend on naturally occurring soluble IL-6 receptors. In the present study we measured the concentrations of IL-6 and its soluble receptors in the sera of patients with CLD related to hepatitis C virus (HCV) infection. We studied 77 patients with varying degrees of HCV-related CLD. Serum levels of IL-6 and its soluble receptors (sIL-6R, sgp130) were measured by enzyme-linked immunosorbent assay. Serum IL-6 and sIL-6R were elevated in patients with CLD compared with healthy subjects. Serum levels of sgp130 did not differ between patients with chronic hepatitis and healthy subjects. However, in patients with liver cirrhosis, sgp130 was significantly elevated and was positively correlated with total bilirubin and negatively correlated with cholinesterase and prothrombin time. Our study demonstrated that in patients with HCV-related CLD, serum IL-6 and its soluble receptor levels are correlated with both liver function impairment and the degree of liver fibrosis. These observations suggest that the balance of IL-6 and its soluble receptors may correspond to the state of liver damage in patients with CLD. *Human Immunology 67, 27–32 (2006).* © American Society for Histocompatibility and Immunogenetics, 2006. Published by Elsevier Inc.

KEYWORDS: Hepatitis C virus; Interleukin-6; Liver cirrhosis; Soluble interleukin-6 receptor

ABBREVIATIONS

CLD	chronic liver disease
CH	chronic hepatitis
IL-6	interleukin-6
sIL-6R	soluble interleukin-6 receptor
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HGF	hepatocyte growth factor
LC	liver cirrhosis
TNF-α	tumor necrosis factor-α

INTRODUCTION

Interleukin-6 (IL-6) is a pleiotropic cytokine stimulating a variety of cell types, including hepatocytes [1–3]. IL-6 also modulates the hepatic expression of acute-phase proteins during inflammation [4,5]. Apart from its role in inflammation, IL-6 has been found to be essential for liver regeneration [6,7]. Results from IL-6 knockout mice have also indicated that IL-6 might be involved in triggering hepatocyte proliferation after hepatectomy [8]. The pathophysiological role of IL-6 in acute or chronic liver disease has been studied intensively [9]. Although IL-6 was consistently found to be elevated in liver diseases, such as chronic hepatitis and cirrhosis [10,11], the clinical relevance and molecular function of IL-6 in the pathogenesis of liver disease are only incompletely understood.

IL-6 mediates its diverse biological effects by interacting with a receptor complex consisting of a specific

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Received October 28, 2005.

Human Immunology 67, 27–32 (2006)
© American Society for Histocompatibility and Immunogenetics, 2006
Published by Elsevier Inc.
0198-8859/06/$–see front matter
doi:10.1016/j.humimm.2006.02.025

TABLE 1 Clinical and biochemical characteristics of patients with HCV infection

Characteristic	CH (*n* = 28)	LC (*n* = 17)	LC + HCC (*n* = 32)
Males (%)	12 (43)	4 (24)	19 (59)
Age	58.6 ± 11.2	65.8 ± 11.0	66.3 ± 7.3
ALP (normal range 98–251 U/l)	258 ± 65.3	404.4 ± 183.9	386.2 ± 117.7
AST (normal range 12–31 U/l)	66.1 ± 49.4	84.8 ± 28.0	86.4 ± 46.9
ALT (normal range 12–35 U/l)	90.3 ± 76.2	75.8 ± 38.2	73.8 ± 36.4
Bilirubin (mg/dl)	0.7 ± 0.2	1.5 ± 1.1	1.1 ± 0.7
Albumin (g/dl)	4.3 ± 1.0	3.6 ± 0.6	3.5 ± 0.5
Prothrombin time (%)	101.6 ± 18.2	73.6 ± 17.3	83.3 ± 20.3
Cholinesterase (normal range 160–400 IU/l)	257.4 ± 74.4	150.0 ± 59.0	141.9 ± 70.7
Total cholesterol (mg/dl)	174.0 ± 36.0	165.9 ± 36.9	142.2 ± 30.0
Platelets ($10^3/\mu l$)	187 ± 65	105 ± 37	91 ± 38
Fibrosis stage	F0:1/F1:14/F2:6/F3:3 (*n* = 24)	F4:9 (*n* = 9)	F3:1/F4:5 (*n* = 6)
HCV serotype	1:17/2:7 (*n* = 24)	1:10/2:2 (*n* = 12)	1:2/2:2 (*n* = 4)

ALP, alkaline phosphatase; AST, aspartate aminotransferase; ALT, alanine aminotransferase. Values expressed are means ± SD.

ligand-binding protein (IL-6R, gp80) and a signal transduction protein (gp130) [12]. When IL-6 binds to a cell through IL-6R (gp80), the complex facilitates its interaction with a second IL-6 receptor molecule, gp130, thereby triggering intracellular signal transduction [13]. In addition to their roles as membrane-bound proteins, IL-6R and gp130 also occur as receptors shed into the serum [14]. In several conditions, such as myeloma, chronic arthritis, and autoimmune diseases, elevated levels of soluble IL-6R (sIL-6R) have been observed [15]. sIL-6R and soluble gp130 (sgp130) have different functions. sIL-6R bound to IL-6 can interact with membrane-bound gp130 and thereby trigger activation of intracellular signaling pathways [16]. This can lead to an enhanced IL-6-mediated response.

The shed signal transducer, sgp130, itself can bind the IL-6/sIL-6R complex [17]. The resulting trimeric complex is no longer able to interact with membrane-bound gp130. In this case, sgp130 acts as an antagonist. Therefore, the biological activity of IL-6 depends on the balance of sIL-6R and sgp130. Previous studies have concentrated only on the behavior of IL-6 circulating levels in various liver diseases [18]. In this study, we evaluated serum levels of IL-6 and soluble forms of IL-6 receptors (sIL-6R, sgp130) in patients with hepatitis C virus (HCV) infections.

MATERIALS AND METHODS

Patients

We studied 77 patients with various degrees of chronic liver disease (CLD) related to HCV infection (Table 1). Of the patients, 28 had chronic hepatitis, 17 liver cirrhosis, and 32 had liver cirrhosis plus hepatocellular carcinoma (HCC). The diagnosis of HCC was made by several imaging modalities and confirmed histologically by sonography-guided fine-needle biopsy specimens in all patients. At the time of the study, none of the patients was receiving or had previously received interferon therapy. All patients were positive for anti-HCV antibodies detected by a third-generation enzyme immunoassay containing HCV antigens from the viral core and from areas of the nonstructural NS3, NS4, and NS5 regions (Ortho HCV SAVe 3.0; Ortho, Raritan, NJ) and were positive for HCV RNA in the serum as assessed by means of nested reverse transcription polymerase chain reaction. Viral serotyping was performed on 40 patients. Liver biopsy was performed on 39 patients; the degree of liver fibrosis was assessed using the METAVIR system [19]. All patients enrolled in this study were regularly followed with liver function tests every month and with ultrasonography or computed tomography of liver every 4 months. Of these, patients with marked fluctuations in these tests were excluded from this study. Patients with other concomitant causes of liver disease, such as autoimmunity or alcohol abuse (more than 40 g alcohol daily intake), or patients with metabolic disease, infections, or renal dysfunctions were not included in the study to avoid possible confounding factors. All patients were negative for hepatitis B surface antigen and had no symptom or sign related to HIV, cytomegalovirus, and *Toxoplasma gondii* infection.

None of the patients suffered from hemolytic anemia or renal failure or manifested features compatible with the presence of disseminated intravascular coagulation. In addition, sera from 23 healthy volunteers were used as controls (10 males and 13 females, mean age 45.5 ± 13.2 years). All healthy volunteers presented as normal in liver function tests, with negative serology for viral hepatitis and no history of liver disease.

Enzyme-Linked Immunosorbent Assay

For duplicate measurements solid-phase Quantikine Immunoassays (R&D Systems, Minneapolis, MN, USA)

were used to measure serum IL-6, sIL-6R, and sgp-130 according to the manufacturer's instructions.

Statistical Analysis

Data are presented as the means ± SD. The differences between quantitative variables were evaluated with the Mann–Whitney *U* test. A *p* value <0.05 for two-sided tests was considered statistically significant. The correlation between two variables was analyzed using the Spearman rank correlation test. Statistical analysis was performed with StatView software (SAS Institute, Inc., Cary, NC, USA). A *p* value <0.05 was required for statistical significance.

RESULTS

A total of 77 patients were studied. Of these, 35 were male and 42 female, with a mean age of 63.4 ± 10.3 years. According to the disease progression, the patients were divided into three groups (chronic hepatitis, liver cirrhosis, and liver cirrhosis plus HCC). The main clinical, biochemical, and functional characteristics of the patients are presented in Table 1.

Serum levels of IL-6 were measured in patients with HCV infections and were significantly higher in these patients than in healthy subject (Figure 1). Furthermore, this analysis revealed that circulating IL-6 levels were significantly higher in patients with liver cirrhosis (LC) than in patients with chronic hepatitis (CH).

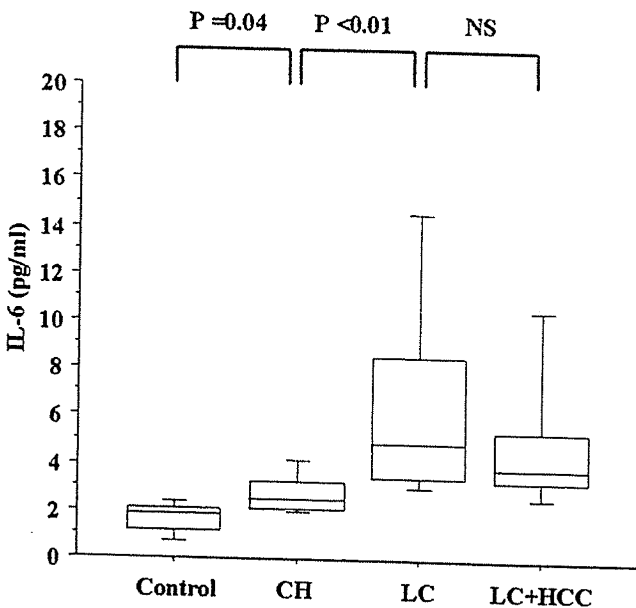

FIGURE 1 Serum IL-6 levels in patients with HCV infection and healthy controls. CH, chronic hepatitis; LC, liver cirrhosis; LC+HCC, liver cirrhosis plus hepatocellular carcinoma. The box contains the values between the 25th and the 75th percentiles and the horizontal line is the median. The error bars stretch to the 10th and the 90th percentiles.

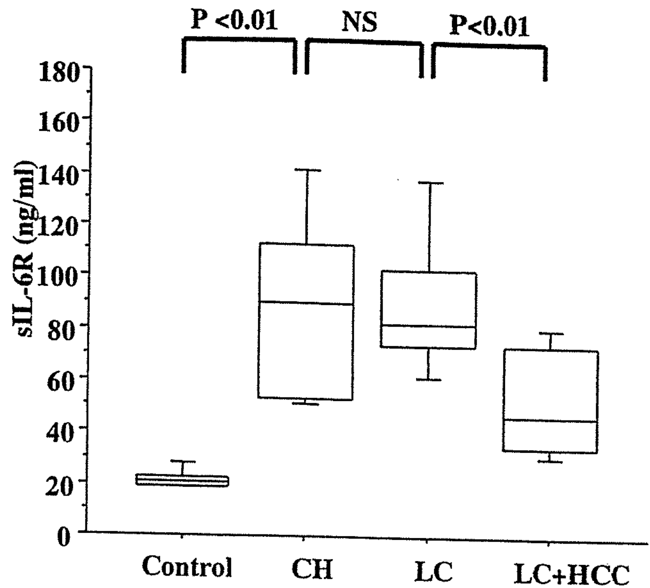

FIGURE 2 Serum sIL-6R levels in patients with HCV infection and healthy controls.

As presented in Figure 2, serum sIL-6R levels of patients with HCV infection were significantly higher than those in healthy subjects. No difference in sIL-6R level was detected between patients with CH and LC. However, sIL-6R levels varied significantly among patients with LC. That is, LC patients with HCC exhibited significantly lower levels of sIL-6R than did LC patients without HCC.

Serum levels of sgp130, which inhibits the action of IL-6, were also measured in patients with HCV infection (Figure 3). Patients with LC either with or without HCC exhibited elevated sgp130 values compared with healthy subjects. Conversely, no significant difference was observed in serum sgp130 levels between healthy subjects and patients with chronic hepatitis.

The relationships among IL-6, sIL-6R, sgp130, and clinicobiochemical parameters were also investigated. As presented in Figure 4, total bilirubin was significantly correlated with circulating sgp130 ($r = 0.49$, $p = 0.001$). In contrast, cholinesterase ($r = -0.48$, $p = 0.002$) and prothrombin time ($r = -0.39$, $p = 0.014$) were significantly inversely correlated with circulating sgp130 (Figure 5). Thus, we observed that in patients with HCV-related CLD, elevated levels of sgp130 were associated with impaired liver functions.

DISCUSSION

IL-6 plays a significant role in liver regeneration in conjunction with additional growth factors such as HGF or TNF-α [20,21]. Previous reports have demonstrated elevated IL-6 levels in patients with acute and chronic

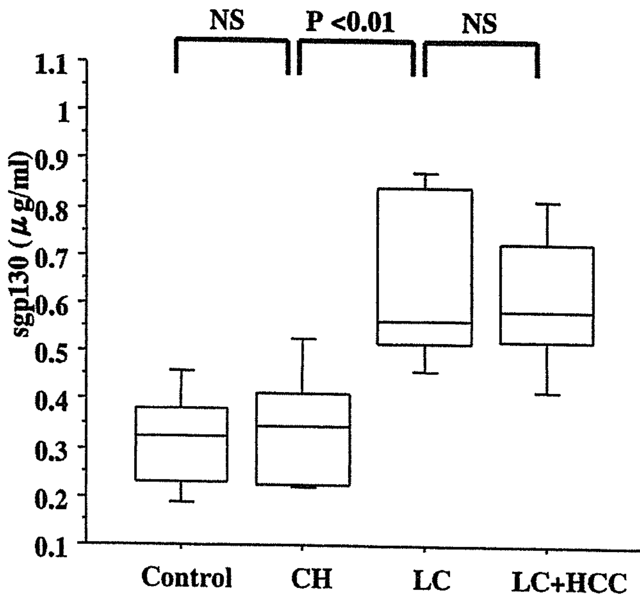

FIGURE 3 Serum sgp130 levels in patients with HCV infection and healthy controls.

liver disease [10,11,18]. The elevated levels of serum IL-6 were also demonstrated in HCV-infected patients [22,23]. However, it is unknown whether this cytokine activation represents only an epiphenomenon. To clarify the role of IL-6 in chronic liver disease, we studied the levels of soluble IL-6 receptors, which affect the biological effects of IL-6 in addition to IL-6 itself. The circulating levels of IL-6 and its soluble receptors have been demonstrated to be modulated by various diseases [24,25]. We therefore measured this cytokine and its soluble receptors in HCV-infected patients without any associated diseases or complications, such as infection, inflammation, or renal dysfunction.

One of the principle findings of this study was that both IL-6 and sIL-6R proteins were elevated in patients with HCV infection. In addition, the levels of IL-6 were significantly higher in patients with LC than in those with CH. Our data are consistent with those of a previous study demonstrating elevated circulating levels of sIL-6R in patients with liver cirrhosis [26].

sIL-6R is a ligand-binding protein that constitutes the extracellular part of the IL-6 receptor. sIL-6R markedly prolongs the IL-6 plasma half-life, and sIL-6R-bound IL-6 can interact with membrane-bound gp130 and thereby lead to activation of the intracellular signaling pathway [27]. This can lead to an enhanced IL-6-mediated response. Additionally, through this mechanism, primary unresponsive cells expressing only gp130 and no gp80 can be activated through the sIL-6R/IL-6 complex. This process has been called "*trans*-signaling" [28]. Considering this enhancing role of sIL-6R, we speculate that high levels of sIL-6R might potentiate the effects of IL-6 in HCV-induced chronic hepatitis or liver cirrhosis.

Results from studies using IL-6 knockout mice have indicated that IL-6 might be involved in hepatocyte proliferation [29]. These observations may suggest that the IL-6/sIL-6R system could be involved in liver regeneration after liver injury. Although the pathophysiological role of elevated IL-6/sIL-6R in HCV infection was not elucidated in this study, it could consist of a regenerative response against liver damage or inflammation.

Additional evidence supporting the idea that IL-6 plays a role in hepatocyte proliferation has come from experiments using double-transgenic mice expressing IL-6 and sIL-6R [30,31]. In these mice, hepatocyte proliferation was evident in the periportal area, and hyperplastic nodules were also observed. It is possible that elevated levels of IL-6/sIL-6R contribute to nodular regenerative changes in patients with HCV-induced liver cirrhosis. However, IL-6/sIL-6R may not be involved in the HCC association, because sIL-6R levels of LC patients with HCC were significantly lower than those without HCC.

Another main finding is that sgp130 levels were significantly higher in LC patients than in patients with chronic hepatitis. We evaluated the relationship between sgp130 levels and liver function tests, such as total bilirubin, cholinesterase, and prothrombin time. We observed that the increase in sgp130 is linked to impaired liver functions, including elevated bilirubin levels, prolonged prothrombin time, and reduced serum cholinesterase levels. Therefore, these findings may suggest that sgp130 elevation is related to progression of liver dysfunction as well as hepatic decompensation in patients with HCV-related CLD.

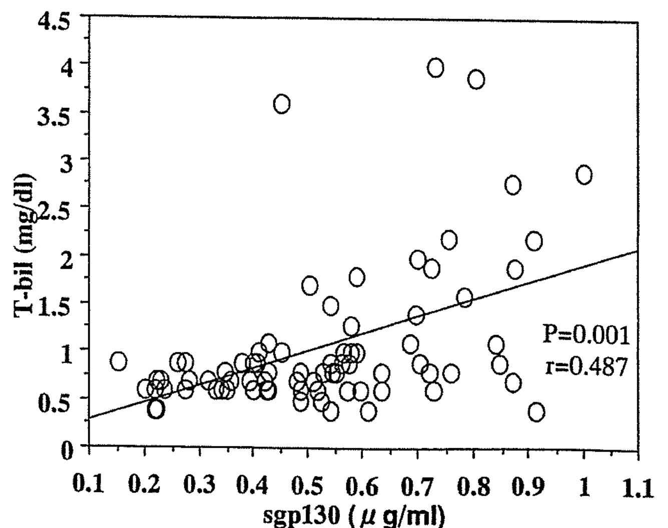

FIGURE 4 Correlation between individual values of total bilirubin and sgp130 in patients with HCV infection. T-bil, total bilirubin.

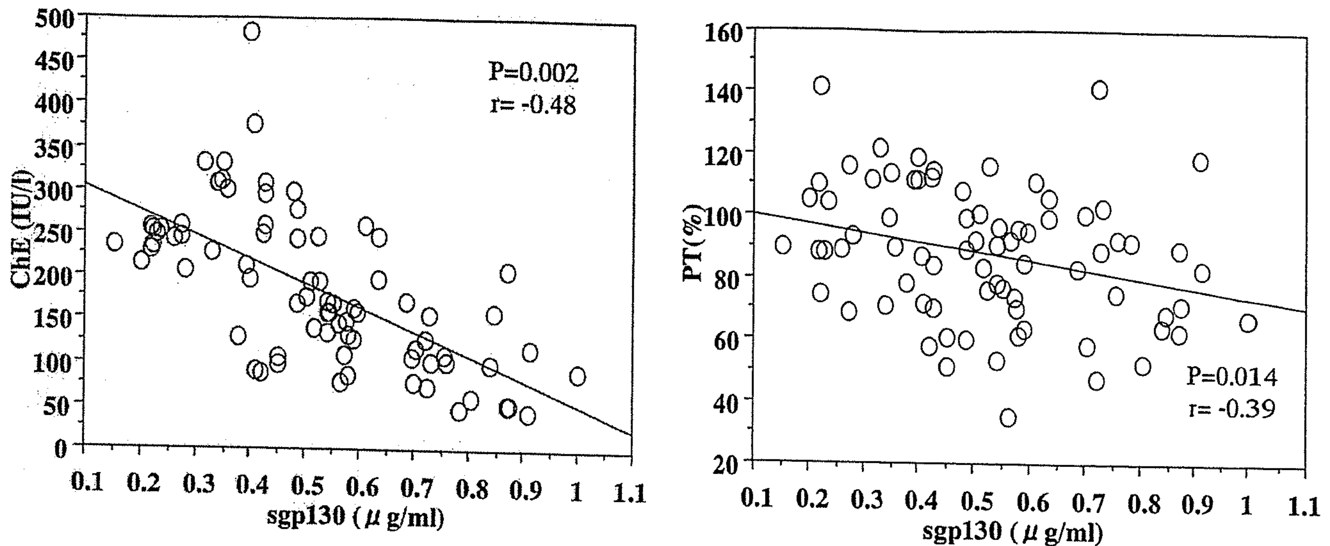

FIGURE 5 (A) Correlation between individual values of cholinesterase and sgp130 in patients with HCV infection. ChE, cholinesterase. (B) Correlation between individual values of prothrombin time and sgp130 in patients with HCV infection. PT, prothrombin time.

sIL-6R and sgp130 have different functions. The shed signal transducer sgp130 can itself bind the IL-6/sIL-6R complex and inhibit the action of IL-6. After injury, the liver has a remarkable capacity to restore major tissue loss through regeneration. IL-6 is an essential cytokine involved in liver regeneration. In fact, our data demonstrated that IL-6 and sIL-6R were significantly elevated in patients with chronic hepatitis and LC. However, under clinical conditions under which circulatory sgp130 is elevated, the IL-6/sIL-6R system may not maintain the liver regeneration. Although the exact mechanism by which sgp130 is elevated in LC patients was not elucidated in this study, this aberrant induction of sgp130 may lead to the abrogated IL-6/sIL-6R biological function and the development of liver dysfunctions and subsequent hepatic insufficiency in LC patients. Elevated levels of IL-6 and sIL-6R in HCV-related CLD likely contribute to compensatory hepatocyte growth during chronic liver injury. However, sgp130 levels in LC patients might antagonize the IL-6-mediated hepatotropic effect within the liver and could contribute to the impaired liver regeneration.

In conclusion, in this study, we have demonstrated that a progressive decline in liver function in patients with HCV-related CLD was paralleled by an increase in circulating sgp130 levels.

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