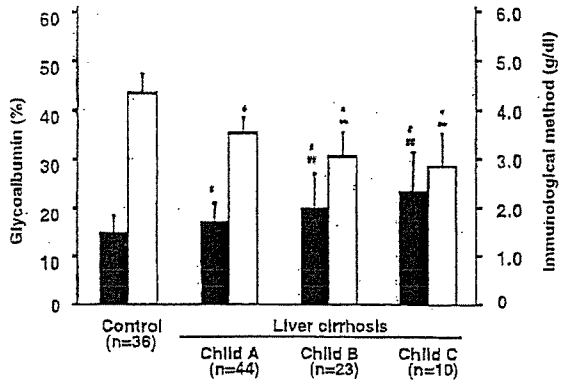


Fig. 7. Glycoalbumin percentage (solid bars) and serum albumin level (open bars) measured by immunoassay and classified by severity (Child's classification) in 78 cirrhotic patients without diabetes. In these patients, the glycoalbumin percentage by Child class increased with progression of the disease. Vertical bars show the standard deviation. Glycoalbumin percentage: control versus liver cirrhosis (Child A, B, and C), $\#P < 0.05$; Child A versus Child B and C, $\#\#P < 0.05$. Albumin level (see Figure 5 for data from control subjects): control versus liver cirrhosis (Child A, B, and C), $*P < 0.05$; Child A versus Child B and C, $**P < 0.05$.

was 27.1%, resulting in a markedly increased percentage independent of the Child class (data not shown).

In cirrhotic patients without diabetes, the oxidized albumin percentage demonstrated a correlation with the glycoalbumin percentage, as shown in Figure 8.

## DISCUSSION

The BCP method was first reported by Pinnell et al. in 1978[20]; at that time, it attracted much interest as a good method highly specific for albumin. Subsequently, however, it was pointed out that the BCP method has various disadvantages,[21,22] such as negative error due to δ-bilirubin,[23] reaction with serum in patients undergoing hemodialysis[24] and a difference in reaction between reduced albumin and oxidized albumin.[25] Our study also showed that some serum albumin levels measured by the BCP method differ markedly from the levels detected by immunoassay and that this difference increased as the oxidized albumin ratio increased. Therefore, the BCP method is not ideal as a method for determination of serum albumin in cirrhotic patients. To solve these problems, Muramoto et al.[26] endeavored to eliminate the difference in reaction between reduced albumin and oxidized albumin by pretreating serum samples. As a result, a modified BCP method using an autoanalyzer (Akua-auto Kainos Albumin Test Kit, Kainos Laboratories, Inc., Tokyo, Japan; lipid-free human serum is used as the reference standard, with CRM470 as the primary standard) is now available.[27] Thus, this modified BCP method (CRM 470) will be more suitable in the future for determination of serum albumin in clinical situations because the method is simple, inexpensive, and reliable.

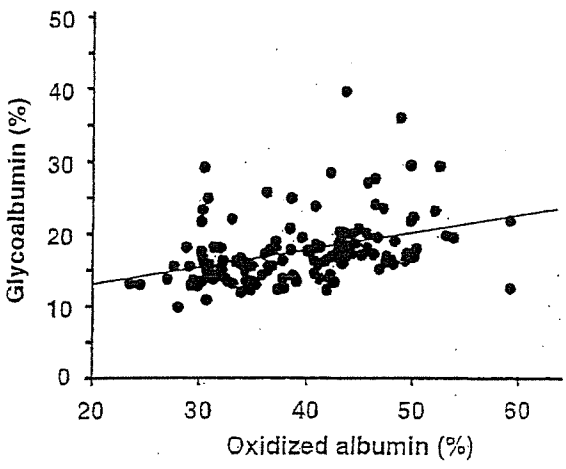


Fig. 8. Correlation between the glycoalbumin percentage and the oxidized albumin percentage in 78 cirrhotic patients without diabetes (solid circles). The glycoalbumin percentage correlated with the oxidized albumin percentage in cirrhotic patients without diabetes. Solid line, regression equation: $y = 23.65x + 8.32$ ($R^2 = 0.13$).

The serum albumin level decreases by 0.1 to 0.2 g/dL annually in most cirrhotic serum. Treatment with BCAA preparations can increase the albumin level by 0.1 to 0.2 g/dL.[28] It is expected that determination of serum albumin will be performed more frequently to assess the prognosis of cirrhotic patients, to decide the timing of liver transplantation or the start of treatment for hypoalbuminemia, and to assess the therapeutic efficacy. Therefore, improvement of methods for albumin determination is strongly desired to minimize variation in the measured values among institutions and to ensure more accurate determination of the albumin level as an important index for deciding when to start treatment, assessing the efficacy of treatment, and predicting the prognosis.

Patients with liver cirrhosis were stratified by serum albumin level in increments of 0.1 g/dL over the range of 2.9 to 4.1 g/dL. There was a significant difference in the survival rate between any two adjacent classes.[29] Because no deaths were observed at serum albumin levels of 3.8 g/dL or higher, this is likely an important threshold. In this study, cirrhotic patients in whom the serum albumin level measured by the BCG method was 3.8 g/dL or higher had complications at a lower frequency than did patients with a serum albumin level below 3.8 g/dL. It is unclear whether a serum albumin level of 3.8 g/dL by the BCG method corresponds to a value of 3.5 g/dL measured around 1964 when Child's classification was developed. However, it is currently appropriate to consider cirrhotic patients with a serum albumin level of 3.8 g/dL as hypoalbuminemic. When the method for serum albumin determination is standardized in the future using the international protein standard CRM 470 supplied by the International Federation of Clinical Chemistry, the definition of hypoalbuminemia should be reconsidered.

The prognosis of cirrhotic patients depends on the serum albumin level.[30] The clinical implications of the serum albumin level have been investigated by many researchers. However, there have been only a few studies on the properties of albumin microheterogeneity on a molecular basis. In cirrhotic patients, the percentage of oxidized albumin increases with a decrease in total albumin concentration, independently of concurrence of diabetes. Further, we found that the increase in the oxidized albumin percentage may cause an increase in the serum albumin level when measured by the BCP method. The absolute levels of oxidized albumin and reduced albumin may decrease with the progression of liver disease. However, the oxidized albumin percentage is thought to increase because the degree of the decrease in the oxidized albumin level is less than that of reduced albumin. In reduced albumin, the cysteine residue at the 34th position from the N-terminal is preserved. In oxidized albumin, this cysteine residue is oxidized or forms a reactive S-S bond with a sulfurous amino acid (−SOH, $-SO_2H$, and $-SO_3H$ are also formed). Because this S-S bond is formed by a reversible reaction within a short time,[31] both molecular species are in dynamic equilibrium in the blood. Accordingly,

oxidized albumin and reduced albumin closely reflect the oxidation reduction state in vivo. Oxidative stress due to aging, nephrotic syndrome, hemodialysis, and diabetes increases the oxidized albumin percentage.[32,33] Human serum albumin preparations that are often used for treatment of hypoalbuminemia in cirrhotic patients include preparations whose major ingredient is oxidized albumin.[34] Effective procedures for converting oxidized albumin to reduced albumin remain unclear. An increased percentage of oxidized albumin may reflect increased scavenging of reactive oxygen species by albumin, indicative of oxidative stress and/or decreased albumin turnover with consequent accumulation of albumin damaged oxidatively.[35,36] The clinical implications of reduced albumin:oxidized albumin ratio in liver disease should be clarified while investigating the pathophysiology and treatment of the disease.

It has been reported that the glycoalbumin level is increased in diabetic patients.[37] However, the method of glycated albumin by a mass spectrometric technique called matrix-assisted laser desorption ionization[38] is based on the assumption that all changes in the molecular mass of albumin in diabetes are due to glycation by glucose. Therefore, the samples determined by a high-performance liquid chromatographic method in our study should be determined simultaneously by the recent new methods.[39] In cirrhotic patients, the percentage of glycated albumin increases with a decrease in total albumin concentration. Glycation of albumin is an irreversible reaction, different from the reversible oxidation reduction reaction of albumin.[40] Unlike oxidized:reduced albumin, glycation of albumin causes an irreversible change in the molecular structure. Glycation of albumin reduces the binding capability of bilirubin to albumin to about half and that of long-chain fatty acids to about one-twentieth.[41] This means that the native function of albumin is attenuated. Therefore, glycated albumin can be considered to be deteriorated albumin. Glycation of albumin in diabetic patients is caused by hyperglycemic stimulation. Interestingly, in this study, the glycoalbumin percentage also was increased in cirrhotic patients without diabetes. Perhaps the hypoalbuminemic status in cirrhotic patients reduces the destruction rate of albumin, which is shown by prolongation of the half-life of albumin,[42] resulting in prolonged stimulation by glucose. This study suggests that this hyperglycemic stimulation is an additional factor because the glycoalbumin percentage in cirrhotic patients accompanied by diabetes was definitely higher than that in patients without diabetes. Reducing sugars, including glucose, bind amino acid residues in protein and form Schiff bases and Amadori products (hemoglobin $A_{1C}$ is a well-known product). These products are gradually changed to irreversible derivatives, called advanced glycation (glycosylation) endproducts (AGEs), over several weeks or several months.[43] However, recent studies[44,45] have suggested that AGEs can form in days by the reaction of $\alpha$-oxaldehydes such as glyoxal, methylglyoxal, and 3-deoxyglucosone with proteins. Fructosamines also cannot degrade over a few days, although they form over 10 to 15 d, depending on the proteins. Plasma AGE level (N$\epsilon$-carboxymethyllysine) in cirrhotic patients (not accompanied by renal disorder and/or diabetes) has been reported to increase with the progression of disease and to return to normal after liver transplantation.[46] Increased N$\epsilon$-carboxymethyllysine levels in cirrhotics may reflect increased oxidative degradation of fructosyllysine to N$\epsilon$-carboxymethyllysine[47] and decreased turnover of albumin. AGEs produced from glycoalbumin have been considered to be attributable to development of various complications in diabetic patients.[48–49] The increased AGEs in patients with liver disease activate various cells in the liver, such as sinusoidal endothelial cells and Kupffer cells, through AGE receptors and stimulate production of cytokines, including transforming growth factor-$\beta_1$, resulting in liver fibrosis and development of complications. Further investigation of the clinical implications of glycoalbumin in the pathophysiology of liver disease is required, as are studies of the kinetics of reduced albumin and oxidized albumin.

## REFERENCES


1. Watson D. Albumin and "total globulin" fraction of blood. Adv Clin Chem 1965;8:237
2. Rosenoer VM, Oratz M, Rothschild MA. *Albumin structure, function and uses.* Elmsford: Pergamon, 1977
3. Doweiko JP, Nompleggi DJ. The role of albumin in human physiology and pathophysiology, part III: albumin and disease states. JPEN 1991;15:212
4. Skrede S, Blomhoff JP, Elgjo K, Gjone E. Serum proteins in diseases of the liver. Scand J Clin Lab Invest 1975;35:399
5. Muto Y, Yoshida T, Sato S, et al. Effect of oral administration of branched-chain amino acid granules (BCAA-G) in patients with liver cirrhosis: a clinical study (in Japanese). JPEN 1989;11:1119
6. Muto Y, Yoshida T, Sato S, et al. Effect of oral administration of branched-chain amino acid granules (BCAA-G) in patients with liver cirrhosis: a double blind study (in Japanese). JPEN 1989;11:1137
7. Muto Y, Yoshida T, Sato S, Suzuki K, Watanabe A. Effect of oral supplementation with BCAA-G on the prognosis of liver cirrhosis (ion Japanese). JPEN 1992;14:765
8. Muto Y, Yoshida T, Kato M, et al. Serum albumin level and prognosis in patients with liver cirrhosis—result of branched-chain amino acid granules (BCAA-G) supplement trial (in Japanese). JPEN 1995;17:1135
9. Japan Society of Clinical Examination Technologists. *Investigative report of accuracy management (Heisei 9th year)* (in Japanese). Kosei Company, Ltd., Tokyo, 1997:66
10. Child CG, III, Trucotte JG. Surgery and portal hypertension. In: Child CG, III, ed. *The liver and portal hypertension,* 3rd ed. Philadelphia: WB Saunders, 1964:90
11. Rodkey FL. Direct spectrophotometric determination of albumin in human serum. Clin Chem 1965;11:478
12. Era S, Kuwata K, Imai H, et al. Age-related change in redox state of human serum albumin. Biochim Biophys Acta 1995;1247:12
13. Era S, Hamaguchi T, Sogami M, et al. Further studies on the resolution of human mercapt- and nonmercaptalbumin and on human serum albumin in the elderly by high-performance liquid chromatography. Int J Pept Protein Res 1988;31:435
14. Ziyadeh FN, Cohen MP. Effects of glycated albumin on mesangial cells: evidence for a role in diabetic nephropathy. Mol Cell Biochem 1993;125:19
15. Association of Clinical Biochemists Ltd. News Sheet No. 186, October 1978
16. Doumas BT, Watson A, Biggs HG. Albumin standards and the measurement of serum albumin with bromcresol green. Clin Chim Acta 1971;31:87
17. Okamoto K. Quantitative method for serum albumin concentration by using bromcresol purple (in Japanese). J Med Technol 1974;18:646
18. Sogami M, Era S. HPLC-studies on nonmercapt-mercapt conversion of human serum albumin. Int J Peptide Res 1985;25:398
19. Shima K, Itoh N. High-performance liquid chromatography assey of serum glycated albumin. Diabetologia 1988;31:627
20. Pinnell AE, Northam BE. New automated dyebinding method for serum albumin determination with bromcresol purple. Clin Chem 1978;24:80
21. Schmidt Von H, Ebeling H, Kraft D. Vergleiche konventioneller methoden zur albumin-, transferrin- und coeruloplasmin- bestimmung in serum mit immunologischen referenzmethoden. Z Klin Chem Klin Biochem 1975;13:117
22. Javed MU, Waqua SN. An enzymatic method for the detection of human serum albumin. Exp Mol Med 2001;33:103
23. Ihara H, Nakamura H, Aoki Y, Aoki T, Yoshida M. Effects of serum-isolated vs synthetic bilirubin-albumin complexes on dye-binding methods for estimating serum albumin. Clin Chem 1991;37:1269
24. Mabuchi H, Nakahashi H. Underestimation of serum albumin by the bromcresol purple method and a major endogenous ligand in uremia. Clin Chim Acta 1987;37:89
25. Muramoto Y, Kushigemachi A, Matsushita M, Irino T. Increase in albumin concentration measured by bromcresol purple method during storage of serum (in Japanese with English abstract). Jpn J Clin Chem 1991;20:13
26. Muramoto Y, Matsushita M, Irino T. Reduction of reaction differences between human mercaptalbumin and human nonmercaptalbumin measured by the bromcresol purple method. Clin Chim Acta 1999;289:69
27. Muramoto Y, Sasaki M, Aoki Y, Matsushita M. Evaluation of a new routine method for the determination of serum albumin with bromcresol purple (in Japanese with English abstract). Med Exam 1997;46:823
28. Peters T, Jr. Clinical aspects: albumin in medicine. In: Peters T, Jr., ed. *All about albumin.* New York: Academic Press, 1996:251
29. Moriwaki H, Tajika M, Miwa Y. Protein-energy malnutrition, glucose intolerance (in Japanese). Acta Hepatol Jpn 2002;43:101
30. Kuroki T, Nishiguchi S, Nakajima S, et al. The management of nutrition for hepatic cirrhosis (in Japanese with English abstract). Jpn J Clin Med 1994;52:197
31. Kuwata K, Era S, Sogami M. The kinetic studies on the intramolecular SH, S-S exchange reaction of bovine mercaptalbumin. Biochim Biophys Acta 1994;1205:317

32. Sogami M, Era S, Nagaoka S, et al. High-performance liquid chromatographic studies on non-mercapt in equilibrium with mercapt conversion of human serum albumin. IL J Chromtogr 1985;332:19
33. Suzuki E, Yasuda K, Takeda N, et al. Increased oxidized form of human serum albumin in patients with diabetes mellitus. Diabetes Res Clin Pract 1992;18:153
34. Yasukawa K. Glycation and oxidation of albumin (in Japanese). Clin Med Technol 2000;44:907
35. Kadota K, Yui Y, Hattori R, Murohara Y, Kawai C. Decreased sulfhydryl groups of serum albumin in coronary artery disease. Jpn Circ J 1991;55:937
36. Murohara Y, Yui Y, Hattori R, Kadota K, Kawai C. Human serum albumin inhibits prostacyclin production by endothelial cells: the relation of the inhibitory activity to sulfhydryl groups in albumin. Jpn Circ J 1991;55:777
37. Lapolla A, Fedele D, Seraglia R, et al. A new effective method for the evaluation of glycated intact plasma proteins in diabetic subjects. Diabetologia 1995;38: 1076
38. Lapolla A, Fedele D, Seraglia R, et al. A new effective method for the evaluation of glycated intact plasma proteins in diabetic subjects. Diabetologia 1995;38: 1076
39. Dobrian A, Simionescu M. Irreversibly glycated albumin alters the physicochemical characteristics of low density lipoproteins of normal and diabetic subjects. Biochim Biophys Acta 1995;1270:26
40. Shaklai N, Garlick RL, Bunn HF. Nonenzymatic glycosylation of human serum albumin alters its conformation and function. J Biol Chem 1984;259:3812
41. Sterling K. Serum albumin turnover in Laennec's cirrhosis as measured by $^{131}$I-tagged albumin. J Clin Invest 1951;30:1238
42. Horiuchi S. The liver is the main site for metabolism of circulating advanced glycation endproducts. J Hepatol 1951;36:123
43. Thornalley PJ. The clinical significance of glycation. Clin Lab 1999;45:263
44. Thornalley PJ, Langborg A, Minhas HS. Formation of glyoxal, methylglyoxal and 3-deoxyglucosone in the glycation of proteins by glucose. Biochem J 1999; 344:109
45. Sebekov K, Kupcov V, Schinzel R, Heidland A. Markedly elevated levels of plasma advanced glycation end products in patients with liver cirrhosis-amelioration by liver transplantation. J Hepatol 2002;36:66
46. Smith PR, Thornalley PJ. Mechanism of the degradation of non-enzymatically glycated proteins under physiological conditions. Studies with the model fructosamine, Ne-(1-deoxy-D-fructos-1-yl) hippuryl-lysine. Eur J Biochem 1992; 210:729
47. Chen S, Cohen MP, Ziyadeh FN. Amadori-glycated albumin in diabetic nephropathy: pathophysiologic connections. Kidney Int 2000;58(suppl 77):S40
48. Schalkwijk CG, Ligtvoet N, Twaalhoven H, et al. Amadori albumin in type I diabetic patients: correlation with markers of endothelial function, association with diabetic nephropathy, and localization in retinal capillaries. Diabetes 1999; 48:2446
49. Cohen MP, Ziyadeh FN. Role of Amadori-modified nonenzymatically glycated serum proteins in the pathogenesis of diabetic nephropathy. J Am Soc Nephrol 1996;7:183

LIVER

# Benefit of interferon therapy in hepatocellular carcinoma prevention for individual patients with chronic hepatitis C

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**Background:** An increase in the incidence of hepatocellular carcinoma (HCC) in Japan since the 1980s suggests an imminent outbreak in other countries where viral spread occurred more recently. Interferon therapy for chronic hepatitis C, in general, has been shown to prevent HCC.
**Aims:** To determine the scale of benefit in individual patients.
**Subjects:** Histologically proven chronic hepatitis C patients in the Inhibition of Hepatocarcinogenesis by Interferon Therapy (IHIT) cohort (*Ann Intern Med* 1999;**131**:174), as updated in March 2003.
**Methods:** The lifetime risk for HCC was calculated based on HCC incidence rates, stratified by sex, age, fibrosis stage, and outcome of interferon therapy. The gain in HCC free survival was defined as the difference between expected HCC free survival with sustained virological response and that without.
**Results:** The gain in HCC free survival was greater when a patient was younger and fibrosis was more advanced. For example, a 30 year old male with F3 fibrosis gained 12.4 years by attaining sustained response while a patient with F1 fibrosis older than 60 years gained less than one year. For a treatment protocol with a given sustained response rate, prior estimation of the gain can be obtained by multiplying the calculated HCC free survival for responders by the response rate.
**Conclusions:** The gain in HCC free survival may serve as an indicator of the benefit of interferon therapy in terms of HCC prevention and be useful in the consideration of indication and selection of treatment protocol for individual patients.

Various studies have indicated that a nationwide spread of hepatitis C virus (HCV) took place in Japan in the 1950s and 1960s.[1 2] As a result, there has been a rapid increase in hepatocellular carcinoma (HCC) incidence since 1980, now claiming more than 30 000 deaths each year. Simultaneously, there was a decline in the number of deaths assigned to cirrhosis. The decline may be partly due to advances in HCC diagnosis but the major cause seems to lie in the increasing risk of HCC as patients are getting older. The time lag of 30 years between the peaks of infection spread and HCC incidence corresponds to the observed interval between the time of blood transfusion and carcinogenesis in typical HCC patients.[3] These data strongly suggest an imminent outbreak of HCC incidence in other countries, including the USA, where HCV infection is thought to have been spread more recently.[4 5]

Shortly after the discovery of HCV in 1989,[6 7] interferon therapy was confirmed to be effective against HCV infection.[8–10] We and other groups have shown that interferon therapy significantly reduces the risk of HCC development among chronic hepatitis C patients.[11–15] In our previous study, 50% relative risk reduction, compared with untreated patients, was observed for conventional interferon monotherapy that showed an overall sustained virological response (SVR) rate of 33%, and a relative risk reduction to 20% was revealed among patients who achieved SVR.[15] As the antiviral efficacy of interferon therapy has been improved by recent advances such as combination with ribavirin[16–18] and introduction of pegylated interferons,[19–22] we can expect that the efficacy on HCC prevention has also been strengthened.

We have also shown that cirrhosis gradually resolves once SVR is achieved,[23] suggesting that interferon therapy will also prevent death due to liver failure or variceal rupture. However, HCC is clearly the dominant cause of death in patients with chronic hepatitis C, at least among our cohort in Japan where the average age is over 50 years and most patients abstain from heavy alcohol consumption.[24] Thus we have focused on HCC prevention as the primary object of interferon therapy.

Considering the current status of therapeutics, interferon therapy is clearly recommended only in a selected group of patients.[25] Since the benefit of antiviral therapy differs among individual patients, the indication as well as the choice of regimen should be decided based on the expected benefit for each patient. Quantification of benefit requires reasonable assessment of the lifetime risk of HCC and the expected reduction in it with treatment. In this study, we propose an indicator, the gain in HCC free survival, to quantify the benefit specific to individual patients. The value is calculated based on both life expectancy and individualised risk of HCC, and applicable to distinct protocols with varying efficacy. It may serve as the gold standard for the benefit of antiviral therapy in terms of HCC prevention.

## MATERIALS AND METHODS

### Incidence rates of HCC

Crude data were obtained from the IHIT (Inhibition of Hepatocarcinogenesis by Interferon Therapy) database,[15 23 24] as updated on 31 March 2003. Every patient underwent liver biopsy in 1990 or later, and liver fibrosis was staged according to the classification system of Desmet and colleagues.[26] Patients had no history of HCC, and were positive for HCV antibody and negative for hepatitis B surface antigen. We excluded those who developed HCC or dropped out of surveillance within one year after liver biopsy, and the start

**Abbreviations:** HCV, hepatitis C virus; HCC, hepatocellular carcinoma; SVR, sustained virological response; NNT, number needed to treat

of observation was set at exactly one year after liver biopsy. Entry into the cohort was closed in 1999. The cohort population analysed in this study consisted of 2392 patients who received interferon monotherapy within one year of liver biopsy, and 395 patients who did not. Among 2392 interferon treated patients, 836 (34.9%) showed SVR, as determined six months after cessation of interferon administration. After undergoing liver biopsy, 90% of patients abstained from alcohol except for infrequent social occasions, and only 2% continued drinking alcohol (>80 g daily).

Patients underwent abdominal ultrasonography every 3–6 months, and contrast enhanced computed tomography was also performed every 6–12 months in patients with advanced fibrosis. A final diagnosis was made based on haemodynamic patterns on contrast enhanced computed tomography, abdominal angiography, or computed tomography during angiography. Ultrasound guided tumour biopsy was performed in ambiguous cases. The SVR group showed 27 events of HCC development during an observation period of 4767 person years; in the non-SVR group, 214 events in 9922 person years; and in the untreated group, 67 events in 2168 person years.

HCC incidence rates, stratified by age, sex, and fibrosis stage at entry, were calculated in each group by the person year method. Risk ratios were analysed using Cox proportional hazard regression. Age, as ranked by 10 years, and fibrosis stage were represented by dummy variables in the analysis. Adjusted HCC incidence rates were calculated so that the sum of squares of differences between the adjusted and observed values, weighted by the number of patients in each category, was minimised while conserving the risk ratios obtained by proportional hazard regression.

### HCC free survival

The probability that a patient remains free of HCC at $n$th year of observation was calculated as:

$$(1-Q1)(1-P1)\times(1-Q2)(1-P2)\times(1-Q3)\times$$
$$(1-P3)\times \ldots \times(1-Qn)(1-Pn)$$

where $Qi$ is the age and sex specific death rate in the general population and $Pi$ is the annual incidence of HCC specific to the patient in the $i$th year. Age and sex specific death rates were those published by the Ministry of Health, Welfare, and Labour of Japan for the vital statistics surveyed in 2000.[27] The gain in HCC free survival by interferon therapy was defined as the area between the cumulative HCC free survival curves. This model is based on an assumption that fibrosis stage remains constant with time (see model limitations in the results section).

### Statistics

Values are expressed as mean (SD) unless otherwise specified. All statistical analyses were performed with SAS Software version 6.12. We used an original program coded in Object Pascal to calculate cumulative HCC free survival.

## RESULTS

### Incidence rates of HCC

Demographic data of the patients analysed in the current study are summarised in table 1, and observed HCC development and deaths are shown in table 2, illustrating that HCC was the major sequela among the cohort. Crude incidences of HCC did not differ between the untreated group and the non-SVR group in the corresponding category (data not shown). Patients with advanced fibrosis (stages F3 or F4) in the non-SVR group or in the untreated group showed a very high incidence rate. In fact, values obtained were greater than those found in 1999,[15] suggesting that the risk of HCC has increased with time. Fibrosis stage was determined at the time of liver biopsy and had possibly progressed during the observation period. As previously described, HCC incidence rates were substantially lower in the SVR group.

Cox proportional hazard regression analysis revealed that male sex, older age, and advanced fibrosis were associated with a higher risk of HCC, both in the non-SVR groups (table 3) and in the untreated group (data not shown). Multivariate analysis showed that the risk ratio of non-SVR to no treatment was 0.835 (95% confidence interval (CI) 0.625–1.1125; $p = 0.2214$). We previously showed that the risk of HCC was decreased in patients who showed normalised serum alanine aminotransferase levels in spite of continued viraemia after interferon therapy.[24] However, active hepatitis recrudesces not infrequently in a short period[28] and the effect on HCC prevention in those patients appears to decline in the long run. Thus we assumed that interferon therapy without attaining SVR had no effect on HCC prevention. Table 4 shows the incidence rates of HCC, as fitted to the crude data by the least squares method. These values were used in modelling of the estimated HCC free survival of individual patients.

### HCC free survival

Using the fitted HCC incidence rates and the age and sex specific death rates, we estimated the lifetime cumulative HCC free survival with or without SVR. Figure 1 shows an example of a 30 year old male patient with chronic hepatitis C with stage F3 fibrosis. The area under the curve indicates the expected HCC free survival and the area between the two curves is the gain in HCC free survival when the patient achieves SVR. The gain, or the area, was calculated to be 12.4 years in this case.

We similarly calculated the gain in HCC free survival under various conditions (see fig 2, table 5). By definition, these values are applicable only after SVR has been achieved. The gain in HCC free survival that can be expected before the virological outcome is known is the product of the value in table 5 and the prior probability of achieving SVR.

The gain in HCC free survival was greater when a patient was younger or fibrosis was more advanced. Judging by the expected gain, indications for treatment are questionable in patients with fibrosis stage F0 or F1 and older than 60 years because they would gain less than one year even if they

**Table 1** Demographic data for the patients analysed in the current study

| | | Interferon treated | |
|---|---|---|---|
| | Untreated | SVR | Non-SVR |
| No of patients | 395 | 836 | 1556 |
| Age (y) | 55.0 (10.7) | 47.7 (11.9) | 50.5 (6.4) |
| Sex (M/F) | 204/191 | 583/253 | 942/614 |
| Fibrosis stage | | | |
| (F0-1/F2/F3/F4) (n) | 128/141/42/84 | 278/331/173/54 | 440/568/381/167 |
| (F0-1/F2/F3/F4) (%) | 32/36/11/21 | 33/40/21/6 | 28/37/24/11 |

SVR, sustained virological response.

**Table 2** Incidence of hepatocellular carcinoma (HCC) and death in the study cohort

| | Untreated | Interferon treated | |
|---|---|---|---|
| | | SVR | Non-SVR |
| No of patients | 395 | 836 | 1556 |
| Follow up (y) | 6.5 (2.8) | 6.7 (3.0) | 7.4 (2.9) |
| HCC development (n) | 67 | 27 | 214 |
| Death (n) | 33 | 11 | 89 |
| With HCC (n)* | 22 | 6 | 59 |
| Hepatic deaths | | | |
| Without HCC (n) | 4 | 1 | 8 |
| Non-hepatic deaths (n) | 7 | 4 | 22 |

*Includes deaths not directly related to HCC in patients who had developed the cancer.
SVR, sustained virological response.

attained SVR. On the other hand, patients with fibrosis stage F3 or F4 and younger than 50 years will gain more than 10 years with SVR, and more than five years even if the probability of attaining SVR is 50%.

Recently, the efficacy of interferon therapy has been improved by the introduction of peginterferon and ribavirin. However, more effective protocols will be accompanied by an increase in cost and incidence of untoward effects. These must be counterbalanced by an increase in expected benefit. While the increase in cost is the same, that in benefit is directly proportional to the values shown in table 5 and differs in each patient. The SVR rate for type 1b genotype high viral load infection was approximately 7% among the current cohort where six months of interferon monotherapy was the main protocol. The combination of peginterferon and ribavirin for 48 weeks, which is still under clinical trials in Japan, is expected to show a response rate of 40% or better for those patients. This difference (33%) corresponds to five years of the gain in HCC free survival in 40 year old patients with fibrosis stage F4 and to approximately one year in 60 year olds with fibrosis stage F2 (one third of the values given in table 5). Although these values are a hypothetical extrapolation, they may be clinically useful in choosing treatment protocols.

## Model limitations

The model described in this article is based on several assumptions. Firstly, we assumed that interferon therapy that failed to achieve SVR had no beneficial effect, as described above, and this may result in underestimation of the benefit. However, the difference cannot be large: a 30 year old male with fibrosis stage F4 has a gain of 16.59 years instead of 15.98 years, and an 80 year old male with fibrosis stage F0/1 has a gain of 0.18 years instead of 0.15 years if we based the calculations on the incidence observed in the untreated group.

Secondly, we assumed that the benefit of interferon therapy was limited to HCC prevention. This is certainly an underestimation: successful interferon therapy improves liver function and may prevent death from liver failure. Several studies, failing to find an effect on HCC incidence, still indicated improvement in liver function with interferon therapy.[29 30] However, hepatic death without developing HCC was rare in the current cohort; one patient in the SVR group (variceal rupture (n = 1)), eight in the non-SVR group (liver failure (n = 4), variceal rupture (n = 3), not specified (n = 1)), and four in the untreated group (liver failure (n = 2), variceal rupture (n = 2)) died of a liver related cause without developing HCC, indicating annual mortality rates of 0.02%, 0.08%, and 0.18%, respectively (table 2). These values were small relative to the observed incidence of HCC.

Thirdly, we assumed that fibrosis stage remained constant, with the risk of HCC unchanged except for the increment due to aging. This may not be true: in fact, we previously indicated fibrosis progression in untreated patients and amelioration in interferon responders.[23] However, we did

**Table 3** Annual hepatocellular carcinoma (HCC) incidence rates according to age and sex

| Age (y) | F0/1 | F2 | F3 | F4 |
|---|---|---|---|---|
| SVR, male | | | | |
| ≤39 | 0.05% (0/65) | 0.09% (0/59) | 0.16% (0/14) | 0.24% (0/4) |
| 40–49 | 0.05% (0/57) | 0.09% (0/66) | 0.16% (1/29) | 0.24% (0/9) |
| 50–59 | 0.39% (0/38) | 0.69% (3/62) | 1.21% (5/51) | 1.86% (1/18) |
| 60+ | 0.70% (3/29) | 1.18% (3/38) | 2.01% (4/35) | 3.20% (1/9) |
| SVR, female | | | | |
| ≤39 | 0.02% (0/32) | 0.05% (0/38) | 0.10% (0/7) | 0.15% (0/1) |
| 40–49 | 0.03% (0/25) | 0.05% (0/23) | 0.10% (0/3) | 0.15% (1/1) |
| 50–59 | 0.23% (0/20) | 0.41% (1/33) | 0.73% (1/20) | 1.12% (1/6) |
| 60+ | 0.40% (0/6) | 0.71% (1/18) | 1.25% (0/14) | 1.93% (1/6) |
| Non-SVR, male | | | | |
| ≤39 | 0.05% (0/83) | 0.13% (0/72) | 0.28% (2/29) | 0.56% (0/6) |
| 40–49 | 0.35% (2/85) | 1.00% (4/101) | 2.16% (7/46) | 4.26% (10/32) |
| 50–59 | 0.82% (6/82) | 2.33% (19/111) | 5.06% (26/74) | 10.0% (17/33) |
| 60+ | 1.03% (4/36) | 2.93% (13/59) | 6.35% (17/64) | 12.5% (15/29) |
| Non-SVR, female | | | | |
| ≤39 | 0.02% (0/37) | 0.07% (0/21) | 0.14% (0/10) | 0.29% (0/2) |
| 40–49 | 0.18% (0/41) | 0.51% (2/44) | 1.10% (3/18) | 2.17% (0/6) |
| 50–59 | 0.42% (1/53) | 1.19% (8/96) | 2.57% (19/80) | 5.08% (5/32) |
| 60+ | 0.52% (1/23) | 1.49% (11/64) | 3.23% (10/60) | 6.37% (12/27) |

The percentages indicate the annual incidence rates fitted by the least squares method using the risk ratios shown in table 4. Numbers in parentheses are the observed events/number at risk in each category.
SVR, sustained virological response; F0–F4, fibrosis stage.

**Table 4** Risk of hepatocellular carcinoma (HCC) development

| Factor | Relative risk (95% CI) | |
|---|---|---|
| | SVR | Non-SVR |
| Male v female | 1.66 (0.67–4.13) | 1.97 (1.48–2.62) |
| Age (y) | | |
| ≤39 | 1 | 1 |
| 40–49 | 1 | 7.61 (1.81–31.93) |
| 50–59 | 7.67 (1.69–34.72) | 17.84 (4.39–72.49) |
| 60+ | 13.20 (2.93–59.53) | 22.36 (5.48–91.26) |
| Fibrosis stage | | |
| F0/1 | 1 | 1 |
| F2 | 1.76 (0.47–6.67) | 2.86 (1.59–5.13) |
| F3 | 3.10 (0.86–11.26) | 6.19 (3.50–10.95) |
| F4 | 4.78 (1.13–20.18) | 12.23 (6.81–21.95) |

Relative risks were calculated by Cox proportional hazard regression separately in each group.

not have enough samples to calculate age stratified rates of fibrosis progression. The long term changes in HCC risk in interferon responders have not yet been clearly elucidated. Thus using the incidence rates observed in the seven year observation period was a compromise. All of the assumptions listed here may have underestimated, but not overestimated, the benefit of interferon therapy.

Lastly, we did not analyse the effect of alcohol consumption as there were very few heavy drinkers among the cohort. Alcohol is one of the major risk factors for HCC development and liver failure. The merit of successful interferon therapy may be greater in drinkers if they wish to continue alcohol. However, we recommend abstention to chronic hepatitis C patients whether or not they receive antiviral therapy.

## DISCUSSION

To date, large scale cohort studies conducted in Japan, including ours, have unanimously indicated that by far the most important sequela of chronic hepatitis C is HCC development, and that interferon therapy significantly reduces its incidence. In contrast, several studies performed in Western countries found that HCC was less common, and interferon therapy did not have significant effects. The reason for this discrepancy has not been clarified. In this study, we showed that the risk of HCC substantially increased with age when patients of the same sex and fibrosis stage were compared (table 2). The prevalence of HCV infection in Japan is highly skewed to the older generations, and this may partly explain the high incidence of HCC in Japan. If this is the case, HCC incidence will increase substantially in Western countries in the near future, as it did in Japan in the 1980s.

The clinical importance of interferon therapy should be measured in terms of its efficacy in HCC prevention, at least in countries where HCC is the predominant complication of HCV infection. A popular indicator of efficacy of therapy in preventing a disease is the number (of patients) needed to treat (NNT), which is identical to the inverse of absolute risk reduction. Mathematically, NNT for one decrement in HCC development during a lifetime is equivalent to the life expectancy divided by the gain in HCC free survival. Supposing that the SVR rate is 100%, NNT is 3.92 (48.7/12.4; table 5) for a Japanese 30 year old male patient with fibrosis stage F3. This value should be divided by the expected SVR rate if the outcome is not known. As NNT is directly proportional to life expectancy, older patients have smaller values for NNT, indicating "higher efficacy", if the gain in HCC free survival is the same. This may not be intuitive for individualised consideration of indications for treatment.

Several authors have performed cost effective analyses of interferon therapy for chronic hepatitis C based on the Markov model.[31–35] In fact, our current model can be considered as a simplified Markov model where a chronic hepatitis C patient either achieves or does not achieve SVR with interferon therapy, and has the corresponding risk of HCC thereafter. Also, the HCC free survival is equivalent to the gain in quality adjusted life year, where a year before HCC development scores 1 and one after it scores 0. Owing to those simplifications, the model is not dependent on assumptive parameters but on observed data.

In conclusion, by using data obtained in a real cohort, we established an indicator of the benefit of interferon therapy—the gain in HCC free survival. This indicator may be useful in assessing the indications for interferon therapy and in selecting the best treatment protocol for individual patients.

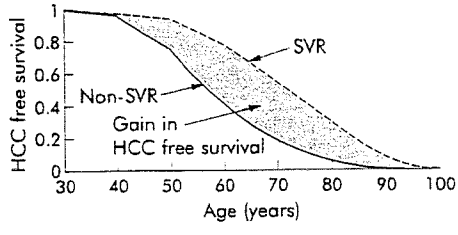


**Figure 1** Gain in hepatocellular carcinoma (HCC) free survival by interferon therapy. The case of a 30 year old male patient with fibrosis stage F3. Cumulative HCC free survival curves were determined based on the patient specific HCC incidence rates and age and sex specific death rates in case of sustained virological response (SVR) and non-SVR. The area surrounded by the two curves indicates the gain in HCC free survival obtained by achieving SVR.

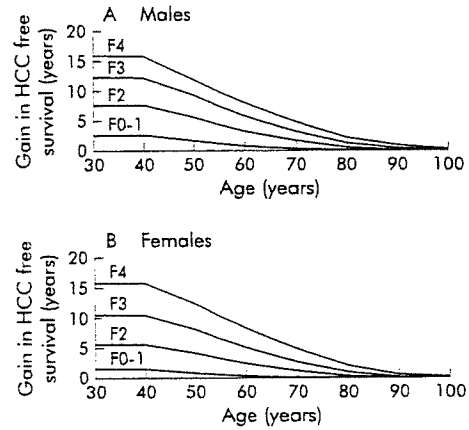


**Figure 2** Gain in hepatocellular carcinoma (HCC) free survival by sustained virological response as a function of age and fibrosis stage.

**Table 5** Gain in hepatocellular carcinoma (HCC) free survival by sustained virological response as a function of age and fibrosis stage

| Age (y) | Life expectancy | F0/1 | F2 | F3 | F4 |
|---|---|---|---|---|---|
| Males | | | | | |
| 30 | 48.7 | 2.48 | 7.66 | 12.40 | 15.98 |
| 40 | 39.1 | 2.52 | 7.68 | 12.41 | 15.96 |
| 50 | 29.9 | 1.68 | 5.75 | 9.45 | 12.14 |
| 60 | 21.4 | 0.84 | 3.38 | 5.95 | 8.14 |
| 70 | 14.0 | 0.40 | 1.70 | 3.26 | 4.98 |
| 80 | 8.0 | 0.15 | 0.67 | 1.40 | 2.38 |
| Females | | | | | |
| 30 | 55.3 | 1.45 | 5.60 | 10.52 | 15.73 |
| 40 | 45.5 | 1.46 | 5.61 | 10.51 | 15.69 |
| 50 | 36.0 | 0.93 | 4.24 | 8.17 | 12.44 |
| 60 | 26.9 | 0.44 | 2.52 | 5.17 | 8.39 |
| 70 | 18.2 | 0.22 | 1.30 | 2.81 | 4.95 |
| 80 | 10.6 | 0.08 | 0.52 | 1.18 | 2.24 |

Expressed in years, life expectancy was that in the Japanese general population in 2000. The gain in HCC free survival was the difference in expected cumulative HCC free survival with and without attaining a sustained virological response.

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## REFERENCES

1 **Yamada G**, Tanaka E, Miura T, *et al.* Epidemiology of genotypes of hepatitis C virus in Japanese patients with type C chronic liver diseases: a multi-institution analysis. *J Gastroenterol Hepatol* 1995;**10**:538–45.
2 **Yano M**, Kumada H, Kage H, *et al.* The long-term pathological evolution of chronic hepatitis C. *Hepatology* 1996;**23**:1334–40.
3 **Moriya T**, Koyama T, Tanaka J, *et al.* Epidemiology of hepatitis C virus in Japan. *Intervirology* 199;**42**:153–8.
4 **Wasley A**, Alter MJ. Epidemiology of hepatitis C: geographic differences and temporal trends. *Semin Liver Dis* 2000;**20**:1–16.
5 **Tanaka Y**, Hanada K, Mizokami M, *et al.* Inaugural article: A comparison of the molecular clock of hepatitis C virus in the United States and Japan predicts that hepatocellular carcinoma incidence in the United States will increase over the next two decades. *Proc Natl Acad Sci U S A* 2002;**99**:15584–9.
6 **Kuo G**, Choo QL, Alter HJ, *et al.* An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. *Science* 1989;**244**:362–4.
7 **Choo QL**, Kuo G, Weiner AJ, *et al.* Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* 19789;**244**:359–62.
8 **Hoofnagle JH**, Mullen KD, Jones DB, *et al.* Treatment of chronic non-A, non-B hepatitis with recombinant human alpha interferon. A preliminary report. *N Engl J Med* 1986;**315**:1575–8.
9 **Di Bisceglie AM**, Martin P, Kassianides C, *et al.* Recombinant interferon alfa therapy for chronic hepatitis C. A randomized, double-blind, placebo-controlled trial. *N Engl J Med* 1989;**321**:1506–10.
10 **Omata M**, Yokosuka O, Takano S, *et al.* Resolution of acute hepatitis C after therapy with natural beta interferon. *Lancet* 1991;**338**:914–15.
11 **Nishiguchi S**, Kuroki T, Nakatani S, *et al.* Randomised trial of effects of interferon-alpha on incidence of hepatocellular carcinoma in chronic active hepatitis C with cirrhosis. *Lancet* 1995;**346**:1051–5.
12 **Mazzella G**, Accogli E, Sottili S, *et al.* Alpha interferon treatment may prevent hepatocellular carcinoma in HCV-related liver cirrhosis. *J Hepatol* 1996;**24**:141–7.
13 **Kasahara A**, Hayashi N, Mochizuki K, *et al.* Risk factors for hepatocellular carcinoma and its incidence after interferon treatment in patients with chronic hepatitis C. Osaka Liver Disease Study Group. *Hepatology* 1998;**27**:1394–402.
14 **Imai Y**, Kawata S, Tamura S, *et al.* Relation of interferon therapy and hepatocellular carcinoma in patients with chronic hepatitis C. Osaka Hepatocellular Carcinoma Prevention Study Group. *Ann Intern Med* 1998;**129**:94–9.
15 **Yoshida H**, Shiratori Y, Moriyama M, *et al.* Interferon therapy reduces the risk for hepatocellular carcinoma: national surveillance program of cirrhotic and non-cirrhotic patients with chronic hepatitis C in Japan. IHIT Study Group. Inhibition of Hepatocarcinogenesis by Interferon Therapy. *Ann Intern Med* 1999;**131**:174–81.
16 **Reichard O**, Norkrans G, Fryden A, *et al.* Randomised, double-blind, placebo-controlled trial of interferon alpha-2b with and without ribavirin for chronic hepatitis C. The Swedish Study Group. *Lancet* 1998;**351**:83–7.
17 **Poynard T**, Marcellin P, Lee SS, *et al.* Randomised trial of interferon alpha2b plus ribavirin for 48 weeks or for 24 weeks versus interferon alpha2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. International Hepatitis Interventional Therapy Group (IHIT). *Lancet* 1998;**352**:1426–32.
18 **McHutchison JG**, Gordon SC, Schiff ER, *et al.* Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. *N Engl J Med* 1998;**339**:1485–92.
19 **Zeuzem S**, Feinman SV, Rasenack J, *et al.* Peginterferon alfa-2a in patients with chronic hepatitis C. *N Engl J Med* 2000;**343**:1666–72.
20 **Manns MP**, McHutchison JG, Gordon SC, *et al.* Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001;**358**:958–65.
21 **Lindsay KL**, Trepo C, Heintges T, *et al.* A randomized, double-blind trial comparing pegylated interferon alfa-2b to interferon alfa-2b as initial treatment for chronic hepatitis C. *Hepatology* 2001;**34**:395–403.
22 **Fried MW**, Shiffman ML, Reddy K, *et al.* Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002;**347**:975–82.
23 **Shiratori Y**, Imazeki F, Moriyama M, *et al.* Histologic improvement of fibrosis in patients with hepatitis C who have sustained response to interferon therapy. *Ann Intern Med* 2000;**132**:517–24.
24 **Yoshida H**, Arakawa Y, Sata M, *et al.* Interferon therapy prolonged life expectancy among chronic hepatitis C patients. *Gastroenterology* 2002;**123**:483–91.
25 **Management of Hepatitis C**. NIH Consensus Statement Online 1997 Mar 24–26;15(3):1–41. http://odp.od.nih.gov/consensus/cons/105/105_statement.htm.
26 **Desmet VJ**, Gerber M, Hoofnagle JH, *et al.* Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology* 1994;**19**:1513–20.
27 The 19th National Complete Life Table (2000). The Ministry of Health, Welfare and Labour of Japan. http://www.mhlw.go.jp/toukei/saikin/hw/life/19th/.
28 **Shindo M**, Hamada K, Oda Y, *et al.* Long-term follow-up study of sustained biochemical responders with interferon therapy. *Hepatology* 2001;**33**:1299–302.
29 **Fattovich G**, Giustina G, Degos F, *et al.* Effectiveness of interferon alfa on incidence of hepatocellular carcinoma and decompensation in cirrhosis type C. European Concerted Action on Viral Hepatitis (EUROHEP). *J Hepatol* 1997;**27**:201–5.
30 **Niederau C**, Lange S, Heintges T, *et al.* Prognosis of chronic hepatitis C: results of a large, prospective cohort study. *Hepatology* 1998;**28**:1687–95.
31 **Bennett WG**, Inoue Y, Beck JR, *et al.* Estimates of the cost-effectiveness of a single course of interferon-alpha 2b in patients with histologically mild chronic hepatitis C. *Ann Intern Med* 1997;**127**:855–65.

32 **Younossi ZM**, Singer ME, McHutchison JG, *et al*. Cost effectiveness of interferon alpha2b combined with ribavirin for the treatment of chronic hepatitis C. *Hepatology* 1999;**30**:1318–24.
33 **Stein K**, Rosenberg W, Wong J. Cost effectiveness of combination therapy for hepatitis C: a decision analytic model. *Gut* 2002;**50**:253–8.
34 **Buti M**, Medina M, Casado MA, *et al*. A cost-effectiveness analysis of peginterferon alfa-2b plus ribavirin for the treatment of naive patients with chronic hepatitis C. *Aliment Pharmacol Ther* 2003;**17**:687–94.
35 **Siebert U**, Sroczynski G, Rossol S, *et al*. Cost effectiveness of peginterferon alpha-2b plus ribavirin versus interferon alpha-2b plus ribavirin for initial treatment of chronic hepatitis C. *Gut* 2003;**52**:425–32.

PRELIMINARY COMMUNICATION

# Role of Vitamin $K_2$ in the Development of Hepatocellular Carcinoma in Women With Viral Cirrhosis of the Liver

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**Context** Previous findings indicate that vitamin $K_2$ (menaquinone) may play a role in controlling cell growth.

**Objective** To determine whether vitamin $K_2$ has preventive effects on the development of hepatocellular carcinoma in women with viral cirrhosis of the liver.

**Design, Setting, and Participants** Forty women diagnosed as having viral liver cirrhosis were admitted to a university hospital between 1996 and 1998 and were randomly assigned to the treatment or control group. The original goal of the trial was to assess the long-term effects of vitamin $K_2$ on bone loss in women with viral liver cirrhosis. However, study participants also satisfied criteria required for examination of the effects of such treatment on the development of hepatocellular carcinoma.

**Interventions** The treatment group received 45 mg/d of vitamin $K_2$ (n=21). Participants in the treatment and control groups received symptomatic therapy to treat ascites, if necessary, and dietary advice.

**Main Outcome Measure** Cumulative proportion of patients with hepatocellular carcinoma.

**Results** Hepatocellular carcinoma was detected in 2 of the 21 women given vitamin $K_2$ and 9 of the 19 women in the control group. The cumulative proportion of patients with hepatocellular carcinoma was smaller in the treatment group (log-rank test, $P=.02$). On univariate analysis, the risk ratio for the development of hepatocellular carcinoma in the treatment group compared with the control group was 0.20 (95% confidence interval [CI], 0.04-0.91; $P=.04$). On multivariate analysis with adjustment for age, alanine aminotransferase activity, serum albumin, total bilirubin, platelet count, α-fetoprotein, and history of treatment with interferon alfa, the risk ratio for the development of hepatocellular carcinoma in patients given vitamin $K_2$ was 0.13 (95% CI, 0.02-0.99; $P=.05$).

**Conclusion** There is a possible role for vitamin $K_2$ in the prevention of hepatocellular carcinoma in women with viral cirrhosis.

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WE PREVIOUSLY REPORTED a 2-year study showing that vitamin $K_2$ (menaquinone) helps to prevent bone loss in women with viral cirrhosis of the liver.[1] Most of the women agreed to participate in a longer study to clarify the long-term effects of vitamin $K_2$ on bone loss associated with cirrhosis. The incidence of hepatocellular carcinoma was found to differ between women who received vitamin $K_2$ and those who did not.

## METHODS

The participants in this study were 50 women with viral liver cirrhosis who were admitted to a university hospital between 1996 and 1998. When the results of abdominal dynamic computed tomography and abdominal ultrasonography suggested the presence of hepatocellular carcinoma, abdominal angiography or needle biopsy was performed to confirm the diagnosis. The diagnosis of cirrhosis was based on histological examination of liver specimens obtained by laparoscopy or needle biopsy performed under ultrasonic guidance.

Hepatocellular carcinoma was confirmed in 3 women in the treatment group and 4 in the control group. These 7 women were excluded from further study. The remaining 43 women were randomly assigned using sealed envelopes to a treatment or control group. The treatment group received 45 mg/d of vitamin $K_2$ (Glakay, Eisai Co, Tokyo, Japan). At the end of the first study, 21 women in the treatment group and 19 in the control group consented to participate in a longer trial. All but 1 woman in each group had hepatitis C

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virus infection; the other 2 women had hepatitis B infection. Seven women, 4 in the control group and 3 in the treated group, had previously received interferon alfa for their hepatitis C virus infections, but hepatitis C virus was not eradicated. No one was given interferon therapy after study entry.

Surveillance for hepatocellular carcinoma was performed according to detailed procedures recommended for follow-up of patients with liver cirrhosis in Japan.[2] Abdominal ultrasonography or abdominal dynamic computed tomography was performed and serum α-fetoprotein levels measured tumors at 3-month intervals. Any abnormality was followed up by tumor biopsy or abdominal angiography to confirm a diagnosis of hepatocellular carcinoma.

Diagnosed cases of hepatocellular carcinoma were classified according to the primary tumor, regional lymph nodes, and distant metastasis (TNM) system of the International Union Against Cancer.[3] Histopathologic diagnosis of hepatocellular carcinoma was performed according to the criteria proposed by Edmondson and Steiner.[4] Compliance with vitamin $K_2$ in the treatment group was good; no patient had adverse reactions or dropped out of the study. This trial was conducted in accordance with the Declaration of Helsinki and was approved by the ethics committee at the Osaka City University Medical School. Written informed consent was obtained from each participant.

Statistical analysis was performed using SAS statistical software (version 8.12, SAS Institute Inc, Cary, NC). The $\chi^2$ test was used to assess homogeneity between the groups. Cumulative incidences were plotted using the Kaplan-Meier method and the statistical significance of differences was analyzed using the log-rank test. Cox regression analysis was used for univariate and multivariate analyses. $P<.05$ was considered significant.

## RESULTS

The 2 groups were similar with respect to age, virus type, platelets, alanine aminotransferase, α-fetoprotein, and other clinical findings (TABLE 1). Risk factors for hepatocellular carcinoma[5,6] were also similar between the groups. After the first study commenced, hepatocellular carcinoma was detected in 2 of the 21 patients given vitamin $K_2$ and in 9 of the 19 patients in the control group (FIGURE 1). The cumulative proportion of women with hepatocellular carcinoma was smaller in the treatment group compared with the control group (log-rank test, $P=.02$; FIGURE 2). The clinical characteristics of the women in whom hepatocellular carcinoma was detected during surveillance are shown in TABLE 2. All newly diagnosed cases of hepatocellular carcinoma were stage I or II according to the International Union Against Cancer classification and were given aggressive anticancer therapy. On univariate analysis, the risk ratio for the development of hepatocellular carcinoma in the treatment group compared with the control group was 0.20 (95% confidence interval, 0.04-0.91; $P=.04$; TABLE 3). On multivariate analysis with adjustment for age, alanine aminotransferase activity, serum albumin, total bilirubin, platelet count, α-fetoprotein, and history of treat-

**Table 1.** Baseline Characteristics*

| | Treatment Group (n = 21) | Control Group (n = 19) | P Value |
|---|---|---|---|
| Age, y | 59.8 (8.7) | 61.4 (7.1) | .54† |
| Hepatitis virus, No. (%) | | | |
| B | 1 (4) | 1 (5) | .94‡ |
| C | 20 (95) | 18 (95) | |
| Albumin, g/dL | 3.9 (0.3) | 3.9 (0.3) | .87† |
| Platelets, $\times 10^3$/mL | 147 (54) | 121 (52) | .13† |
| Total bilirubin, mg/dL | 0.8 (0.2) | 0.9 (0.4) | .47† |
| Alanine aminotransferase, U/L | 81.7 (42.7) | 70.4 (33.4) | .36† |
| α-Fetoprotein, mg/mL | 13.4 (17.7) | 13.3 (8.7) | .99† |
| Interferon, No. (%) | | | |
| Prior to enrollment§ | 4 (19) | 3 (16) | .79‡ |
| At enrollment | 17 (81) | 16 (84) | |

SI conversion units: To convert bilirubin to μmol/L, multiply by 17.1.
*Values expressed as mean (SD) unless otherwise indicated.
†Values calculated using the Mann-Whitney U test.
‡Values calculated using the $\chi^2$ test.
§Hepatocellular carcinoma developed in 1 of 4 patients in the treatment group and 1 of 3 patients in the control group who received interferon prior to enrollment.

**Figure 1.** Flow of Participants Through Trial

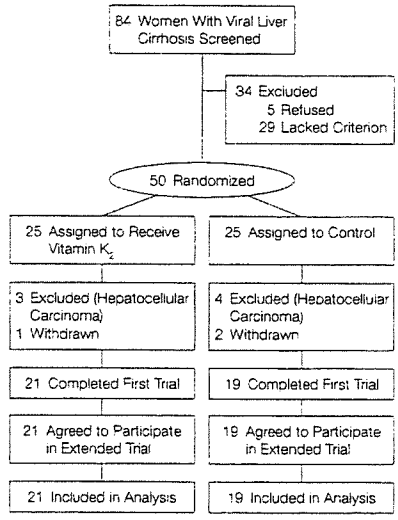


**Figure 2.** Cumulative Incidence of Hepatocellular Carcinoma Diagnosed in Women Treated With Vitamin $K_2$

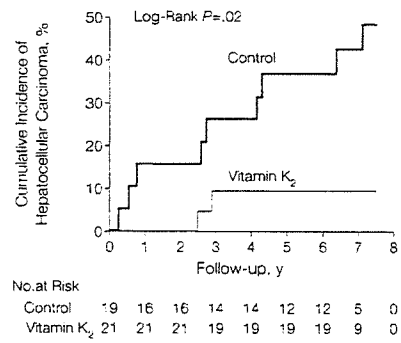

**Table 2.** Profiles of Women With Hepatocellular Carcinoma

| Case No./ Age, y | Group | Type of Hepatitis Virus | No. of Days Diagnosis Occurred After Enrollment | No. of Tumors | Diameter of Largest Tumor, mm | UICC Cancer Stage | Method of Diagnosis | Histological Grade | Type of Therapy |
|---|---|---|---|---|---|---|---|---|---|
| 1/70 | Control | C | 200 | 1 | 15 | 1 | Biopsy | 1* | PEIT |
| 5/62 | Control | C | 2333 | 2 | 20 | 2 | AAG | Unknown | TAE |
| 6/59 | Control | C | 282 | 1 | 9 | 1 | Biopsy | 1* | PEIT |
| 9/70 | Control | C | 91 | 1 | 13 | 1 | Biopsy | 1* | PEIT |
| 21/43 | Control | C | 1516 | 1 | 30 | 2 | Biopsy | 2* | MCT |
| 25/59 | Control | C | 1569 | 2 | 18 | 2 | AAG | Unknown | TAE |
| 26/67 | Control | B | 949 | 1 | 32 | 2 | AAG | Unknown | TAE |
| 33/57 | Control | C | 2600 | 1 | 30 | 2 | AAG | Unknown | TAE |
| 40/68 | Control | C | 1002 | 1 | 21 | 2 | Biopsy | 3* | Operation |
| 4/64 | Vitamin $K_2$ | C | 907 | 1 | 30 | 2 | AAG | Unknown | TAE |
| 27/68 | Vitamin $K_2$ | C | 1054 | 1 | 11 | 1 | Biopsy | 1* | PEIT |

Abbreviations: AAG, abdominal angiography; MCT, microwave coagulation therapy; PEIT, percutaneous ethanol injection therapy; TAE, transcatheter hepatic arterial embolization; UICC, International Union Against Cancer.
*Based on Edmondson and Steiner classification system (scale of 1-4).[4]

**Table 3.** Crude Odds Ratios for Development of Hepatocellular Carcinoma

| | Odds Ratio (95% Confidence Interval) | *P* Value |
|---|---|---|
| Group* | 0.20 (0.04-0.91) | .04 |
| Total bilirubin | 1.23 (0.33-4.64) | .76 |
| Albumin | 3.80 (0.80-18.06) | .09 |
| Platelets | 1.55 (0.46-5.19) | .48 |
| Alanine aminotransferase | 0.62 (0.17-2.36) | .49 |
| α-Fetoprotein | 1.67 (0.36-7.79) | .51 |
| Interferon | 1.00 (0.22-4.65) | >.99 |

*Vitamin $K_2$ group compared with control group.

**Table 4.** Adjusted Odds Ratios for Development of Hepatocellular Carcinoma*

| | Odds Ratio (95% Confidence Interval) | *P* Value |
|---|---|---|
| Group† | 0.13 (0.02-0.99) | .05 |
| Total bilirubin | 0.29 (0.04-2.04) | .22 |
| Albumin | 33.43 (2.36-473.35) | .009 |
| Platelets | 2.24 (0.46-10.90) | .32 |
| Alanine aminotransferase | 0.39 (0.07-2.16) | .28 |
| α-Fetoprotein | 1.69 (0.31-9.34) | .55 |
| Interferon | 1.26 (0.20-7.90) | .81 |

*Values are adjusted for age and all other variables in this table.
†Vitamin $K_2$ group compared with control group.

ment with interferon alfa, the risk ratio for the development of hepatocellular carcinoma in women given vitamin $K_2$ was 0.13 (95% confidence interval, 0.02-0.99; $P = .05$; TABLE 4).

## COMMENT

Vitamin K is a cofactor for the enzyme γ-glutamyl-carboxylase, which converts glutamate residues into γ-carboxyglutamate. Vitamin K–dependent proteins include prothrombin II and the coagulation factors VII, IX, X, proteins C and S, osteocalcin, surfactant-associated proteins, and bone matrix protein. The vitamin K family of molecules comprises the natural forms vitamin $K_1$ (phylloquinone) and vitamin $K_2$ (menaquinones) and the synthetic form of vitamin $K_3$-(menadione). These naphthoquinone-containing molecules inhibit tumor cell growth in culture, with vitamin $K_3$ being more potent than either vitamin $K_1$ or $K_2$. Vitamin $K_2$ inhibits growth of human cancer cell lines and induction of differentiation in various human myeloid leukemia cell lines.[7,8] Clinically, vitamin $K_2$ has successfully treated myelodysplastic syndrome.[9]

A number of findings indicate that vitamin K may play a role in controlling cell growth. Underlying mechanisms possibly involve (1) cycling of oxidation and reduction (as known for vitamin $K_3$), (2) proteins with growth-inhibitory properties induced by vitamin K, such as prothrombin,[10] (3) previously unidentified pathways involving arylation,[11] (4) or growth arrest genes such as gas 6.[12] Geranylgeraniol, which is a side chain of vitamin $K_2$, strongly induces apoptosis of tumor cells, suggesting that geranylgeraniol might play an important role in inhibiting cell growth.[13] The mechanisms responsible for the inhibition of cell growth mediated by vitamin $K_2$ remain unexplained. These or other hypothetical mechanisms may have contributed to the reduced hepatocellular carcinoma incidence among patients receiving vitamin $K_2$. Indeed, the annual incidence of hepatocellular carcinoma in the control group was 8.8%, which is similar to the incidence of hepatocellular carcinoma (7.9%; 32/107) in cirrhotic patients in Japan[5] compared with 1.6% in the treatment group.

As shown in Table 4, the albumin level showed the highest odds ratio for the development of hepatocellular carcinoma. The serum albumin level is considered an important prognostic factor in liver cirrhosis.[14-17] Low serum albumin levels in patients with liver cirrhosis are associated with disease progression, poor nutritional status, and compromised immunity, which increases the risk of carcinogenesis. The importance of low serum albumin levels as a risk factor for hepatocellular carcinoma should be confirmed in larger studies.

The original goal of our trial was to assess the long-term effects of vitamin $K_2$ on bone loss in women with viral liver cirrhosis. Our trial had several important limitations when the data were used to assess the value of vitamin $K_2$ for the primary prevention of hepatocellular carcinoma in patients with liver cirrhosis, resulting from the small study group,

the inclusion of only women, and the participation of only 1 center. However, similar to previously reported randomized controlled studies of cirrhosis in which the primary end point was the development of hepatocellular carcinoma, patients with evidence of hepatocellular carcinoma on highly sensitive imaging studies were excluded, and the 2 study groups were similar with respect to risk factors for hepatocellular carcinoma, such as age, severity of cirrhosis, history of interferon therapy, and type of hepatitis virus infection. The procedures used for the surveillance and diagnosis of hepatocellular carcinoma were also similar to those used in our study. The sensitivity of these procedures for the detection of hepatocellular carcinoma was underscored by the fact that all of the detected cases of hepatocellular carcinoma were stage I or stage II. Our results must also be tempered by the fact that 3 cases of hepatocellular carcinoma were diagnosed in the control group within a year of enrollment. These patients may have harbored occult disease at the time of enrollment. Nonetheless, despite its small size, our study indicates that vitamin $K_2$ decreases the risk of hepatocellular carcinoma to about 20% compared with the control group, suggesting that vitamin $K_2$ may delay the onset of hepatocarcinogenesis. Moreover, the safety, relatively low cost, and ease of use of vitamin $K_2$ led to good compliance with treatment. The results of this preliminary trial are intriguing and suggest that a potential role for vitamin $K_2$ to prevent hepatocarcinogenesis in patients with liver cirrhosis. These results must be confirmed by multicenter randomized controlled studies with the prevention of hepatocellular carcinoma by vitamin $K_2$ as the primary end point.

**Author Contributions:** Dr Shiomi had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
*Study concept and design:* Habu, Shiomi, Kubo, Nishiguchi.
*Acquisition of data:* Habu, Shiomi, Tamori, Takeda, Nishiguchi.
*Analysis and interpretation of data:* Habu, Shiomi, Tamaka, Kubo, Nishiguchi.
*Drafting of the manuscript:* Habu, Shiomi, Tamori, Takeda, Kubo, Nishiguchi.
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*Statistical Analysis:* Habu, Tamaka.
*Obtained funding:* Habu, Shiomi, Tamori, Takeda, Nishiguchi.
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*Supervision:* Shiomi, Nishiguchi, Kubo.
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REFERENCES

1. Shiomi S, Nishiguchi S, Kubo S, et al. Vitamin K2 (menatetrenone) for bone loss in patients with cirrhosis of the liver. *Am J Gastroenterol.* 2002;97:978-981.
2. Nishiguchi S, Kuroki T, Nakatani S, et al. Randomised trial of effects of interferon-α on incidence of hepatocellular carcinoma in chronic active hepatitis C with cirrhosis. *Lancet.* 1995;346:1051-1055.
3. International Union Against Cancer *TNM Classification of Malignant Tumors.* Sobin LH, Wittekind Ch, eds. 5th ed. New York, NY: Wiley-Liss; 1997.
4. Edmondson HA, Steiner PE. Primary carcinoma of the liver: a study of 100 cases among 48,900 necropsies. *Cancer.* 1954;7:462-503.
5. Yoshida H, Shiratori Y, Moriyama M, et al. Interferon therapy reduces the risk for hepatocellular carcinoma: national surveillance program of cirrhotic and noncirrhotic patients with chronic hepatitis C in Japan. *Ann Intern Med.* 1999;131:174-181.
6. Ikeda K, Saitoh S, Koide I, et al. A multivariate analysis of risk factors for hepatocellular carcinogenesis: a prospective observation of 795 patients with viral and alcoholic cirrhosis. *Hepatology.* 1993;18:47-53.
7. Sasaki I, Hashimoto S, Yoda M, et al. Novel role of vitamin K2: a potent inducer of differentiation of various human myeloid leukemia cell lines. *Biochem Biophys Res Commun.* 1994;205:1305-1310.
8. Nishimaki J, Miyazawa K, Yamaguchi M, et al. Vitamin K2 induces apoptosis of a novel cell line established from a patients with myelodysplastic syndrome in blastic formation. *Leukemia.* 1999;13:1399-1405.
9. Takami A, Nakao S, Ontachi Y, et al. Successful therapy of myelodysplastic syndrome with menatetrenone, a vitamin K2 analog. *Int J Hematol.* 1999;69:24-26.
10. Carr BI, Wang Z, Kar S, Wang M. Prothrombin inhibits hepatocyte DNA synthesis (DNA-S) and expression of the α5-integrin gene. *Proc Am Assoc Cancer Res.* 1995;36:266.
11. Kar S, Carr BI. Growth inhibition and protein tyrosine phosphorylation in MCF breast cancer cells by a novel K vitamin. *J Cell Physiol.* 2000;185:386-393.
12. Varnum BC, Young C, Elliott G, et al. Axl receptor tyrosine kinase stimulated by the vitamin K-dependent protein encoded by growth-arrest-specific gene 6. *Nature.* 1995;373:623-626.
13. Ohizumi H, Masuda Y, Nakajo S, et al. Geranylgeraniol is a potent inducer of apoptosis in tumor cells. *J Biochem.* 1995;117:11-13.
14. Pere G, Enrique Q, Vicente A, et al. Compensated cirrhosis: natural history and prognostic factors. *Hepatology.* 1987;7:122-128.
15. Marco Z, Maria RC, Guulio M, et al. Prognostic indicators in compensated cirrhosis. *Am J Gastroenterol.* 1991;86:1508-1513.
16. Jerome G, Lawrence S, Anne MB, Olivier C, Renee EP, Raoul P. Prognostic value of serum hyaluronan in patients with compensated HCV cirrhosis. *J Hepatol.* 2000;32:447-452.
17. Orimo H, Shiraki M, Tomita A, Morii H, Fujita T, Ohata M. Effects of menatetrenone on the bone and calcium metabolism in osteoporosis: a double-blind placebo-controlled study *J Bone Miner Metab.* 1998; 16:106-112.

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## LETTERS TO THE EDITOR

# Adefovir Dipivoxil to Prevent Exacerbation of Lamivudine-resistant Hepatitis B Infection during Chemotherapy for Non-Hodgkin's Lymphoma

TO THE EDITOR: Lamivudine is a potent inhibitor of hepatitis B virus (HBV) replication and is widely used for the treatment of chronic hepatitis B (1). In HBV carriers receiving cytotoxic or immunosuppressive chemotherapy for hematological malignancies, acute exacerbation of hepatitis following viral reactivation is well documented; therefore, prophylactic or therapeutic use of lamivudine is highly recommended (2, 3). However, prolonged treatment may induce the emergence of lamivudine-resistant variants with mutations in the reverse transcriptase (rt) domain of the HBV polymerase gene. There is currently no consensus on the management of exacerbations of chronic HBV infection in patients with lamivudine-resistant variants during chemotherapy. Adefovir dipivoxil, another nucleotide analogue, has recently demonstrated potency against HBV, including lamivudine-resistant strains (4–6). We hereby report a patient with lamivudine-resistant HBV who was treated with adefovir dipivoxil during chemotherapy for non-Hodgkin's lymphoma.

A 44-yr-old woman with malaise and generalized lymphadenopathy was admitted to our hospital in December 2002. About 5 yr previously, chronic hepatitis B had been diagnosed. Her mother and all four siblings were also carriers of HBV. Treatment with lamivudine was started at a daily dose of 100 mg in June 2001. The subsequent clinical course is shown in Figure 1. Shortly after the start of therapy, the serum HBV DNA level declined, and transaminase activity normalized by wk 20. At wk 40 of therapy, viral DNA became undetectable by transcription-mediated amplification assay (< 3.7 $\log_{10}$ copies/ml). At wk 64, however, lamivudine-resistant HBV variants emerged, and viral DNA became detectable again in serum. On admission (at wk 70), alanine aminotransferase (ALT) activity was 21 IU/L, aspartate aminotransferase (AST) activity 45 IU/L, lactate dehydrogenase activity 872 IU/L, and $\gamma$-glutamyltransferase activity 29 IU/L. The hepatitis B e (HBe) antigen was positive and the anti-HBe negative, with an HBV DNA level of 6.7 $\log_{10}$ copies/ml. The genotype of the HBV was type C. The stop codon mutation at nucleotide (nt) 1,896 in the precore region of HBV DNA was not found, but mutations were found at nt1762 and nt1764 in the basal core promoter. Mutations related to lamivudine resistance were detected from leucine to methionine at amino acid rt180 and from methionine to valine at rt204.

On the basis of the findings of lymph node biopsy, ultrasonography, computed tomography, and gallium scintigraphy, diffuse large-B-cell non-Hodgkin's lymphoma (stage III) was diagnosed. Five courses of chemotherapy with adriamycin, cyclophosphamide, and vincristine were administered

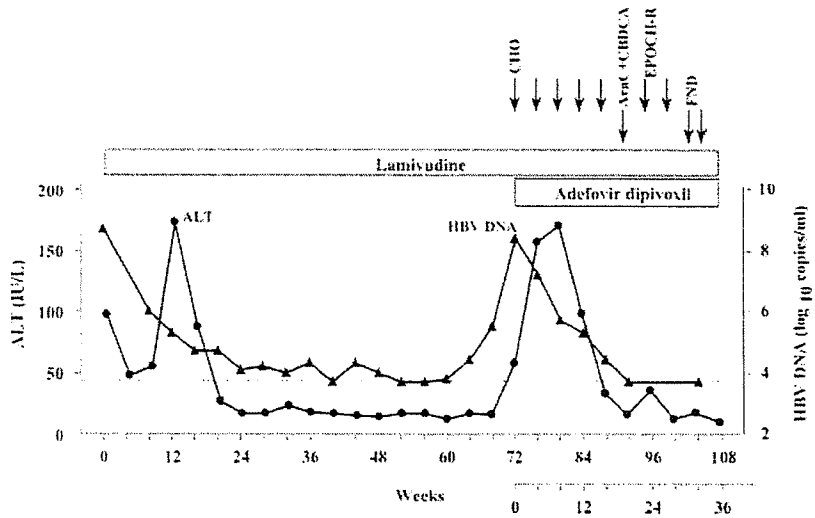


**Figure 1.** ALT and HBV DNA levels during the clinical course of the patient from the time of initiation of lamivudine treatment. CHO, adriamycin, cyclophosphamide, and vincristine; AraC, cytarabine; CBDCA, carboplatin; EPOCH-R, rituximab plus etoposide, prednisone, vincristine, cyclophosphamide, and doxorubicin; FND, fludarabine, mitoxantrone, and dexamethasone. Broken line shows the detection limit of the transcription-mediated amplification assay (3.7 $\log_{10}$ copies/ml of HBV DNA).

between December 2002 and April 2003. After three courses of chemotherapy, lymph nodes regressed considerably in size. However, lymphoma recurred after the fifth course; involvement of the liver and spleen were found on gallium scintigraphy. The patient did not respond to subsequent intensive chemotherapy with a combination of cytarabine and carboplatin in May 2003, two courses of rituximab plus etoposide, prednisone, vincristine, cyclophosphamide, and doxorubicin between June and July 2003, or two courses of fludarabine, mitoxantrone, and dexamethasone in August 2003. On September 25, the patient died suddenly of rupture of splenic lesions.

At the start of antineoplastic chemotherapy, the ALT activity was 58 IU/L, and the AST activity 71 IU/L with an HBV DNA level of 8.3 $\log_{10}$ copies/ml. To prevent chemotherapy-induced exacerbation of lamivudine-resistant HBV infection, treatment with adefovir dipivoxil at 10 mg daily was added to the lamivudine. The serum HBV DNA level fell immediately after the commencement of adefovir dipivoxil and became undetectable at wk 20 of therapy. HBe antigen did not seroconvert to anti-HBe. ALT activity was 170 IU/L at wk 8 of therapy, decreased thereafter, and normalized at wk 16 of therapy. During 36 wk of therapy with adefovir dipivoxil, suppression of HBV replication was sustained, allowing chemotherapy to proceed without delay or modification, albeit the response to chemotherapy was not favorable. There were no clinically significant adverse events related to adefovir dipivoxil, such as nephrotoxicity, which has been reported with higher doses ($\geq$ 30 mg daily).

Ohmoto *et al.* described a case of lamivudine-resistant HBV reactivation that was successfully treated with a combination of lamivudine and interferon-$\alpha$ during chemotherapy for non-Hodgkin's lymphoma (7). However, randomized controlled trials have not shown long-term beneficial effects of this combination (8, 9). Interferon-$\alpha$ sometimes causes serious adverse effects, including hemopoietic toxicity. Adefovir dipivoxil is generally well tolerated. Resistance to adefovir dipivoxil was not found in large, placebo-controlled 48-wk studies (5, 6), although a novel mutation associated with resistance has more recently been identified in the HBV polymerase gene (10).

There remain two unsolved issues related to the treatment schedule. First, should adefovir dipivoxil be initiated as prophylaxis in patients with lamivudine-resistant HBV at the start of chemotherapy? Second, how long should lamivudine be continued after the initiation of adefovir dipivoxil? Delayed treatment due to acute exacerbation of HBV may preclude the subsequent completion of chemotherapy protocols. Withdrawal of lamivudine can cause breakthrough of wild-type HBV strains. We believe that prophylactic use of adefovir dipivoxil with continued use of lamivudine (at least for the first few wk) is indicated in the management of hematological malignancies by chemotherapy.

In conclusion, adefovir dipivoxil should be considered to prevent viral reactivation in patients who carry lamivudine-resistant HBV variants during intensive chemotherapy. Further studies are needed to establish optimal treatment regimens.

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## REFERENCES

1. Lau DT, Khokhar MF, Doo E, et al. Long-term therapy of chronic hepatitis B with lamivudine. Hepatology 2000;32:828–34.
2. Ahmed A, Keeffe EB. Lamivudine therapy for chemotherapy-induced reactivation of hepatitis B virus infection. Am J Gastroenterol 1999;94:249–51.
3. Mancuso A, Pagliaro L. Lamivudine for chemotherapy-induced reactivation of HBV: Prophylaxis or treatment? Am J Gastroenterol 2002;97:1268–9.
4. Benhamou Y, Bochet M, Thibault V, et al. Safety and efficacy of adefovir dipivoxil in patients co-infected with HIV-1 and lamivudine-resistant hepatitis B virus. Lancet 2001;358:718–23.
5. Marcellin P, Chang TT, Lim SG, et al. Adefovir dipivoxil for the treatment of hepatitis B e antigen-positive chronic hepatitis B. N Engl J Med 2003;348:808–16.
6. Hadziyannis SJ, Tassopoulos NC, Heathcote EJ, et al. Adefovir dipivoxil for the treatment of hepatitis B e antigen-negative chronic hepatitis B. N. Engl J Med 2003;348:800–7.
7. Ohmoto K, Tsuduki M, Yamamoto S. Combined lamivudine and interferon-alpha therapy for chemotherapy-induced reactivation of hepatitis B virus. Am J Gastroenterol 2003;98:1215–6.
8. Mutimer D, Naoumov N, Honkoop P, et al. Combination alpha-interferon and lamivudine therapy for alpha-interferon-resistant chronic hepatitis B infection: Results of a pilot study. J Hepatol 1998;28:923–9.
9. Tatulli I, Francavilla R, Rizzo GL, et al. Lamivudine and alpha-interferon in combination long term for precore mutant chronic hepatitis B. J Hepatol 2001;35:805–10.
10. Angus P, Vaughan R, Xiong S, et al. Resistance to adefovir dipivoxil therapy associated with the selection of a novel mutation in the HBV polymerase. Gastroenterology 2003;125:292–7.

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SURGERY TODAY


# Previous or Occult Hepatitis B Virus Infection in Hepatitis B Surface Antigen-Negative and Anti-Hepatitis C-Negative Patients with Hepatocellular Carcinoma

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**Abstract**

***Purpose.*** We investigated the clinical and virologic findings in hepatitis B surface antigen (HBsAg)-negative and anti-hepatitis C virus antibody (anti-HCV)-negative patients with hepatocellular carcinoma (HCC) to investigate the role of previous or occult hepatitis B virus (HBV) infections in the development of HCC.

***Methods.*** We examined sera and HCC samples from 40 HBsAg-negative and anti-HCV-negative patients. Sera were tested for some viral markers, and genomic DNA was extracted from the HCC samples. HBx RNA was also extracted from the HCC and amplified by a polymerase chain reaction with reverse transcription (RT-PCR).

***Results.*** Hepatocellular carcinomas from five patients with anti-HBc (group 1, 25 patients) and nine patients without anti-HBc (group 2, 15 patients) were examined for HBx RNA. HBx RNA was detected in four of the five HCC samples from group 1 and in four of the nine HCC samples from group 2.

***Conclusion.*** These findings suggested that previous or occult hepatitis B virus infection is common in HBsAg-negative and anti-HCV-negative patients with HCC.

**Key words** Hepatocellular carcinoma · Hepatitis B virus · Hepatitis C virus · Occult infection · Hepatitis Bx RNA

## Introduction

The major causes of hepatocellular carcinoma (HCC) are hepatitis B virus (HBV) and hepatitis C virus (HCV). In fact, in Japan, HB surface antigen (HBsAg) or anti-HCV antibody (anti-HCV) is detected in the sera of about 90% of patients with HCC.[1,2] In the other 10%, the cause of carcinogenesis and the relationship between viral hepatitis and HCC remain unclear. In patients infected with HBV, integration of the HBV genome and the production of HBx protein in the liver play important roles in the development of HCC.[3] HBV sequences have been found in HCC samples from patients without HBsAg,[4-15] possibly indicating an occult HBV infection.[16] Other studies have shown that HBV DNA is still present in blood or tissue after seroconversion to HBsAg-negativity in patients with chronic hepatitis B or transient HBV infection.[17-21] The role of occult HBV infection in chronic liver disease was recently clarified.[16] In Japan, the anti-HB core antibody (anti-HBc), which indicates present or past HBV infection, is detected in about 60% of HBsAg-negative and anti-HCV-negative patients with HCC.[12-14] However, the clinical features related to HBV infection in such patients have not been clarified.

The hepatitis G virus (HGV)/GB virus C and the TT virus (TTV), a novel DNA virus, were recently reported to cause liver disease.[22-24] However, few studies have investigated the prevalence of HGV and TTV in HBsAg-negative and anti-HCV-negative patients with HCC, and the role of these viruses in the development of HCC is still unclear.[12,25-30] We studied the clinical and virologic findings of HBsAg-negative and anti-HCV-negative patients with HCC to investigate the role of previous or occult HBV, HGV, and TTV infections in the development of HCC.

## Patients and Methods

### *Patients*

Between April 1990 and December 1999, 391 patients underwent liver resection for HCC at the Second

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Department of Surgery, Osaka City University Medical School. Sera from 40 of these patients were negative for both HBsAg and anti-HCV. We investigated the clinicopathologic findings and hepatitis markers in these 40 patients, comprised of 33 men and 7 women, with a mean age of 61 years (range 29–80). Clinicopathologic findings and hepatitis markers in these 40 patients were investigated. The medical and family history of chronic hepatitis B, and alcohol intake were established by interviews with the patients and their families. Clinical records were also reviewed if possible. Alcohol abuse was defined as the consumption of an estimated mean of 86 g or more of ethanol per day for at least 10 years, according to the Liver Cancer Study Group of Japan.[31]

We were able to use unfixed frozen sections of resected tissue specimens to detect HBx RNA, from five anti-HBc positive patients (group 1) and nine anti-HBc negative patients (group 2). There were no remarkable differences in clinicopathologic findings between the subjects of this study and other patients in either group.

This study was conducted in accordance with the Helsinki Declaration, adhering to the guidelines of the Ethics Committee of our institution. Informed consent was obtained from each patient.

### *Viral Markers in Serum and Tissue*

Serum was obtained before surgery and frozen at −80°C until assayed. Sera was tested for anti-HCV by an enzyme-linked immunosorbent assay (Ortho Diagnostic Systems, Tokyo, Japan). Serum HCV RNA was examined by a polymerase chain reaction with reverse transcription (RT-PCR) using primers derived from a conserved 5′-untranslated region of the viral genome[32] and by a branched DNA probe method (>1.0 × $10^5$ copy/ml, Quantiplex HCV-RNA, Chiron, Emeryville, CA, USA). Sera were also tested for HBsAg, anti-HBe antibody (anti-HBe), anti-HBs antibody (anti-HBs), and anti-HBc using an enzyme immunoassay (International Reagents, Kobe, Japan). A titer for anti-HBc with >70% inhibition was scored as positive, and was defined as high when there was greater than 70% inhibition after a 200-fold dilution of the test serum. We measured serum HBV DNA as described previously (>4.0 × $10^5$ copy/ml).[33] Sera were tested for anti-hepatitis D virus by an enzyme immunoassay (Abbott Laboratories, North Chicago, IL, USA).

Hepatitis G virus/GB virus-C RNA was tested by a nested RT-PCR as described previously.[1] TT virus was tested by PCR using second- and third-generation primers as described previously.[30,34,35] For a semi-nested PCR assay (second generation), we used primers NG059 (outer, sense, 5′-ACAGACAGAGGAGAA GGCAACATG-3′), NG061 (inner, sense, 5′-GGCA ACATGTTATGGATAGACTGG-3′), and NG063 (antisense, 5′-CTGGCATTTTACCATTTCCAAAG TT-3′). We also performed a single-round PCR assay (third generation) using primers T801 (sense, 5′-GCTACGTCACTAACCACG-3′) and T935 (antisense, 5′-CTBCGGTGTGTAAACTCACC-3′; B = mixture of G, C, and T).

Resected samples of HCC were frozen and stored at −80°C. Genomic DNA was extracted from both tissues by standard proteinase-K digestion, followed by phenol-chloroform extraction. RNA was also extracted from 100 mg of tissue by the acid guanidinium thiocyanate-phenol-chloroform method[36] followed by treatment with ribonuclease-free DNase (Stratagene Cloning Systems, La Jolla, CA, USA) at 37°C for 15 min. After inactivation of the DNase, the samples were incubated with reverse transcriptase (Gibco BRL, Gaithersburg, MD, USA) and 50 pmol of the downstream primer specific for HBx (1818–1799).[37] For the negative control, 10 μl of the cDNA and RNA samples (equivalent to 1 μg) were amplified in 50 μl of a reaction buffer containing 25 pmol of the two appropriate primers, deoxynucleotides, each at a concentration of 0.2 mmol/l, PCR buffer, and 2.5 units of Gold Taq polymerase (Roche Molecular Systems, Branchburg, NJ, USA). Thirty-five cycles of amplification were done (95°C for 30 s, 55°C for 60 s, and 72°C for 90 s) for the first PCR. With 2 μl of the first PCR product, a second PCR was done to detect HBx RNA.[14] Gel electrophoresis was done in 1.5% agarose and direct sequencing of the HBx gene was done with a DNA sequencing system (373A, Applied Biosystems, Tokyo, Japan) as described previously.[14]

### *Pathologic Examination*

Resected specimens were cut into serial slices 5 mm thick, then fixed in 10% formalin, and stained with hematoxylin and eosin. The severity of active hepatitis (grade) and the degree of fibrosis (stage) in the noncancerous hepatic tissue was determined according to the definition of Desmet et al.[38] Grade 0 indicated no activity; grade 1, minimal activity; grade 2, a low level of activity; grade 3, a moderate level of activity; and grade 4, a high level of activity. Stage 0 indicated no fibrosis; stage 1, portal fibrous expansion (mild fibrosis); stage 2, portal-portal septa without architectural distortion (moderate fibrosis); stage 3, portocentral septa with architectural distortion (severe fibrosis); and stage 4, cirrhosis.

**Table 1.** Clinical features and serologic markers for hepatitis in HBsAg-negative and anti-HCV-negative patients with hepatocellular carcinoma

| | Anti-HBc-positive (group 1, $n$ = 25) | Anti-HBc-negative (group 2, $n$ = 15) |
|---|---|---|
| Age (mean ± SD) | 61.0 ± 8.3 | 60.4 ± 11.8 |
| Sex (Male:Female) | 22:3 | 11:4 |
| History of chronic hepatitis B | 15 | 0 |
| Family history of chronic hepatitis B | 8 | 1 |
| History of blood transfusion | 4 | 1 |
| Alcohol abuse | 9 | 7 |
| Anti-HBe | 12 | 0 |
| Anti-HBs | 15 | 0 |
| HBV DNA | 2 | 0 |
| Anti-HDV | 0 | 0 |
| HCV RNA | 0 | 0 |
| HGV (GBV-C) RNA | 2 | 0 |
| TT virus DNA | | |
| Second-generation primers | 8 | 2 |
| Third-generation primers | 18 | 9 |
| Associated liver disease | | |
| Autoimmune hepatitis | 1 | 1 |
| Budd-Chiari syndrome | 1 | 0 |

Anti-HBe, anti-hepatitis B e antibody; anti-HBs, anti-hepatitis B surface antibody; anti-HDV, anti-hepatitis D virus antibody; HGV, hepatitis G virus; GBV-C, GB virus C

## Results

Sera from 40 of the total 391 patients were negative for HBsAg and anti-HCV (Table 1). The sera from 25 of these 40 patients were positive for anti-HBc (group 1). In group 1, the sera from 12 patients were positive for anti-HBe and the sera from 15 patients were positive for anti-HBs. The sera from the other 15 patients were negative for anti-HBe, anti-HBs, and anti-HBc (group 2). The sera from all 40 patients were negative for HCV RNA and anti-hepatitis D virus antibody. HGV RNA was detected in the sera from two patients in group 1 but none in group 2. TT virus DNA was detected in 10 of the 40 patients by second-generation primers, and in 27 of the 40 patients by third-generation primers.

Sera from two patients in group 1 were positive for HBV DNA and the titers for anti-HBc in these 2 patients were high. In group 1, eight patients had a family history of chronic hepatitis B, and 15 patients, including the 2 with HBV DNA and high titers of anti-HBc, had been treated for chronic hepatitis B (Table 1). These findings suggest strongly that HCC was detected after seroconversion to HBsAg-negativity during chronic hepatitis B in at least 15 patients in group 1. Figure 1 shows the typical clinical course of one patient in whom HCC was detected after seroconversion to HBsAg-negativity. In group 2, HBV DNA was not detected in the sera of any patients. None of the group 2 patients had a history of chronic hepatitis B, and only one patient had a family history of chronic hepatitis B. Nine patients in group 1 and seven patients in group 2 had a history of alcohol abuse. Four patients in group 1 (two of whom had signs of chronic hepatitis B)

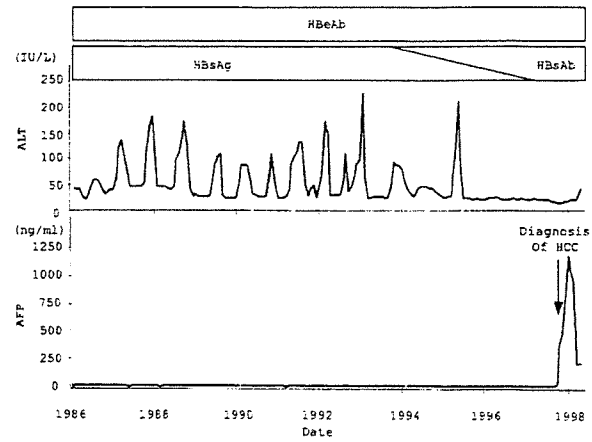


**Fig. 1.** Typical clinical course of one patient in whom HCC was detected after seroconversion of hepatitis B surface antigen. *HBeAg*, hepatitis B e antigen; *HBsAg*, hepatitis B surface antigen; *HBsAb*, hepatitis B surface antibody; *AFP*, α-fetoprotein; *ALT*, alanine aminotransferase. *Arrow* indicates the diagnosis of HCC. HBsAg disappeared with the remission of ALT fluctuations. HCC was detected after seroconversion of HBsAg

**Table 2.** Laboratory test results and histologic findings of noncancerous hepatic tissue from HBsAg-negative and anti-HCV-negative patients with hepatocellular carcinoma

| | Anti-HBc-positive (group 1, $n$ = 25) | Anti-HBc-negative (group 2, $n$ = 15) |
|---|---|---|
| AFP (>20 ng/ml) | 9 | 5 |
| PIVKA II (>40 ng/ml) | 12 | 8 |
| Albumin (g/dl) | 3.6 (3.2/4.1) | 3.9 (3.8/4.1) |
| AST (IU/l) | 49 (28/76) | 44 (31/73) |
| ALT (IU/l) | 52 (34/76) | 45 (26/70) |
| $ICGR_{15}$ (%) | 12.3 (5.5/23.6) | 11.0 (8.3/19.0) |
| HAI score | | |
| Grade (≧3) | 1 | 3 |
| Stage (≧3) | 13 | 6 |
| Tumor differentiation | | |
| Well differentiated | 2 | 2 |
| Other | 23 | 13 |

Laboratory test results are expressed as the median with 10th and 90th percentiles
AFP, α-fetoprotein; PIVKA II, protein-induced vitamin K absence or antagonist II; AST, aspartate aminotransferase; ALT, alanine aminotransferase; $ICGR_{15}$, indocyanine green retention rate at 15 min; HAI score, histologic activity index score, defined by Desmet et al.[35]; Grade, the severity of active hepatitis in the noncancerous hepatic tissue; Stage, the degree of fibrosis in the noncancerous hepatic tissue

**Table 3.** HBx RNA in hepatocellular carcinoma and noncancerous hepatic tissue

| Patient no. | Hepatocellular carcinoma HBx RNA | Noncancerous hepatic tissue HBx RNA |
|---|---|---|
| Group 1 | | |
| 5 | + | + |
| 10 | − | − |
| 20 | − | − |
| 24 | + | + |
| 25 | − | + |
| Group 2 | | |
| 2 | − | − |
| 3 | − | − |
| 4 | − | − |
| 5 | − | − |
| 6 | + | + |
| 7 | − | − |
| 8 | − | − |
| 13 | + | + |
| 14 | − | − |

and one patient in group 2 had a history of blood transfusion. One patient in group 1 and one patient in group 2 had autoimmune hepatitis, and one patient in group 1 had Budd-Chiari syndrome. Although the serum albumin concentration was significantly higher in group 2 than in group 1 ($P$ = 0.002), there were no differences in other laboratory test results or histologic findings of the cancerous and noncancerous hepatic tissues (Table 2).

Resected specimens from five patients in group 1 and nine patients in group 2 were tested for HBx RNA (Table 3 and Fig. 2). HBx RNA was detected in four of the five group 1 samples of HCC and in four of the nine group 2 samples of HCC.

HBx RNA was also detected in two of six patients in group 2 with severe fibrosis or cirrhosis. The other four of these six patients were heavy drinkers.

## Discussion

The clinical significance of previous HBV infection in HCC development, especially in patients with HCV-related HCC, has been the subject of many recent studies.[14,30–45] However, there have been few reports on the clinical features and significance of previous or occult HBV infection in HBsAg-negative and anti-HCV-negative patients with HCC. Negative HBsAg and

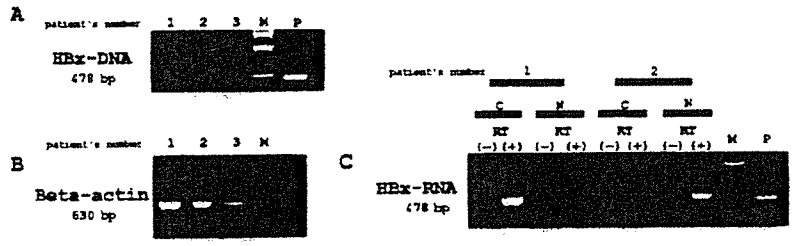


**Fig. 2A–C.** Nucleotides from HCC samples (*C*) and noncancerous hepatic tissues (*N*). **A** HBx DNA amplification by nested polymerase chain reaction (PCR). **B** β-Actin mRNA amplification as the housekeeping gene. **C** HBx mRNA amplification by nested reverse transcription (*RT*)-PCR. The extracted RNA was treated with reverse transcriptase [RT (−) or not RT (−)]. Only samples treated with RT were amplified. The expected molecular weights of the PCR products are given as base pairs (*bp*). on the left. *P*. cloned HBx gene as the positive control: *M*. size marker

positive anti-HBc usually indicates seroconversion in chronic hepatitis B or previous transient HBV infection. In this study, 15 of 25 patients with anti-HBc (group 1) had a history of chronichepatitis B. and the sera from 2 of these 15 patients were positive for HBV DNA and the titer of anti-HBc was high. These findings indicate that HBsAg seroconversion occurred before HCC was detected in at least 15 patients. In fact. the detection of HCC after seroconversion has been reported before.[44] In patients with chronic hepatitis B. screening for signs of HCC is necessary even after the disappearance of serum HBsAg. Although four patients in group 1 and one patient in group 2 had a history of blood transfusion. the route of HBV infection could not be identified in most patients.

Occult HBV and its oncogenic potential are considered a consequence of the potential of this virus to be integrated into the host genome. HBx RNA was detected in the HCC samples from four of the five group 1 patients and four of the nine group 2 patients. which suggests that many. if not most HBsAg-negative and anti-HCV-negative patients with HCC have been infected with HBV. Free episomal HBV genomes or viral particles may persist in the liver during occult infection. Other investigators have reported that Southern hybridization of genomic DNA in HCC tissues enabled the detection of HBV DNA in some HBsAg-negative and anti-HCV-positive patients.[12-15] HBV DNA might have been integrated in the early stage of hepatocarcinogenesis. but then lost during clonal evolution.[15] On the other hand. HBx-coding plasmids can transform NIH 3T3 cells.[45] and HBx protein induces the transformation of liver cells in transgenic mice.[46] HBx protein binds to and inactivates wild-type p53 protein.[47,48] HBx protein also transactivates viral and cellular genes through transcription regulatory factors such as AP-1. AP-2. NF-κB. and CREB.[48-50] Hepatitis B virus DNA was not detected in normal liver tissue without serum markers for viral hepatitis and liver cancer. in donor for living-related liver transplants.[50] These findings indicate that HBx RNA expression. which is necessary for producing the protein, is critical in the development of HCC in patients with occult HBV infection. although the reason that HBx RNA is present in HCC tissue is still unclear. In six of our group 2 patients with severe fibrosis or cirrhosis. HBx RNA was detected in two. and the other four were heavy drinkers. passively contributing to their hepatocarcinogenesis.[51]

Several investigators have reported a poor or weak association between HGV/GBV-C infection and HCC.[1,25-28] Accordingly. we detected HGV RNA in only 2 of the 40 patients. Thus. HGV did not contribute strongly to the development of HCC in patients without HBsAg and anti-HCV. TT virus was recently cloned from patients with post-transfusion hepatitis of unknown etiology;[24] however. TTV DNA is detected commonly in the adult population of Japan. indicating that it is an unlikely causative agent of chronic liver disease.[30,35] In this study. TTV was detected in 10 patients by second-generation primers and in 27 patients by third generation primers. Moreover. the prevalence of TTV was similar to that in patients with chronic liver disease without HCC and in the healthy population.[30,35] These findings indicate that TTV is not related to hepatocarcinogenesis.

In conclusion. HBx RNA was often detected in HCC samples from HBsAg-negative and anti-HCV-negative patients. suggesting that previous or occult HBV infection may be critical in the development of HCC. Hepatitis G virus and TTV are not causative agents for HCC in HBsAg-negative and anti-HCV-negative patients.

## References

1. Kubo S, Nishiguchi S, Kuroki T, Hirohashi K, Tanaka H, Kinoshita H, et al. Poor association of GBV-C viremia with hepatocellular carcinoma. J Hepatol 1997;27:91–5.