or down-regulated before IFN therapy were similar to those of healthy volunteers 6 months after the end of IFN therapy (figure 2*A*, CR group). On the other hand, in the NR group, expression of genes that were either up- or down-regulated before IFN therapy tended to remain up- or down-regulated 6 months after the end of IFN therapy (figure 2*A*, NR group). This suggests that the changes in gene-expression profiles of patients with CH-C before IFN therapy reflect the state of HCV infection.

We performed real-time PCR to corroborate the microarray data. Real-time PCR revealed that *CD69* was up-regulated in patients with CH-C and that *CCR2* and *IL7R* were down-regulated in patients with CH-C (figure 2*B* and table 2).

Relationship between PBMC gene-expression profiles and IFN response. We then analyzed the relationship between PBMC gene-expression profiles before the start of IFN therapy and IFN response. Because the regimen of IFN treatment was different in group A and group B patients, we first focused on group A patients (table 1). In hierarchical clustering analysis using all genes before IFN therapy, no clustering was seen in the CR, BR, or NR groups. Conventional supervised learning methods, such as support vector machine and nearest neighbor (BRB-ArrayTools), could not discriminate between the CR, BR, and NR groups. Therefore, we applied the FNN-SWEEP method to predict the outcome of IFN therapy. Before FNN-SWEEP analysis, nonspecific genes or genes with errors were eliminated by the PART method. The 32 genes screened by PART are shown in figure 3. Topoisomerase (DNA) I *(TOP1)* and interleukin 2 receptor β *(IL2RB)* were up-regulated in the CR group, hemoglobin γG *(HBG2)* and monocyte chemotactic protein were up-regulated in the BR group, and chemokine (C-C motif) ligand 4 and ras-related C3 botulinum toxin substrate 2 *(RAC2)* were up-regulated in the NR group. Genes selected by PART were subjected to the FNN-SWEEP method to construct a class prediction model. Consequently, we selected 10 gene combinations by the SWEEP operator method for CH-C class prediction before the start of IFN therapy (table 4). The most effective gene combination for the prediction of an IFN response was *TOP1;* catenin (cadherin-associated protein) β1 (88 kD); and *RAC2*. The accuracy of the training and test sets were high, at 91.0% and 89.1%, respectively.

Changes in gene-expression profiles over the course of IFN therapy. We next focused on the changes in gene-expression profiles over the course of IFN therapy and their relationship with IFN response. Using PART, 86 genes with changes in expression between before and 2 weeks after the start of IFN therapy were selected. To investigate the relationship between the 86 genes with changes due to IFN therapy and the efficacy of IFN therapy, changes in the expression of the 86 genes were determined for the CR group. On the basis of self-organizing maps, changes in gene expression in the CR group were classified into the following 5 patterns (figure 4): pattern A, up-regulated 2 weeks after the start of IFN therapy and then down-regulated after the end of IFN therapy; pattern B, down-regulated 2 weeks after the start of IFN therapy and then up-regulated after the end of IFN therapy; pattern C, up-regulated 2 weeks after the start of IFN therapy and also up-regulated after the end of IFN therapy; pattern D, up-regulated at 2 weeks after the start of IFN therapy and then returned to normal after the end of IFN therapy; and pattern E, down-regulated at 2 weeks after the start of IFN therapy and also down-regulated after the end of IFN therapy. Patterns A and B represent gene groups with temporary changes during IFN therapy, whereas patterns C, D, and E represent gene groups with changes after the end of IFN therapy and are thought to be attributable to viral eradication or normalization of hepatic function. Interestingly, very little change was seen in the patterns for the NR group. Therefore, changes in gene expression are also useful in predicting therapeutic efficacy. From the 86 genes isolated by PART, the SWEEP operator method was used to identify 10 gene combinations, and therapeutic efficacy was predicted according to the FNN-SWEEP method (table 5). The results showed that the accuracy for gene combinations 7, 8, and 9 was high, at 90.2%. LOOCV confirmed the high accuracy (87.9%) of prediction using these gene combinations. These combinations included the following genes that are important for predicting therapeutic efficacy: *CDC20* was classified as belonging to pattern A; cyclin G1 and differentiation 6 were as belonging to pattern B; and *MIHC* and apoptosis inhibitor 1 were as belonging to pattern E (figure 4).

IFN and ribavirin combination therapy. We then investigated the usefulness of the above-mentioned genes in predicting the efficacy of IFN and ribavirin combination therapy. It has been shown that concurrent ribavirin administration improves the rate of CR. In addition, the changes in gene expression during combination therapy are due not only to IFN but also to ribavirin. Thus, the results for monotherapy may not be applicable to combination therapy. However, changes in the expression of several genes—CD2 antigen (p50), *IL2RB*, *HBG2*, myeloid cell nuclear differentiation antigen *(MNDA)*, and *STAT1AB*—were shown to be extremely useful for distinguishing CR from NR in IFN and ribavirin combination therapy (tables 3 and 4).

q15 q16 q17 q18 q19

DISCUSSION

HCV load, genotype, and fibrosis have been listed as factors that influence the effectiveness of IFN therapy [4, 5], but these factors are not sufficient, and other predictive factors are needed. Unlike liver-biopsy specimens, PBMCs can be easily collected, and collection can be repeated as necessary. We analyzed the gene-expression profiles of PBMCs in patients with CH-C by use of cDNA microarrays under the hypothesis that

gene expression in PBMCs is indicative of IFN efficacy and CH-C disease state. In addition, changes in the gene-expression profiles of PBMCs were analyzed during the course of IFN therapy to clarify the relationship between gene-expression profiles of PBMCs and IFN response.

Interestingly, the gene-expression profiles of PBMCs from patients with CH-C and from healthy volunteers were different, and this was confirmed by hierarchical clustering analysis and supervised learning analysis using support vector machine. When patients with CH-C and healthy volunteers were compared, gene expression in the JAK-STAT cascade, humoral immune response, and G protein–coupled receptor protein signaling pathway differed markedly. In most patients with CH-C, expression of these genes is activated, and HCV infection is thought to bring about changes in the gene expression in PBMCs. Several chemokine- and cytokine-related genes, such as *CCR2* and *IL7R*, were down-regulated. Although the reason for this was not clear, expression of these genes in liver-infiltrating lymphocytes was up-regulated. Therefore, the down-regulation of immune-related genes may represent increased levels of liver-infiltrating lymphocytes accompanying hepatitis. Interestingly, when the chronological changes in PBMC gene-expression profiles were analyzed for the CR group, the profiles at 6 months after the end of therapy were similar to those of healthy volunteers. Therefore, the changes in gene-expression profiles before IFN therapy were due to HCV infection. On the other hand, the gene-expression profiles of the NR group before IFN therapy were similar to those at 6 months after the end of IFN therapy (figure 2*A*).

Unfortunately, it was not possible to differentiate between CR, BR, and NR patients on the basis of gene-expression profiles of PBMCs by use of nonsupervised learning methods, such as hierarchical clustering, before IFN therapy. Therefore, we used FNN theory for CH-C class prediction. The most attractive feature of FNN is that causality between input and output variables can be described very accurately as explicit if-then roles obtained from the constructed model. For the purpose of analyzing numerous genes in a short time, FNN combined with the SWEEP operator method was developed (FNN-SWEEP method) and has been shown to be a precise, simple tool for predicting patient survival on the basis of microarray data [28, 29]. In addition, by first filtering genes by use of PART, the accuracy of the FNN-SWEEP method was further increased [30]. In the present study, a total of 32 genes were identified by PART on the basis of genetic changes before therapy, and, in the CR group, expression of genes such as *TOP1*, *IL2RB*, prothymosin α *(PTMA)*, and ADP-ribosyltransferase was up-regulated, thus indicating active cellular proliferation. In the NR group, the expression of genes indicating activated cytotoxic T cells—such as granzyme, CD2 antigen, *RAC2*, and natural killer cell transcript 4—was up-regulated. Because these genes were up-regulated by IFN therapy in the CR group, they were thought to be up-regulated before therapy in the NR group. Lempicki et al. reported elevated expression of endogenous IFN/innate immune response genes in PBMCs from NR patients coinfected with HCV and HIV [31]. This suggests that, in many NR patients, few immune effector cells are active or that these effector cells cannot infiltrate the liver and remain in the peripheral blood.

To further investigate the above-mentioned points, changes in the gene-expression profiles of PBMCs were determined during the course of IFN therapy. On the basis of expression profiles before and 2 weeks after the start of IFN therapy, 86 genes were selected. These genes did not include as many IFN-α–stimulated genes as were noted in liver [25–27] (table 6), but they included valuable immune regulatory genes.

On the basis of self-organizing maps, changes in gene expression in the CR group were then classified into 5 patterns (figure 4). These gene groups represent genes with temporary changes due to IFN therapy and those with changes after the end of IFN therapy. Gene groups with changes after the end of IFN therapy are thought to be involved in viral eradication or the normalization of hepatic function. Interestingly, little change was seen in any of the patterns in the NR group. In efficacy prediction by the FNN-SWEEP method, the accuracy for the gene combinations 7, 8, and 9 was high, at 90.2%, thus suggesting that changes in gene expression 2 weeks after the start of IFN therapy are also useful in predicting therapeutic efficacy.

We also investigated whether these genes are useful in predicting the efficacy of IFN and ribavirin combination therapy. Changes in gene expression during combination therapy were due not only to IFN but also to ribavirin, and the results for monotherapy could not simply be applied to combination therapy. However, changes in the expression of several genes—CD2 antigen (p50), *IL2RB*, *HBG2*, *MNDA*, and *STAT1AB*—were shown to be extremely useful for distinguishing CR from NR in IFN and ribavirin combination therapy.

Unfortunately, because the number of subjects in the present study was small, the genes that were identified as predictors for IFN monotherapy were not necessarily predictors for IFN and ribavirin combination therapy. However, the present study was the first to show that responses to IFN therapy could be predicted on the basis of changes in gene expression by PBMCs, and further investigations in greater numbers of patients are required.

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QUERIES TO THE AUTHOR

1 Au: Your article has been edited for grammar, clarity, consistency, and adherence to journal style. To expedite publication, we no longer ask authors for approval of routine grammatical and style changes. Please read the article to make sure your meaning has been retained; any layout problems (including table and figure placement) will be addressed after we have incorporated corrections. Note that we may be unable to make changes that conflict with journal style, obscure meaning, or create grammatical or other problems. If you are writing corrections by hand, please print clearly, and be aware that corrections written too close to the edges of the paper may not transmit by fax. Finally, please note that a delayed, incomplete, or illegible response may delay publication of your article. Thank you!

2 Au: Correct, here and at the beginning of Subjects, Materials, and Methods, to edit "7 healthy volunteers" to read "6 healthy volunteers," as at the beginning of Results and in figure 1?

3 Au: Please expand SWEEP, if possible?

4 Au: Note that *JID* style, in accordance with HUGO nomenclature, calls for gene symbols to be italicized, for punctuation to not be included (with a few exceptions), and for Greek letters to not be used; please check that such edits were made accurately and that your meaning was not corrupted. Also, note that *JID* style calls for Greek characters to be used and not spelled out (including in gene names); please check for accuracy. (Some exceptions were made, however, for the figures, for it is not possible for us to extensively revise copy in figures themselves.)

5 Au: Okay to edit to read "IFN-α and IFN-β," or is this not your meaning?

6 Au: Is "IFN-α2b" correct here, or were the boxes in the manuscript meant to indicate something else?

7 Au: Should "(600 mg for $\leq$60 kg of body weight" read "600 mg for <60 kg of body weight," because 60 is included in the following 60–80 range?

8 Au: Your tables and/or figures have been edited in accordance with journal style. Please check carefully to ensure that all edits are acceptable and that the integrity of the data has been maintained. Please also confirm, where applicable, that units of measure are correct, that table column heads accurately reflect the information in the columns below, and that all material contained in figure legends and table footnotes (including definitions of symbols and abbreviations) is correct.

9 Au: (1) Is "HCV was classified by a serologic genotyping assay that has been shown" okay as edited? (2) In table 1, okay to edit to read "Histology score"? Also, could brief definitions of F1–F4 and A1–A3 be supplied, to be added in a footnote (for clarity)?

10 Au: Should "volunteer" here read "healthy volunteer"?

11 Au: Please provide version numbers for QuantArray and BRB-ArrayTools, if possible.

12 Au: Should "log transferred" here read "log transformed"? Also, correct to edit to read "class prediction"?

13 Au: In tables 2, 4, and 5, okay to omit the few gene symbols that followed the names, for consistency with the names for which symbols were not indicated?

14 Au: (1) Has JAK-STAT been expanded correctly? (2) In table 3, please expand LS and KS.

15 Au: In the figure 3 and 4 legends, is "Asterisks indicate genes that present similar expression patterns during IFN and ribavirin combination therapy" correct as added (as in tables 4 and 5)? If not, please specify what they indicate.

16 Au: Okay to edit to read "catenin (cadherin-associated protein) β1 (88 kD)," as in table 4?

17 Au: In figure 4, it's unclear to me what the colored symbols to the left of the gene names indicate (they are not

in the figure panels themselves); can copy be added to the legend explaining these, for clarity? If the symbols have no real meaning, *JID* would strongly prefer to omit them, to avoid confusion. Please advise.

18 Au: With respect to gene symbols, okay to edit to read "*MNDA*," per HUGO? Also, okay to render "STAT1-alpha/beta" as "*STAT1AB*," per HUGO guidelines? Perhaps "*STAT1A* and *STAT1B*" would be better?

·19 Au: Okay/correct to edit to read "tables 3 and 4" here?

20 Au: Is "increased levels of liver-infiltrating lymphocytes" okay as edited?

21 Au: Correct/okay to edit the gene symbol for prothymosin α to *PTMA*, per HUGO?

22 Au: Is "elevated expression of" okay as edited?

23 Au: In table 6, please expand ISG and Huh7. Also, does "Another PBMC study" require a reference? If so, please provide one. Additionally, please check the heads for this table especially carefully and advise if further edits are required; your meaning wasn't quite clear to me. (If need be, copy can be added to a note explaining what the data are.) Last, please indicate what the boldface type indicates.

24 Au: Note that ref. 25 was updated, on the basis of PubMed; please check that it is okay.

CORRESPONDENCE

External Validation of FIB-4: Diagnostic Accuracy Is Limited in Elderly Populations

To the Editor:

We read with interest the articles by Stering et al.[1] and Vallet-Pichard et al.[2] The former authors developed the FIB-4 index, a noninvasive method for assessing liver fibrosis in patients with HIV/HCV coinfection. The variables used are age, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and platelet (PLT) count, and the formula is as follows: (age [yr] × AST [U/L])/ ((PLT[10^9/L]) × (ALT[U/L])$^{1/2}$). They showed that over 70% of patients could be classified into either absence or presence of advanced fibrosis by cutoff of <1.45 or >3.25 respectively, with diagnostic accuracy of 87%. The latter authors expanded the applicability of the FIB-4 index to HCV-monoinfected patients and showed that 73% of patients were classified with diagnostic accuracy of 93%, an excellent performance in both classification and accuracy of diagnosis.

Because the mean age of patients was young in these studies (40 years[1] and 44 years[2]), we wondered whether this index could also fit to Japanese patients who are rather older than the Western patients. We validated the FIB-4 index in a retrospective cohort of 1,405 patients who underwent liver biopsy at our hospital. The mean age was 55 ± 12 years. The distribution of METAVIR fibrosis scores was as follows: 1.6% showed no fibrosis (F0), 44.8% showed mild fibrosis (F1), 29.5% showed moderate fibrosis (F2), 20.2% showed severe fibrosis (F3), and 3.9% showed cirrhosis (F4). The proportion of advanced fibrosis (F3 or F4) was slightly higher in our population compared to the former studies (24.1% vs. 20.7% [1] and 17.2% [2]). As shown in Table 1, only 53% of patients were classified to either <1.45 or >3.25, a much lower rate than previous reports. The diagnostic accuracy was excellent in patients with a FIB-4 index <1.45 (94%), however, it was relatively poor in patients with a FIB-4 index >3.25 (50%) making the overall accuracy as low as 67%.

We supposed this discordance with previous reports may be derived from the older age of our populations and thus we categorized patients into three groups according to age and analyzed separately. In patients with age ≤50 years, 64% of patients were classified, and the diagnostic accuracy was 94% for a FIB-4 index <1.45 and 68% for a FIB-4 index >3.25 making the overall accuracy of 90%, a result comparable to previous reports. In older patients, however, diagnostic accuracy was significantly low compared to those with age ≤50 years (56% for age 51-60 years, $P < 0.0001$ and 51% for age ≥60 years, $P < 0.0001$). Because patients with a FIB-4 index >3.25 increased according to age (6%, 34%, and 53% for ages ≤50, 51-60 and >60 years), and the diagnostic accuracy was low in these patients (48% to 50%), these results suggest that, in elderly patients, a variable "age" generates excessively high FIB-4 index leading to misclassification of no-moderate fibrosis (F0-F2) into a FIB-4 index >3.25.

In conclusion, the FIB-4 index could accurately differentiate advanced fibrosis in young Japanese patients with chronic hepatitis C but the diagnostic accuracy is limited in the elderly. Thus, in elderly patients, some sort of adjustment for the effect of age on FIB-4 index may be necessary for more precise classification.

Table 1. Comparison of FIB-4 Index and Liver Biopsy Results in Terms of Age

		METAVIR Fibrosis Score			
	FIB-4	F0-2	F3-4	Total	Diagnostic Accuracy
All patients					
	<1.45	283 (20%)	18 (1%)	301 (21%)	94%
	>3.25	228 (16%)	226 (16%)	454 (32%)	50%
	1.45-3.25	556 (40%)	94 (7%)	650 (47%)	
	Total	1067 (76%)	338 (24%)	1405 (100%)	67%
Age ≤50	<1.45	240 (54%)	16 (4%)	256 (58%)	94%
(Mean 40 yrs)	>3.25	9 (2%)	19 (4%)	28 (6%)	68%
	1.45-3.25	126 (28%)	38 (8%)	164 (36%)	
	Total	375 (84%)	73 (16%)	448 (100%)	90%
Age 51-60	<1.45	30 (7%)	2 (1%)	32 (8%)	94%
(Mean 56 yrs)	>3.25	76 (18%)	69 (16%)	145 (34%)	48%
	1.45-3.25	215 (50%)	36 (8%)	251 (58%)	
	Total	321 (75%)	107 (25%)	428 (100%)	56%
Age >60	<1.45	13 (2%)	0 (0%)	13 (2%)	100%
(Mean 66 yrs)	>3.25	143 (27%)	138 (26%)	281 (53%)	49%
	1.45-3.25	215 (41%)	20 (4%)	235 (45%)	
	Total	371 (70%)	158 (30%)	529 (100%)	51%

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Reply:

We thank Kurosaki and colleagues for interest in our article that developed a simple noninvasive index, FIB-4, to accurately assess fibrosis in patients coinfected with hepatitis C virus (HCV) and human immunodeficiency virus (HIV).[1] This model was recently validated in a large cohort of patients monoinfected with HCV, and had similar accuracy and was found to be as good as or better than other noninvasive models that use nonroutine tests.[2] In these 2 studies, the mean ages were 40 and 44 years, respectively. Therefore, the utility of this index in older patients is not known.

The current report in a large cohort of Japanese patients with HCV found that although FIB-4 had excellent accuracy (90%) in those < 50 years of age, it did not perform well in those > 50 (56% in those 51-60 years of age and 51% in those > 60 years old) despite an increasing proportion of advanced fibrosis in the older patients (25%-30% versus 16% in those < 50). The reason for the drop in performance of FIB-4 in older patients in the present study is not clear but may be due in part to patient demographics. Although the overall proportion of patients with advanced fibrosis were similar in the 3 cohorts, the proportion of patients with cirrhosis in the Japanese cohort was only 3.9% compared to 7.2% in the study by Vallet-Pichard[2] and 15% in our coinfected population.[1] Furthermore, we are not told the distribution of F4 cirrhosis among the 3 age groups in the current study, which could have affected their results.

Whenever an index includes both a numerator and denominator, changes in either can affect the results. Age was included in the numerator due to the observation that increasing age is associated with increasing fibrosis.[3] This is supported in the current analysis, which found increasing advanced fibrosis in those > 50 years of age. Another

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The presence of steatosis and elevation of alanine aminotransferase levels are associated with fibrosis progression in chronic hepatitis C with non-response to interferon therapy ☆

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Background/Aims: Interferon (IFN) therapy leads to regression of hepatic fibrosis in chronic hepatitis C patients who achieve sustained virologic response (SVR), while the beneficial effect is limited in those who fail therapy. The aim of the present study was to define factors associated with progression of fibrosis in patients who do not achieve a SVR.

Methods: Fibrosis staging scores were compared between paired liver biopsies before and after IFN in 97 chronic hepatitis C patients who failed therapy. The mean interval between biopsies was 5.9 years. Factors associated with progression of fibrosis were analyzed.

Results: Fibrosis progressed in 23%, remained unchanged in 47% and regressed in 29%. Steatosis and a high average alanine aminotransferase (ALT) between biopsies were independent factors for progression of fibrosis with risk ratios of 5.53 and 4.48, respectively. Incidence and yearly rate of progression of fibrosis was 64% and 0.22 ± 0.29 fibrosis units per year in those with both risk factors compared to 8% and −0.04 ± 0.17 fibrosis units per year in those negative for both factors.

Conclusions: Hepatic steatosis and elevated ALT levels are risk factors for progression of fibrosis in chronic hepatitis C patients who fail to achieve a SVR to IFN therapy and therefore may be therapeutic targets to halt the potentially progressive disease independent of antiviral therapy.

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Keywords: Steatosis; ALT; Fibrosis

1. Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver disease worldwide. Mortality associated with HCV infection results from the development of liver cirrhosis and hepatocellular carcinoma, which now is the leading indication for liver transplantation [1]. Treatment with interferon (IFN), alone or in combination with ribavirin (RBV), can eradicate HCV infection in some patients, leading to sustained normalization of

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liver functions, improvement of hepatic inflammation and fibrosis and a decreased risk of the development of hepatocellular carcinoma [2,3]. The problem is that only 50% of patients achieve a sustained virological response (SVR) to therapy even with the most highly developed regimens of IFN [4,5]. The remaining patients who fail to clear the virus are left with the risk of progressive disease. In order to halt this potentially progressive disease, there is the need to establish an effective target of therapeutic intervention independent of antiviral therapy. Therefore, it is important to define risk factors for the progression of fibrosis among chronic hepatitis C patients who do not achieve a SVR to IFN therapy.

Several factors that may affect the rate of progression of fibrosis have been investigated extensively, including older age at infection, male gender, obesity, heavy alcohol consumption, and a high grade of necroinflammation [6–8]. Several cross-sectional and longitudinal studies suggested that hepatic steatosis, which is a common histological feature of chronic hepatitis C [9], influence the progression of hepatic fibrosis [10–14], while other studies did not find such an association [15–18]. Besides these conflicting results, no study has to date reported the effect of steatosis on longitudinal progression of fibrosis among patients who fail to respond to IFN therapy. Therefore, we studied factors associated with progression of fibrosis in those who failed IFN therapy by comparing paired pretreatment and post-treatment liver biopsies.

2. Methods

2.1. Patients

The principle of the study was to identify risk factors associated with progression of fibrosis in chronic hepatitis C patients who failed IFN therapy. To be included in this retrospective study, patients must have undergone liver biopsy before and after therapy, been treated with IFN and had not achieved a SVR. Patients with alcohol consumption of more than 20 g per day, co-infected with HBV or HIV, and those with another known etiology of liver disease, such as autoimmune hepatitis or metabolic disorders, were excluded. A database of patients who had undergone liver biopsy at Musashino Red Cross Hospital between 1990 and 2004 was reviewed, retrospectively and a total of 1241 chronic hepatitis C patients treated with IFN were identified; of these, 407 had SVR and 834 had not achieved a SVR. Among those with treatment failure 104 fulfilled the above criteria but seven patients with cirrhosis before treatment were excluded because the endpoint of the study was progression of fibrosis. Therefore, this study cohort consisted of 97 patients. In these patients, second liver biopsies were performed before the second course of IFN therapy. Otherwise, there were no standardized indications for the second liver biopsy which may be the limitation of our study. Demographic characteristics of patients at the time of initial biopsy are shown in Table 1. The time between the paired biopsies was 5.9 years on average, with a range of 1.2–11.6 years. The median interval between first biopsy and IFN therapy was 3 days (range 2–93 days), and that between completion of IFN therapy and second biopsy was 5.4 years (range 0.8–11.2 years). Laboratory tests were performed monthly or bimonthly in all patients and all measurements were taken at our single hospital.

Table 1
Demographic characteristics of patients

Number of patients	97
Age (years)	52 ± 9
Gender: male/female	50/47
BMI (kg/m^2)	23.9 ± 3.2 (median 24.0, range19–33)
BMI <25/25–30/30 ⩽ (kg/m^2)	55/37/5
Route of transmission	
Blood transfusion/unknown	38/59
Duration of infection (years)	30.4 ± 9.2 (median 33.5, range 3–48)
Genotype 1b/2a/2b	85/4/8
Serum HCV-RNA (Meq/ml)	7.7 ± 9.7
Pretreatment AST (IU/l)	73 ± 40
Pretreatment ALT (IU/l)	104 ± 69
Pretreatment GGT (IU/l)	51 ± 44
Histological variables at first biopsy	
Stage of fibrosis 1/2/3	33/38/26
Grade of activity 0/1/2/3	15/36/41/5
Grade of steatosis 0/1/2/3	21/37/25/14
Size of steatosis macro/micro/mixed	16/17/64
Localization of steatosis centrilobular/diffuse	3/94

BMI, body mass index; AST, aspartate aminotransferase, normal range is 7–38 IU; ALT, alanine aminotransferase, normal range is 4–43 IU/l; GGT, gamma-glutamyltransferase, normal range is 0–73 IU/l.

2.2. Histological evaluation

Median length of biopsy specimen and number of portal tracts were 13.0 mm (range 10–40 mm) and 12 (range 6–34). All liver biopsy specimens were evaluated separately by three independent pathologists who were blinded to the clinical details. If there was discordance, the scores assigned by two pathologists were used for the analysis. Fibrosis and activity were scored according to the METAVIR scoring system [19]. Fibrosis was staged on a scale of 0–4: F0 (no fibrosis), F1 (mild fibrosis: portal fibrosis without septa), F2 (moderate fibrosis: few septa), F3 (severe fibrosis: numerous septa without cirrhosis) and F4 (cirrhosis). Activity of necroinflammation was graded on a scale of 0–3: A0 (no activity), A1 (mild activity), A2 (moderate activity) and A3 (severe activity). Percentage of steatosis was quantified by determining the average proportion of hepatocytes affected by steatosis and was graded on a scale of 0–3: grade 0 (no steatosis), grade 1 (0–9%), grade 2 (10–29%), and grade 3 (over 30%). Size of steatosis was categorized into micro-vesicular, macro-vesicular and mixed types. Localization of steatosis was categorized into either centrilobular or diffuse pattern. Definition of changes in the grade of steatosis was as follows: worsening as 1 point or more increase, improvement as 1 point or more decrease, and stability as no change.

2.3. Changes in fibrosis-staging score overtime

Changes in progression of fibrosis were defined as follows: progression of fibrosis was defined as a 1 point or more increase, regression as a 1 point or more decrease and stability as no change in the METAVIR fibrosis-staging score. In addition, because the time between paired biopsies was variable, the yearly rate of progression of fibrosis was calculated as the change in fibrosis-staging score divided by the time between paired biopsies, as originally described by Poynard et al. [6].

2.4. Statistical analysis

The STAT View software package was used for statistical analysis. Categorical data were analyzed using the Fisher's exact test. Continuous variables were compared with the Student's t test. Variables that

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130 were statistically significant in univariate analysis were included in multivariate analysis using logistic regression analysis. The Kaplan–Meier method and log-rank test were used to analyze the time to occurrence of fibrosis progression. A p-value of less than 0.05 was considered statistically significant.

3. Results

Factors associated with the initial stage of fibrosis (cross-sectional study).

All three pathologists assigned the same scale in 85% for fibrosis staging and 95% for steatosis-grading. In cases with discordance, at least two pathologists assigned the same scale. The stage of fibrosis in the initial liver biopsy was F1 in 33, F2 in 38 and F3 in 26 patients. Various clinical factors were analyzed in association with the advanced stage of fibrosis. As a result, the presence of F3 fibrosis was associated with older age, (51 ± 9 in F1–2 vs. 55 ± 9 in F3, $p = 0.03$), higher grade of histological activity (A2–3 was 35% in F1–2 vs. 84% in F3, $p = 0.0001$) and higher grade of steatosis (steatosis grade 2–3 was 34% in F1–2 vs. 58% in F3, $p = 0.04$).

The grade of steatosis was 0 in 21, 1 in 37, 2 in 25 and 3 in 14 patients. A higher grade of steatosis was associated with female gender (the male/female ratio was 35/23 in grade 0–1 vs. 15/24 in grade 2–3, $p = 0.04$), increased BMI (BMI over 25 kg/m^2 was 31% in grade 0–1 vs. 62% in grade 2–3, $p = 0.006$), and higher grade of histological activity (A2–3 was 38% in grade 0–1 vs. 62% in grade 2–3, $p = 0.03$). Multivariate logistic regression analysis revealed that increased BMI and female gender were independent factors associated with a high grade of steatosis (Table 2).

3.1. Change in fibrosis-staging scores over time (longitudinal study)

Fibrosis-staging progressed in 23% (progression by 2 points in 5% and 1 point in 18%), remained unchanged in 47% and regressed in 28% (regression by 2 points in 2% and 1 point in 27%). At first liver biopsy, laparoscopy was performed in 73 patients and the presence of cirrhosis (F4) was carefully excluded. In another 24 patients, the possibility of misdiagnosis of F4 as F3 remains. However, the incidence of fibrosis progression did not differ according to the initial stage of fibrosis (21.2% in F1, 26.3% in F2 and 19.2% in F3, $p = 0.78$) which indicates that misdiagnosis of F4 as F3 at initial biopsy is unlikely.

Among various factors, as shown in Table 3, a higher grade of steatosis, higher levels of ALT and AST (average value for the period between the paired liver biopsies) were associated with progression of fibrosis. Since there was significant correlation between ALT and AST levels ($r = 0.684$, $p < 0.0001$), these two variables could not be analyzed together in multivariate analysis. Thus, average level of ALT was used for the following analysis. The probability of progression of fibrosis was 14%, 8%, 36% and 50% in patients with steatosis grades of 0, 1, 2 and 3, respectively ($p = 0.008$), and 17%, 17%, 13%, 21% and 53% in patients with average ALT values of <40, 40–59, 60–79, 80–99 and over 100 IU/l, respectively ($p = 0.02$) (Fig. 1). Multivariate logistic regression

Table 2
Multivariate logistic regression analysis of factors associated with hepatic steatosis

	Odds	95% C.I.	p Value
BMI			
⩾25 kg/m^2	4.23	1.63–10.95	0.003
Gender			
Female	2.75	1.06–7.14	0.04
Activity grade			
2–3	2.30	0.85–6.26	0.10
Fibrosis stage			
3	1.63	0.53–4.97	0.39

Table 3
Factors associated with the progression of fibrosis over time

	Progression $n = 22$	Non-progression $n = 75$	p Value
Gender: male/female	9/13	41/34	0.33
Age at biopsy: <60/ ⩾60 years	14/8	59/16	0.17
HCV genotype: 1b/non-1b	18/3	65/9	0.72
BMI: <25/⩾25 kg/m^2	11/11	42/31	0.63
Duration of infection (years)	32.1 ± 5.2	29.9 ± 10.0	0.56
Activity on first biopsy			
Grade: 0–1/2–3	8/14	43/32	0.10
Steatosis on first biopsy			
Grade: 0–1/2–3	6/16	52/23	0.001
Size: macro/micro/mixed	4/4/14	12/13/50	0.96
Location: centrilobular/diffuse	1/21	2/73	0.54
Evolution of steatosis			
Worsening/improvement/stable	2/2/18	9/8/58	0.09
Average ALT: <100/⩾100 IU/l	13/9	67/8	0.003
Average AST: <75/⩾75 IU/l	10/12	59/14	0.002
Interval between biopsies (years)	5.1 ± 3.2	6.2 ± 2.4	0.09
Interval between completion of IFN and second biopsy (years)	4.6 ± 3.2	5.7 ± 2.4	0.10
Treatment regimen			
RBV−/RBV+	22/0	71/4	0.27
Response to IFN			
Relapser/non-responder	16/6	53/22	0.99
Evolution of weight			
Gain/loss/stable	5/8/9	29/21/25	0.38

macro, macro-vesicular steatosis; micro, micro-vesicular steatosis; RBV−, interferon monotherapy; RBV+, interferon plus ribavirin combination therapy.
Duration of infection was determined in 38 patients whose source of infection was blood transfusion.

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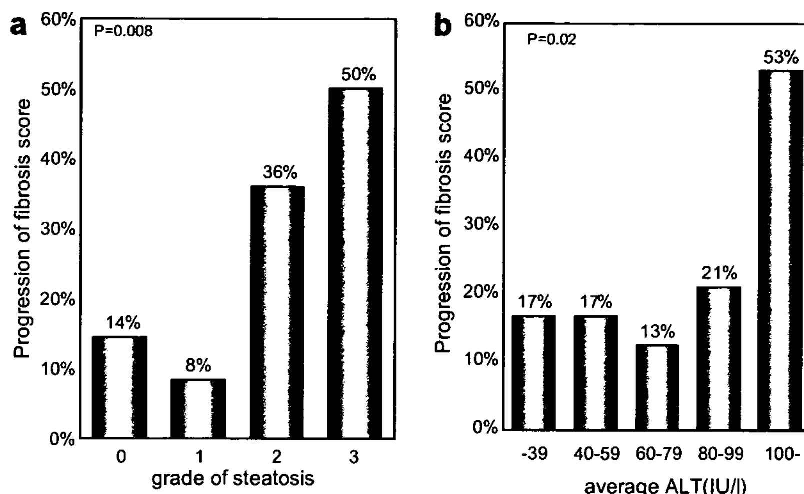

Fig. 1. Progression of fibrosis stage, hepatic steatosis and the average level of ALT. The progression of the fibrosis score over time is illustrated using bar charts. (a) Steatosis grades of 2 or 3 at initial liver biopsy were associated with the increased progression of fibrosis over time. (b) High average ALT levels during the observation period were associated with progression of fibrosis at the threshold of 100 IU/l.

analysis revealed that these two were independent risk factors associated with the progression of fibrosis with risk ratios of 5.14 for steatosis ($p = 0.004$) and 5.21 for ALT ($p = 0.01$) (Table 4).

When patients were categorized in terms of these two risk factors, the incidence of progression of fibrosis was as high as 64% in those with both risk factors, compared to 8% in those negative for these factors. Conversely, the incidence of fibrosis regression was only 9% in those with both risk factors, compared to 37% in those negative for these factors ($p = 0.0003$) (Fig. 2).

In order to adjust for the effect of variable intervals between paired biopsies, the yearly rate of progression of fibrosis was calculated as the change in the fibrosis-staging score divided by the time between paired biopsies. The average of all patients was 0.02 ± 0.22 fibrosis units per year. Again, a higher grade of steatosis ($p = 0.004$) and higher average level of ALT ($p = 0.0005$) were associated with a higher rate of progression of fibrosis (Table 5). In addition, the yearly rate of progression of fibrosis was 0.22 ± 0.29 fibrosis units per year in those with both risk factors, 0.12 ± 0.37 in those with elevated ALT alone, 0.05 ± 0.16 in those with steatosis alone and -0.05 ± 0.17 in those negative for these two factors ($p = 0.001$). Time to progression of fibrosis at second biopsy was also analyzed by the Kaplan–Meier method. The cumulative probabilities of progression of fibrosis at five years were 58% in those with both risk factors, 33% in those with elevated ALT alone, 18% in those with steatosis alone and 2% in those negative for these two factors ($p < 0.0001$) (Fig. 3).

Table 4
Multivariate logistic regression analysis of factors associated with progression of fibrosis over time

	Odds	95% C.I.	*p* Value
Steatosis grade ⩾2	5.14	1.67–15.77	0.004
Average ALT ⩾100 IU/l	5.21	1.49–18.20	0.01

4. Discussion

In the present study, we found that a higher grade of hepatic steatosis at baseline and a higher average value of ALT are independent risk factors for the progression of fibrosis over time in chronic hepatitis C patients who fail to achieve a SVR to IFN therapy. These two factors

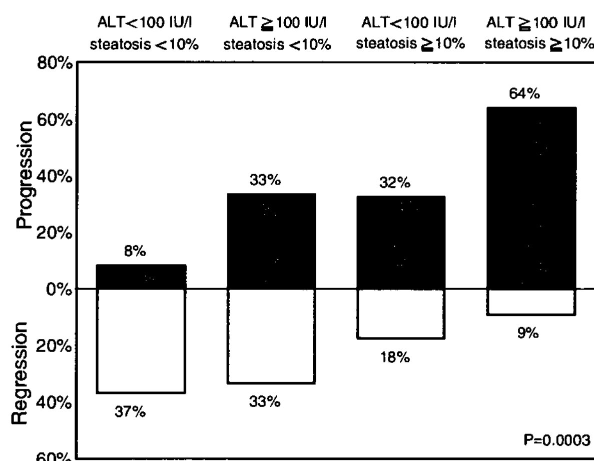

Fig. 2. Evolution of fibrosis stage in terms of risk factors. Patients were categorized into four groups according to the presence or absence of two risk factors. The upper bar chart (dark gray) indicates the progression of fibrosis while the lower bar chart (light gray) indicates the regression of fibrosis.

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Table 5
Factors associated with the yearly rate of fibrosis progression

	n	Mean	SD	p Value
Gender				
Male	50	−0.01	0.19	0.12
Female	47	0.06	0.23	
Age at biopsy				
<60 years	73	−0.0002	0.21	0.06
⩾60 years	24	0.10	0.23	
HCV genotype				
1b	83	0.02	0.20	0.37
non-1b	14	0.08	0.32	
BMI				
<25 kg/m^2	53	0.004	0.24	0.32
⩾25 kg/m^2	42	0.05	0.19	
Steatosis on first biopsy				
0–1	58	−0.03	0.20	0.004
2–3	39	0.10	0.21	
Activity on first biopsy				
0–1	51	−0.001	0.21	0.24
2–3	46	0.05	0.22	
Fibrosis on first biopsy				
1–2	71	0.03	0.20	0.43
3	26	−0.01	0.25	
Average ALT between paired biopsies				
<100 IU/l	80	−0.01	0.17	0.0005
⩾100 IU/l	17	0.18	0.31	

227 may co-operate to promote the progression of fibrosis.
228 The association between steatosis and progression of
229 fibrosis in untreated patients had been suggested by pre-
230 vious studies but this study is the first to demonstrate a
231 similar association for treated patients. These findings
232 are particularly important to establish a rationale for
233 identifying therapeutic targets to halt potentially pro-
234 gressive disease independent of antiviral therapy.
235 There have been many studies that analyzed the
236 association between steatosis and progression of liver

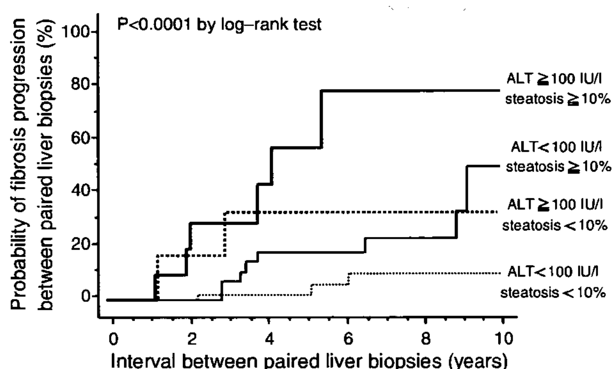

Fig. 3. Probability of fibrosis progression according to the presence of risk factors. Patients were categorized into four groups according to the presence or absence of two risk factors and the time to progression of fibrosis was analyzed.

fibrosis in HCV-infected patients, and the majority have 237
shown a positive association [10–13], including a large 238
scale meta-analysis [14]. However, some studies did 239
not report this association [15–18]. There are two possi- 240
ble reasons for these conflicting results. First, longitudi- 241
nal studies, rather than cross-sectional studies, are 242
particularly important in the analysis of the role of ste- 243
atosis in time-dependent progression of hepatic fibrosis, 244
because cross-sectional studies involve patients with an 245
unknown duration of steatosis. Three of four longitudi- 246
nal studies that analyzed the progression of fibrosis 247
through paired biopsies in untreated patients showed 248
that the presence or worsening of steatosis was associ- 249
ated with the progression of fibrosis [12,13,20], and the 250
probability of progression of fibrosis was significantly 251
related to the grade of steatosis [13]. In one study, how- 252
ever, progression of fibrosis was correlated with older 253
age, periportal necroinflammation and ALT elevations 254
but not with steatosis [17]. Interestingly, steatosis was 255
associated with older age, higher body mass index and 256
ALT elevations in that study, indicating an indirect 257
association of steatosis and fibrosis progression. The 258
authors assumed that steatosis was the result rather than 259
the cause of inflammation. This observation highlights 260
the second reason for the controversies over a correla- 261
tion between the presence of steatosis and progression 262
of fibrosis, that is, there are so many confounding fac- 263
tors associated with both steatosis and fibrosis progres- 264
sion such as older age, advanced stage of fibrosis, higher 265
degree of inflammation, elevated ALT, increased body 266
mass index and insulin resistance. Because it is very dif- 267
ficult to prove a causal relationship between these con- 268
founding factors through clinical observations, 269
steatosis may be a hallmark of the progression of fibro- 270
sis but it is unclear whether the effect of steatosis on pro- 271
gression of fibrosis is direct or mediated by other 272
confounding factors. 273
Hepatic steatosis is a common pathological finding in 274
patients with chronic hepatitis C [9]. Because the pro- 275
portion of patients with steatosis is higher than would 276
be expected from a chance association, a direct role of 277
HCV in the pathogenesis of steatosis is suggested, at 278
least in some patients with genotype 3 infection [21]. 279
Furthermore, other observations suggest that steatosis 280
may be metabolic; it is correlated with a high body mass 281
index, visceral adiposity and insulin resistance, espe- 282
cially in non-3a genotypes and metabolic steatosis also 283
is correlated with progression of fibrosis [11,22]. The 284
most reliable evidence that metabolic steatosis is associ- 285
ated with progression of fibrosis is shown by a study 286
indicating that weight reduction in patients with chronic 287
hepatitis C leads to a reduction in steatosis and an 288
improvement in fibrosis, despite the persistence of 289
HCV infection. A reduction in steatosis was significantly 290
associated with a decrease in stellate cell activation and 291
regression of hepatic fibrosis in 56% of patients. Thus, 292

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weight reduction may provide an important new adjunct treatment strategy for patients with chronic hepatitis C [23]. Separately, a recent study showed that the administration of pioglitazone led to metabolic and histological improvement in subjects with non-alcoholic steatohepatitis [24]. Whether amelioration of insulin resistance could improve steatosis and fibrosis in chronic hepatitis C awaits future investigation.

The mechanism by which steatosis could aggravate hepatic fibrosis in chronic hepatitis C patients remains largely hypothetical. Steatosis related insulin resistance may contribute to hyperinsulinemia and increased hepatic expression of connective tissue growth factor leading to progression of fibrosis [25]. Alternatively, a steatohepatitis-like pathway may be involved where steatosis requires a second hit for progression to fibrosis [26]. The most likely candidate is an oxidative stress with subsequent lipid peroxidation which is reported to correlate with the stage of fibrosis [27]. Another important candidate is an antiviral inflammatory response. It is reported that steatotic liver has increased susceptibility to inflammatory response [28] and that a higher grade of steatosis is correlated with a higher degree of inflammation or elevated ALT [14,15,17]. Higher degree of inflammation or elevated ALTs are associated with the progression of fibrosis [29,30], but hepatic steatosis may be responsible for the amplification of hepatic inflammation and vice versa, and the co-existence of these two factors may lead to further progression of fibrosis, as in patients with non-alcoholic steatohepatitis. In our study, average value of ALT between two biopsies was associated with fibrosis progression, whereas histological inflammation at first liver biopsy was not. The reason for this discordance may be explained by the dynamic process of hepatic necroinflammation. Severity of histological inflammation at the time of biopsy may not reflect subsequent inflammation process, whereas average value of regularly determined ALT may reflect entire fluctuation of hepatic inflammation. If so, our finding may support the hypothesis that co-operation of steatosis as the first hit and dynamic process of hepatic inflammation as the second hit promotes fibrosis progression. On the other hand, elevation of ALT may not be a mere reflection of hepatic inflammation so much as hepatocellular death such as apoptosis. Since it is reported that apoptotic caspase activation is elevated in HCV-associated steatosis [31] and that steatotic liver has increased susceptibility to apoptosis [28], elevation of ALT may also reflect an apoptosis amplified by steatosis which may lead to fibrosis progression.

Regardless of the precise mechanism, the results of the present study suggest that lowering of ALT levels may be beneficial in preventing progression of fibrosis inpatients who failed to achieve a SVR. In our population, all patients received 24 weeks of IFN therapy and none received long-term maintenance therapy aiming to ameliorate hepatic inflammation. However, we speculate that amelioration of hepatic inflammation and lowering ALT levels by long-term IFN may prevent fibrosis progression in patients who remain viremic since it has been reported that IFN slowed the natural progression of fibrosis in patients who failed IFN therapy when the rate of progression of fibrosis after IFN therapy was compared to the estimated rate of progression before therapy [2,32], and that treatment duration was associated with the reduction of fibrosis independent of virological response [2]. Another possible approach to lower ALT levels may be the use of ursodeoxycholic acid, which has been reported to induce an almost 30% decrease in serum ALT levels [33,34]. The long-term efficacy of therapies targeted to the reduction of hepatic fibrosis needs future verifications.

Some factors related to fibrosis progression in previous studies such as obesity [35] and worsening of steatosis [20] were not significant in our study. In our study where majority of population having normal body weight and very few having obesity (BMI $\geqslant$ 30 kg/m^2), impact of increased BMI on fibrosis progression may not be evaluated. Also, a smaller number of patients with worsening of steatosis (11.3% in present study and 34% in previous study [20]) may be the reason for the discrepancy. This may be due to difference in patient selection since no patients in that study had antiviral treatment between two biopsies

In conclusion, the presence of hepatic steatosis and elevated ALT levels are risk factors for progression of fibrosis in chronic hepatitis C patients who failed to achieve SVR to IFN therapy. These two factors may be a therapeutic target to halt the potentially progressive disease independent of antiviral therapy.

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Comparison of complete sequences of hepatitis B virus genotype C between inactive carriers and hepatocellular carcinoma patients before and after seroconversion

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Background. Most patients who acquire chronic hepatitis B virus (HBV) infection by perinatal transmission become inactive carriers (IC) after hepatitis B e (HBe) antigen seroconversion, whereas some patients have persistent abnormal serum transaminase levels and develop hepatocellular carcinoma (HCC) in the anti-HBe-positive phase. The aim of this study was to investigate the HCC-related mutations of HBV. ***Methods.*** Complete sequences of HBV were examined among eight IC and eight HCC patients infected with HBV genotype C before and after seroconversion. ***Results.*** The frequency of the T1653 mutation tended to be higher among HCC patients after seroconversion (16.7% vs. 62.5%; $P = 0.086$). The prevalence of a basal core promoter double mutation (T1762/A1764) was high among both IC and HCC patients after seroconversion (83.3% vs. 87.5%; $P = 0.825$). Among the HCC patients, a pre-S deletion mutant was detected in 62.5% patients before seroconversion, and in 37.5% patients after seroconversion. The core deletion mutant was also detected in 50% of HCC patients only before seroconversion. Deletion mutants of the pre-S or core region before seroconversion were significantly associated with HCC patients (0% vs. 62.5%; $P = 0.007$, 0% vs. 50%; $P = 0.021$, respectively). ***Conclusions.*** Our data showed a significant association of pre-S and core deletion mutants before seroconversion with HCC development. The T1653 mutation after seroconversion was frequently found in HCC patients infected with HBV genotype C. These results suggest that mutations may be predictive factor for development of HCC.

Key words: hepatocellular carcinoma, core deletion, pre-S deletion mutant, T1653 mutation

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most frequent cancer and the third leading cause of cancer-related death in the world, with an estimated annual prevalence of >500000 cases worldwide.[1] It is now accepted that HBV infection has hepatocarcinogenic potential in humans. Several mutations in the HBV genome have been reported to occur during the course of persistent viral infection, and there is increasing evidence of an association between these molecular alterations and the development of end-stage liver disease in patients with HBV infection.[2–6] Nevertheless, it is still unclear whether a specific mutation or a specific combination of mutations is associated with the development of severe disease, because previous studies focused on only a few mutations such as pre-S deletion, basal core promoter (BCP) double mutation, and precore (PC) mutation. Recently, several lines of evidence have indicated that complex HBV variants with deletions in the pre-S or core region and mutations in the enhancer II region are associated with end-stage liver disease.[7–9] Both the pre-S and core regions play an essential role in the interaction with immune responses because they contain B- and T-cell epitopes.[10–12] Pre-S and core deletion mutants with altered epitopes may survive despite the host immune system.

During persistent HBV infection, carriers frequently undergo seroconversion from hepatitis B e antigen (HBeAg) to the corresponding antibody (anti-HBe). Most patients who acquire chronic HBV infection with HBV genotype C (which is the common genotype in East Asian countries) by perinatal transmission become inactive carriers (IC) after seroconversion. A subgroup of patients have persistent abnormal serum transaminase levels and develop HCC in the anti-HBe-positive phase. Because most previous studies examined only a serum sample collected at one time point in each patient,

that is, they were cross-sectional studies, the association between different clinical events after seroconversion and specific HBV genomic mutations has not been clearly defined. To investigate this issue, complete HBV sequences were examined in eight IC and eight HCC patients before and after HBeAg seroconversion.

Materials and methods

Serum samples

Serum samples were obtained from 16 patients (eight patients were IC and the other eight were HCC patients) at the Nagoya City University Graduate School of Medical Sciences and National Hospital Organization Osaka National Hospital before and after seroconversion. Sixteen patients were infected with HBV genotype C. IC were defined as individuals who were hepatitis B surface antigen (HBsAg) positive with normal alanine aminotransferase (ALT) and α-fetoprotein levels over a 5-year period (with at least 12 evaluations at 3-month intervals) and without the presence of portal hypertension. HCC patients were diagnosed on the basis of results of abdominal ultrasonography, angiography, computed tomography, magnetic resonance imaging, or liver biopsy as well as by their having an elevated serum α-fetoprotein level (>400 ng/ml).

HBV Genotyping

HBV genotypes were determined by the restriction fragment length polymorphism method from the *S* gene sequence amplified by polymerase chain reaction (PCR)[13] or enzyme immunoassay (EIA) with monoclonal antibodies for distinct epitopes in the pre-S2 region products,[14] with commercial kits (HBV genotype EIA; Institute of Immunology, Tokyo, Japan). The genotypes were also confirmed by a phylogenetic tree analysis.

HBV DNA extraction

Serum samples were stored at −80°C until the assay. DNA was extracted from 100 μl of serum by using QIAamp DNA blood kits (Qiagen, Hilden, Germany).

Determination of the complete nucleotide sequences of HBV/C

The complete nucleotide sequences of 30 HBV/C isolates from 16 patients (HBV DNA in two serum samples from IC after seroconversion could not be amplified) were determined by a method reported previously[15] with a slight modification. In brief, two overlapping fragments of HBV genome were amplified by PCR, and eight overlapping HBV DNA fragments were amplified further by PCR with nested primers. Amplification was performed in a 96-well cycler (GeneAMP9600; Perkin-Elmer Cetus, Norwalk, CA, USA), and the PCR products were electrophoresed in 3.0% (wt/vol) agarose, stained with ethidium bromide, and observed under UV light.[3] Standard precautions were taken to avoid contamination during PCR. A negative control serum was also processed and included in each run to ensure specificity. Twelve overlapping HBV DNA fragments thus amplified were sequenced directly with a Prism BigDye kit (Applied Biosystems, Foster City, CA) in an ABI 3100 DNA automated sequencer.

Statistical analysis

Statistical analyses were performed with χ-squared and Fisher's exact tests for categorical variables. The Mann-Whitney *U* test was used for continuous variables, as appropriate. Differences were considered to be significant with *P* values <0.05. The statistical analysis software used was Stata software, version 8.0 (Statacorp LP, College Station, TX, USA).

Results

Table 1 compares age, ALT level, platelet count, HBV DNA, and rate of cirrhosis before and after HBeAg seroconversion, as well as age at seroconversion and the intervals between two sampling points for all patients. ALT level, platelet count, HBV DNA, and rate of cirrhosis after seroconversion were significantly higher among HCC patients than in IC.

The alignment of sequences covering the enhancer II and core promoter regions is shown in Fig. 1. We could not amplify HBV DNA in two serum samples from IC because of the small amount of HBV DNA in the samples. The box alpha and basal core promoter contained mutational hot spots, but box beta did not. The frequency of the T1653 mutation tended to be higher among HCC patients after seroconversion [IC vs. HCC: 1/6 (16.7%) vs. 5/8 (62.5%); $P = 0.086$] (Fig. 1 and Table 2), whereas the T1653 mutation did not differ between the two groups before seroconversion [IC vs. HCC: 1/8 (12.5%) vs. 2/8 (25%); $P = 0.522$]. The prevalence of the BCP double mutation was high among both IC and HCC patients after seroconversion [IC vs. HCC: 5/6 (83.3%) vs. 7/8 (87.5%); $P = 0.825$]. The prevalence of S1753 was low among IC and HCC patients before and after seroconversion (Fig. 1 and Table 2). The S1753 mutant was not recognized in the patients who were infected with the T1653 mutation clone. Deletion mutants of the core or pre-S region before seroconversion were significantly associated with HCC patients

Table 1. Comparison of clinical characteristics between IC and HCC patients before and after HBeAg seroconversion

	Before seroconversion			After seroconversion		
Features	Inactive carriers (n = 8)	HCC patients (n = 8)	Differences P value	Inactive carriers (n = 8)	HCC patients (n = 8)	Differences P value
Male, n (%)				4 (50)	7 (87.5)	0.106
Age (years)[a]	31.8 ± 8.4	40.0 ± 10.6	0.246	43.6 ± 10.0	51.6 ± 13.8	0.317
ALT (U/L)[a]	199.6 ± 220.5	234.6 ± 242.2	0.875	20.3 ± 7.2	40.4 ± 16.9	0.009*
Platelet count (×10^4/mm^3)[a]	17.5 ± 2.0	15.1 ± 4.3	0.268	17.5 ± 3.3	11.5 ± 5.4	0.027*
HBV DNA (LGE/ml)[a]	7.2 ± 0.5	7.2 ± 0.5	0.869	4.3 ± 0.7	5.7 ± 1.2	0.022*
Cirrhosis (%)	0 (0)	2 (25)	0.131	0 (0)	5 (62.5)	0.007*
Age at seroconversion (years)				37.8 ± 8.1	47.3 ± 14.2	0.226
Intervals between two sampling points (years)				11.8 ± 2.9	11.6 ± 4.4	1.0

IC, inactive carriers; HCC, hepatocellular carcinoma; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; HBV, hepatitis B virus; LGE, log genome equivalents
*Statistically significant
[a]Mean ± SD

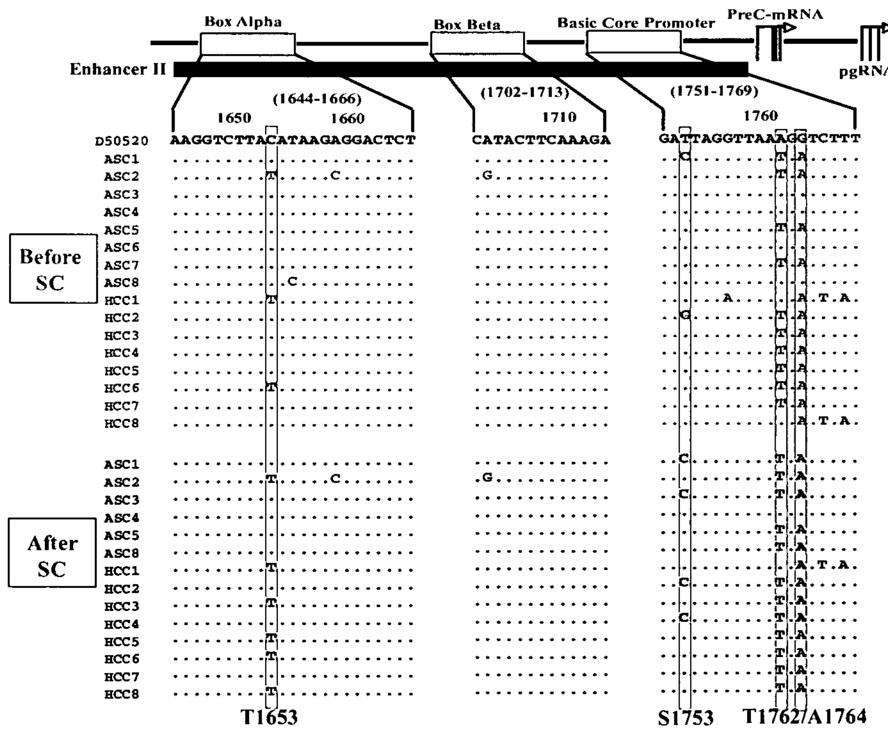

Fig. 1. Nucleotide sequences that cover enhancer II and core promoter regions in inactive carriers (IC) and hepatocellular carcinoma (HCC) patients. The wild-type sequence for genotype C is represented by D50520. *Dots* indicate nucleotides identical to the wild type. *SC*, seroconversion

(core deletion mutant: 0/8 [0%] vs. 4/8 [50%]; $P = 0.021$, pre-S deletion mutant 0/8 [0%] vs. 5/8 [62.5%]; P = 0.007, respectively) (Table 2).

The pre-S deletion mutant was detected in only one IC as a minor clone (Fig. 2), whereas among HCC patients, deletion mutants (major clones) were detected in five of eight (62.5%) patients before seroconversion, and in three of eight (37.5%) after seroconversion. Only two patients (patients 4 and 5) had pre-S deletion mutants before and after seroconversion. Two HCC patients (patients 1 and 2) had pre-S deletion mutants only before seroconversion (Fig. 3). Three HCC patients (patients 4–6) before seroconversion and one patient (No. 7) after seroconversion were coinfected with wild-type virus and pre-S deletion mutants (the deletion mutants were the major clones). Most deletions were identified in the 3′ terminus of the pre-S1 region and the 5′ terminus of the pre-S2 region.

Table 2. Comparison of HBV mutations between IC and HCC patients before and after HBeAg seroconversion

Features	Before seroconversion			After seroconversion		
	Inactive carriers ($n = 8$) n (%)	HCC patients ($n = 8$) n (%)	Differences P Value	Inactive carriers ($n = 8$) n (%)	HCC patients ($n = 8$) n (%)	Differences P Value
T1653 mutation	1 (12.5)	2 (25)	0.522	1/6 (16.7)	5/8 (62.5)	0.086
S1753 mutation	1 (12.5)	1 (12.5)	1.0	2/6 (33.3)	2/8 (25)	0.730
A1896 mutation	3 (37.5)	0 (0)	0.055	4/6 (66.7)	3/8 (37.5)	0.280
BCP double mutation	4 (50)	7 (87.5)	0.106	4/6 (66.7)	7/8 (87.5)	0.325
Core deletion Mutant	0 (0)	4 (50)	0.021*	0/6 (0)	0/8 (0)	1.0
Pre-S deletion Mutant	0 (0)	5 (62.5)	0.007*	1/8 (12.5)	3/8 (37.5)	0.248

BCP, basal core promoter
* Statistically significant

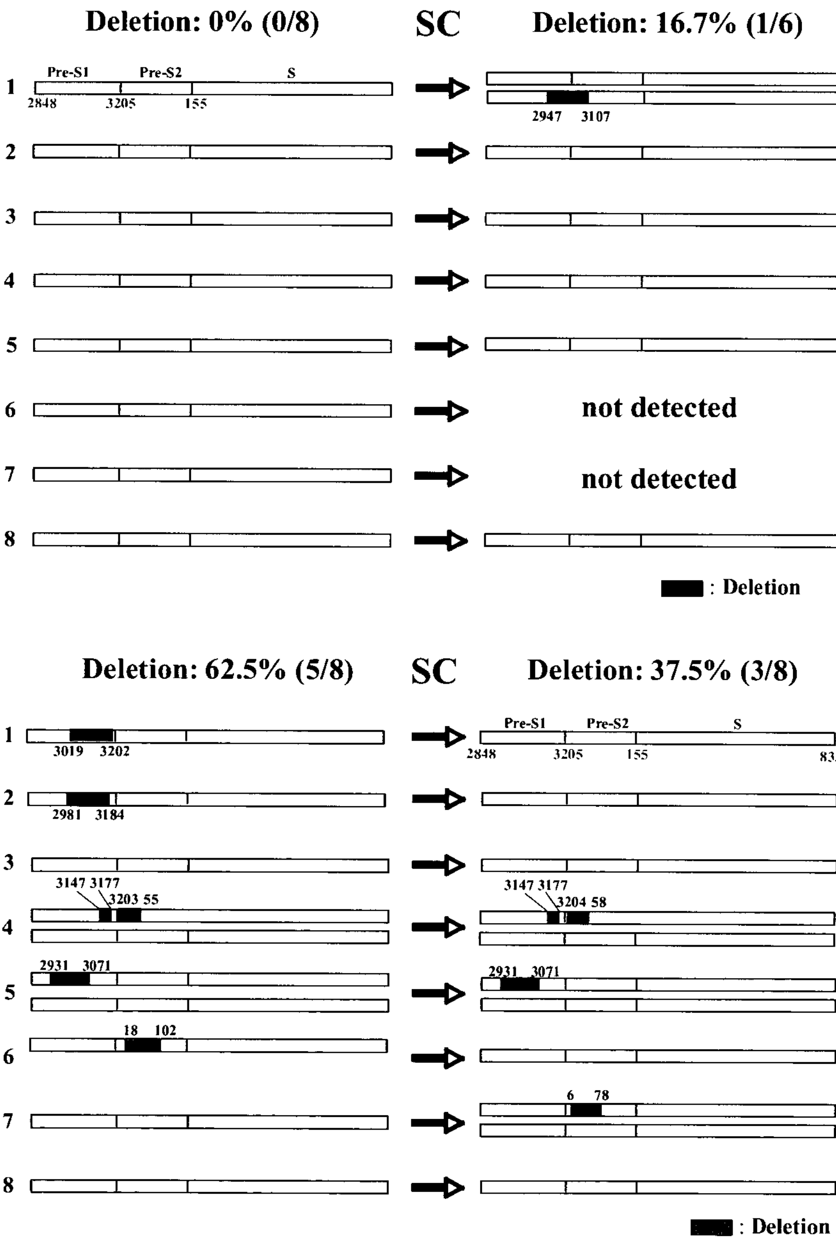

Fig. 2. Pre-S region deletion mutant in IC. The nucleotide sequences of the pre-S1, preS-2 and S are shown as bars. *Shading* of a bar indicates a deletion region. A pre-S deletion mutant was detected in only one IC after seroconversion as a minor clone (the *lower bar* shows the minor clone)

Fig. 3. The pre-S region deletion mutant in HCC patients. Pre-S deletion mutants were identified in five of eight (62.5%) HCC patients before seroconversion as a major clone (*upper bars* show major clones). Three of five deletion mutants before seroconversion were undetectable after seroconversion. Deletions were often in the C terminus of the pre-S1 domain and in the N terminus of the pre-S2 domain

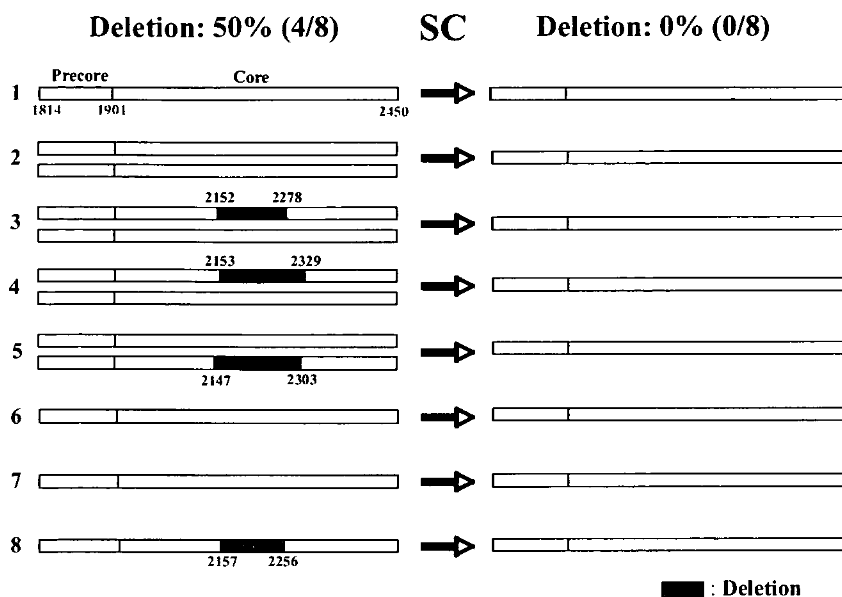

Fig. 4. Precore core region deletion mutant in HCC patients. The nucleotide sequences of precore and core are shown as bars. Core deletion mutants were identified in four of eight (50%) patients, only before seroconversion. All core deletion mutants around the center of the core domain were undetectable after seroconversion

Table 3. Clinical and virological characteristics among IC with HBV genotype C

Patient	Sex	Status of SC	Age (Years)	T1653 mutation	S1753 mutation	A1896 mutation	BCP double mutation	Core deletion mutant	Pre-S deletion mutant
1	M	Before	36	(–)	(+)	(–)	(+)	(–)	(–)
		After	45	(–)	(+)	(+)	(+)	(–)	(+)
2	M	Before	41	(+)	(–)	(+)	(+)	(–)	(–)
		After	55	(+)	(–)	(+)	(+)	(–)	(–)
3	M	Before	31	(–)	(–)	(+)	(–)	(–)	(–)
		After	46	(–)	(+)	(+)	(+)	(–)	(–)
4	M	Before	24	(–)	(–)	(–)	(–)	(–)	(–)
		After	34	(–)	(–)	(–)	(–)	(–)	(–)
5	M	Before	28	(–)	(–)	(+)	(+)	(–)	(–)
		After	35	(–)	(–)	(+)	(+)	(–)	(–)
6	M	Before	41	(–)	(–)	(–)	(–)	(–)	(–)
		After	56	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
7	M	Before	36	(–)	(–)	(–)	(+)	(–)	(–)
		After	49	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
8	F	Before	17		(–)	(–)	(–)	(–)	(–)
		After	29		(–)	(–)	(–)	(–)	(–)

SC, seroconversion

Figure 4 shows the deletion mutants in the precore/core region among HCC patients. Core deletion mutants were detected in four patients with HCC only before seroconversion. Core deletion mutants were identified around the center of the core region in these four patients. Three HCC patients (patients 3–5) before seroconversion were coinfected with wild-type virus and core deletion mutants. One HCC patient (patient 8) before seroconversion was infected with only the core deletion mutant.

Table 3 summarizes the virological characteristics of IC. We could not amplify HBV DNA in two serum samples of IC after seroconversion. No IC was infected with the core deletion mutant. The pre-S deletion mutant was identified in only one IC after seroconversion. BCP double mutations and A1896 mutations were identified in four of six (66.7%) IC after seroconversion. Table 4 shows the virological characteristics of HCC patients. The T1653 mutation was negatively correlated with the S1753 mutation in this population. The prevalence of T1762/A1764 was high in HCC patients. Of interest, a core or pre-S deletion mutant was detected in seven of eight (87.5%) HCC patients before seroconversion.

Table 4. Clinical and virological characteristics of HCC patients with HBV genotype C

Patient	Sex	Status of SC	Age (years)	T1653 mutation	S1753 mutation	A1896 mutation	BCP double mutation	Core deletion mutant	Pre-S deletion mutant
1	M	Before	40	(+)	(–)	(–)	(–)	(–)	(+)
		After	56	(+)	(–)	(+)	(–)	(–)	(–)
2	M	Before	31	(–)	(+)	(–)	(+)	(–)	(+)
		After	38	(–)	(+)	(+)	(+)	(–)	(–)
3	M	Before	38	(–)	(–)	(–)	(+)	(+)	(–)
		After	54	(+)	(–)	(+)	(+)	(–)	(–)
4	M	Before	55	(–)	(–)	(–)	(+)	(+)	(+)
		After	72	(–)	(+)	(–)	(+)	(–)	(+)
5	M	Before	28	(–)	(–)	(–)	(+)	(+)	(+)
		After	34	(+)	(–)	(–)	(+)	(–)	(+)
6	M	Before	36	(+)	(–)	(–)	(+)	(–)	(+)
		After	44	(+)	(–)	(–)	(+)	(–)	(–)
7	M	Before	35	(–)	(–)	(–)	(+)	(–)	(–)
		After	46	(–)	(–)	(–)	(+)	(–)	(+)
8	F	Before	57	(–)	(–)	(–)	(–)	(+)	(–)
		After	69	(+)	(–)	(–)	(+)	(–)	(–)

Discussion

Many previous studies have reported that the clinical course of chronic HBV infection may be modified by several specific viral mutations,[6,16–19] but most studies examined only serum samples collected from each patient at one time point. In this study, we compared viral mutations in IC and HCC patients before and after HBeAg seroconversion. ALT, HBV DNA levels, and rate of cirrhosis were significantly higher among the HCC patients than among IC only after seroconversion. Platelet count was lower among HCC patients than among IC only after seroconversion. Interestingly, even though clinical characteristics did not differ before seroconversion, deletion mutants of the core or pre-S region were significantly more associated with HCC patients than with IC. Core deletion mutants detected before seroconversion become undetectable in serum samples derived from the same patients after seroconversion. As well, pre-S deletion mutants were undetectable in three patients after seroconversion. However, the core and pre-S deletions being undetectable after seroconversion by direct sequencing does not exclude the possibility that they remained as minor clones. Preikschat et al.,[9] who sequenced cloned HBV genomes, reported the existence of deletion mutants as only minor clones; deletions and insertions in core promoter/enhancer II regions, deletions in the *C* gene, or deletions in the pre-S region were distributed on individual genomes in various combinations. Although it is unclear why major deletion mutants decreased after seroconversion, both core and pre-S deletion mutants may be predictive factors for HCC at an early stage in chronic HBV carriers.

Recently, Chen et al.[7] reported that combinations of HBV mutations (deletion in the pre-S region and/or mutations in the BCP and/or PC regions) were strongly associated with liver disease progression; a combination of mutations rather than a single mutation was associated with the development of progressive liver diseases, and pre-S deletion and BCP mutations in particular were significantly associated with the development of progressive liver diseases. In the present study, BCP mutation was identified frequently in HCC patients but also frequently in IC. The combination of a pre-S deletion with other mutants was not significantly associated with the development of HCC, owing to the small sample size in this study.

In our previous case–control study, a BCP double mutation was frequently found in each clinical group (40 IC, 40 chronic hepatitis, and 40 HCC patients), but the frequency of the T1653 mutation was significantly higher among HCC patients than among IC.[20] In this study, the T1653 mutation was identified in five of eight (62.5%) HCC patients after seroconversion and in only one of six (16.7%) IC after seroconversion, suggesting that the T1653 mutation is one of the factors promoting HCC development. However, the combination of pre-S or core deletion mutants with the T1653 mutation was not significantly associated with HCC development.

Both pre-S and core regions play an essential role in the immune response interaction because they contain B- and T-cell epitopes. Pre-S deletion and core deletion mutants thus allow escape from the host's immune function. In this study, most pre-S and core deletion regions in the HCC group encompassed B- and T-cell epitopes: most of the pre-S deletions in the 3′-terminus of the pre-S1 region and the 5′-terminus of the pre-S2 region

in HCC patients, and all of the core deletions around the center of the core region in HCC patients. These pre-S and core deletion sites including B- and T-cell epitopes[21–25] were consistent with those reported in previous papers describing patients infected with pre-S and core deletion mutants.[7,26–28]

Previous studies have shown that pre-S deletion mutants tend to accumulate at the later stage of HBV infection[3,4,29,30] and have demonstrated a marked decrease in the synthesis and secretion of small surface protein leading to retention of envelope protein and viral particles within hepatocytes, resulting in the ground-glass appearance of hepatocytes.[4] Recently, Hsieh et al.[31] identified two types of ground-glass hepatocytes containing two types of mutant L proteins with deletion within the pre-S1 and pre-S2 regions, respectively. They found that these pre-S deletion mutants accumulate in the endoplasmic reticulum (ER), resulting in strong ER stress. They concluded that the pre-S mutation causes ground-glass hepatocytes to induce oxidative DNA damage and mutations in hepatocytes in the late stages of HBV infection.

Yuan et al.[28] described the characteristics of a core deletion mutant: (1) Deletion often occurs within core amino acids 80 to 120. It does not usually extend into the partially overlapping polymerase. (2) The exact end points and sizes of deletions vary from variant to variant. (3) Deletions appear to be more often in frame than out of frame. (4) Internal deletions coincide with a potent T-cell epitope, suggesting an immune system escape function for this mutation. In the present study, the features of the core deletion mutant were mostly consistent with these characteristics.

In conclusion, our data showed a significant association of pre-S deletion and core deletion mutants before seroconversion with HCC development. The T1653 mutation after seroconversion was frequently found in HCC patients infected with HBV genotype C. These results suggest that these mutations may be a predictive factor for HCC development.

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