Table 1 Baseline characteristic of the patients studied

Characteristics	$n = 602$
Age (years)	52 (21–79)
Sex (M:F)	381:221
Nucleotide analogs (Ent : Lam→Ent : Lam + Ade)	405:67:56:74
Duration of nucleotide analog therapy (months)	90 (12–204)
Disease (CH : LC)	492:110
Family history of HCC (yes : no : unknown)	64:375:163
AST (IU/L)	58 (14–1752)
ALT (IU/L)	69 (9–2821)
Albumin (mg/dL)	4.2 (1.7–5.5)
Platelet count ($\times 10^4/\mu L$)	16.1 (3.1–41.7)
HBV genotype (A : B : C : D : NT)	4:23:231:2:342
HBeAg status (positive : negative)	295:305
HBV DNA level (log copies/mL)	6.8 (2.3–9.1)

Median (range).
Ade, adefovir; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CH, chronic hepatitis; Ent, entecavir; HBV, hepatitis B virus; HBeAg, hepatitis B e-antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; Lam, lamivudine; LC, liver cirrhosis; N, no; NT, not tested; Y, yes.

3.1–41.7); HBV genotype A : B : C : others : unknown, 4:23:231:2:342; HBeAg positive : negative status, 295:305; and median HBV DNA level, 6.8 log copies/mL (range, 2.3–9.1).

Of 602 patients at the final follow up during NA therapy, 90.4% showed a normal ALT level, 55.4% lost serum HBV DNA, 16.3% showed HBeAg seroconversion, 2.2% revealed HBsAg seroclearance and 6.1% developed HCC during therapy.

Risk factors of development of HCC during NA therapy

The risk factors associated with the development of HCC during therapy were identified to be pretreatment disease status (LC) ($P < 0.001$), duration of NA therapy ($P < 0.001$), ALT levels ($P < 0.001$) and platelet counts ($P < 0.001$) by univariate analyses (Table 2). By multivariate analyses, LC status and duration of therapy were demonstrated to be the most significant risk factors ($P < 0.001$ and $P < 0.001$, respectively).

Cumulative incidence of development of HCC

The cumulative incidence of the development of HCC was analyzed by the Kaplan–Meier method (Fig. 1a).

Table 2 Significant risk factors related with development of HCC during NA therapy

Characteristics	Development of HCC during therapy		Analysis	
	Yes $n = 37$ (6.1%)	No $n = 565$ (93.9%)	Univariate P	Multivariate P
Age (years)	57.2 (41–76)	51.5 (21–79)	0.036	0.446
Sex (M : F)	25:12	356:209	0.725	
Disease (CH : LC)	13:24	479:86	<0.001	<0.001
Duration of NA therapy (months)	37 (12–98)	94.6 (12–204)	<0.001	<0.001
Family history of HCC (yes : no)	6:21	58:354	0.509	
Pretreatment data				
ALT (IU/L)	55 (17–274)	72 (9–2821)	<0.001	0.401
Platelet count ($\times 10^4/\mu L$)	10.9 (4.0–30.3)	16.4 (3.1–41.7)	<0.001	0.146
HBeAg status (positive : negative)	17:20	280:285	0.798	
HBV DNA level (log copies/mL)	6.6 (2.5–8.9)	6.8 (2.3–9.1)	0.090	
HBsAg level >250 IU/mL (Y : N : NT)	24:6:7	410:61:94	0.519	
Final data during therapy				
ALT (<40 : ≥40 IU/L)	28:9	501:64	0.098	
HBeAg seroconversion in HBeAg positive patients (Y : N)	2:6	46:106	0.749	
Loss of HBV DNA by real-time PCR (Y : N)	14:17	247:193	0.330	
HBsAg seroclearance (Y : N)	0:37	13:552	0.351	

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CH, chronic hepatitis; HBeAg, hepatitis B e-antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; LC, liver cirrhosis; N, no; NA, nucleoside/nucleotide therapy; NT, not tested; PCR, polymerase chain reaction; Y, yes.

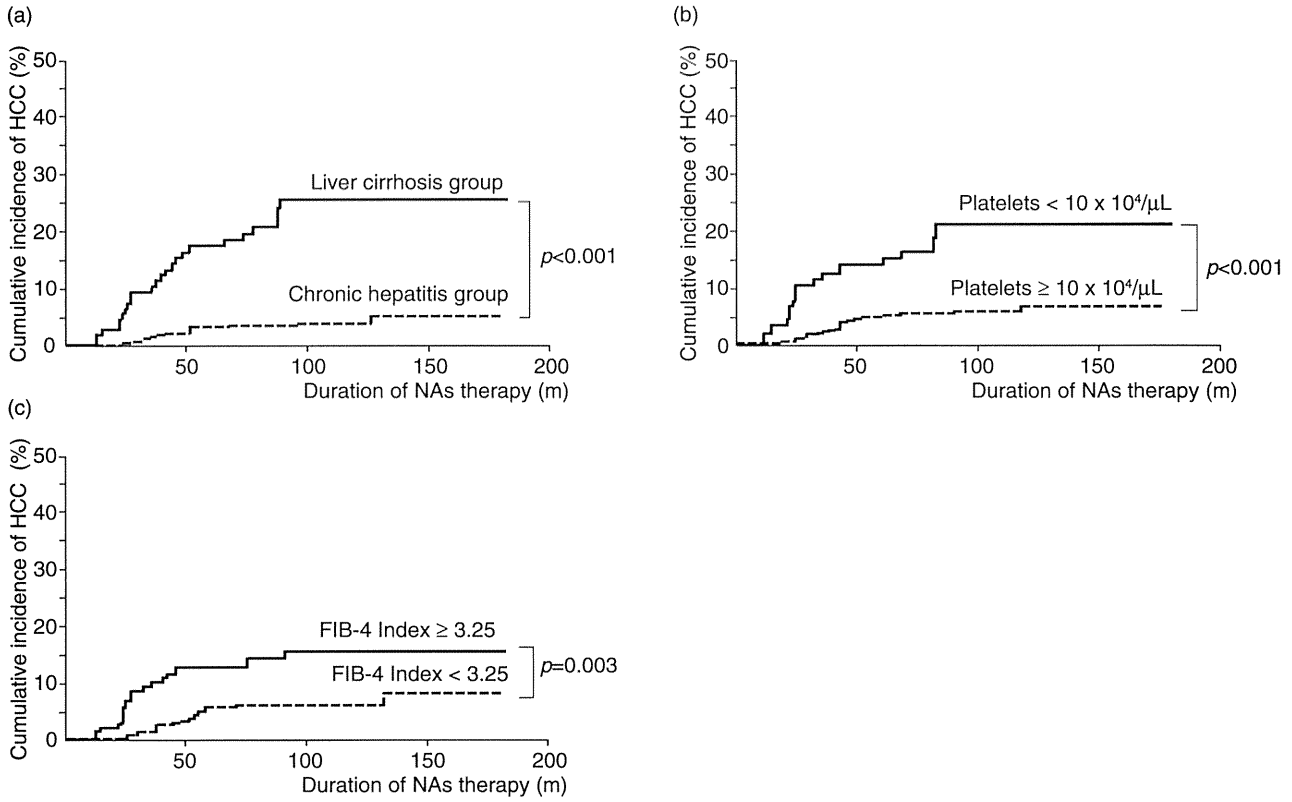

Figure 1 Cumulative incidence of development of hepatocellular carcinoma (HCC) during nucleoside/nucleotide analog (NA) therapy by Kaplan–Meier analysis. (a) Stratification by clinical or histological diagnosis, liver cirrhosis (LC) or chronic hepatitis (CH). (b) Stratification by platelet count. (c) Stratification by the FIB-4 Index. All these analyses indicated that cirrhotic patients carried a higher risk of HCC than non-cirrhotic patients.

The incidence of HCC was significantly higher in the LC group than in the CH group ($P < 0.001$). The annual incidence of development of HCC in the LC group and the CH group was 2.53%/year and 0.34%/year, respectively.

Only approximately 35% of the patients were diagnosed by liver biopsy. Hence, we employed two other methods to confirm the higher risk of HCC in cirrhotic patients with advanced liver fibrosis. By the first method of a stratification according to platelet count ($\geq 10 \times 10^4/\mu L$ or $<10 \times 10^4/\mu L$), the cumulative incidence of HCC was significantly high in the patients with a platelet count of less than $10 \times 10^4/\mu L$, compared with those with a platelet count of $10 \times 10^4/\mu L$ or more ($P < 0.001$) by the Kaplan–Meier analysis (Fig. 1b). Second, when the incidence of HCC was stratified by the FIB-4 index, the patients with a FIB-4 index of 3.25 or more had a significantly high risk, compared with those with a FIB-4 index of less than 3.25 ($P = 0.003$) (Fig. 1c).

Relationship between response to NA and incidence of HCC

To identify the goal of NA therapy for suppression of development of HCC, the relationship between response to NA therapy and incidence of HCC is important. First, when the relationship between normalization of ALT (<40 IU/L) and incidence of HCC was compared, there was no significant difference in the incidence of HCC between the abnormal ALT group and the normalized ALT group in patients with CH and those with LC (Fig. 2a). Second, when the relationship between loss of serum HBV DNA by real-time PCR and incidence of HCC was compared, there was no significant difference between positive and negative HBV DNA groups in CH patients and LC patients (Fig. 2b).

Third, when the relationship between HBeAg seroconversion and incidence of HCC was compared in the patients with positive HBeAg at commencement of the

therapy, there was no significant difference between the group with HBeAg seroconversion and the group without HBeAg seroconversion (Fig. 2c).

When the patients achieved these three goals of NA therapy, namely, normalization of ALT, loss of HBV DNA and HBeAg seroconversion, the incidence of HCC reduced only to some extent.

Finally, the relationship between HBsAg seroclearance and incidence of HCC was compared, none of the patients who achieved HBsAg seroclearance developed HCC in this study (Fig. 2d).

Relationship between duration of NA therapy and incidence of HCC

Duration of NA therapy was a significant risk factor associated with the development of HCC by multivariate analysis (Table 2). Thus, the relationship between duration of NA therapy and incidence of HCC was studied by receiver–operator curve (ROC) analysis. The area under the ROC was 0.802 (95% confidence interval [CI], 0.749–0.856; $P < 0.001$). Duration of therapy of less than 57 months was demonstrated to be the nearest cut-off value with a sensitivity of 76.1% and specificity of 76.3%. When compared with the groups with therapy duration of 57 months or more, and less than 57 months, a significantly higher incidence of development of HCC was observed in the group with therapy duration of less than 57 months than the group with therapy duration of 57 months or more in CH patients and LC patients ($P < 0.001$).

DISCUSSION

IN THE PRESENT study, we indicated that LC patients have a significantly higher risk of development of HCC than CH patients during NA therapy; and that the risk of HCC still existed even if the conventional goals of therapy like normalization of ALT, loss of serum HBV DNA or HBeAg seroconversion were achieved during therapy. However, it was demonstrated that none of the patients who achieved HBsAg seroclearance during therapy developed HCC in this study. It was confirmed that the ultimate goal of antiviral therapy for patients with chronic HBV infection should be HBsAg seroclearance.

Generally, patients with chronic HBV infection are at a high risk of development of HCC.[18] Even during antiviral therapy, a proportion of patients develop HCC.[7,10,11] Furthermore, some patients whose serum HBV DNA levels are under the detection limit level and ALT levels are within normal range develop HCC.

It has been widely stated previously that the conventional goals of therapy for patients with chronic HBV infection are normalization of ALT level, loss of serum HBV DNA and HBeAg seroconversion.[19–21] Of course, the ultimate goal of the therapy should be HBsAg seroclearance. However, HBsAg seroclearance is not common during antiviral therapy. Therefore, achievable conventional goals like normalization of ALT, serum HBV DNA and HBeAg seroconversion are usually pursued.

To achieve these goals, knowing the risk factors of development of HCC during NA therapy is important. We identified LC status and short duration of therapy to be the most significant factors associated with HCC during therapy. That is, the incidence of HCC during therapy in CH patients with a favorable virological response was very low, compared with cirrhotic patients with a favorable response.

Recently, it was reported that the risk of HCC in the patients undergoing NA therapy was reduced, compared with untreated patients, and that LC status was a significant factor of HCC during NA therapy.[10,11] Therefore, NA therapy is thought to be useful for reducing risk of development of HCC in CH and LC patients. However, some risk of HCC during NA therapy was noted in only LC patients.

In our study, the number of patients whose histological diagnosis was confirmed by liver biopsy was limited. The remainder of patients were diagnosed by clinical findings and CT or MRI. Thus, to confirm that LC status was a risk factor for HCC, all patients were analyzed by stratification of platelet count and FIB-4 index.[14–17] It was indicated that lower platelet count or higher FIB-4 index were strongly associated with cirrhotic condition. Using these two additional methods, cirrhotic patients with advanced liver fibrosis were confirmed to have a higher risk of HCC than non-cirrhotic patients.

So far, the relationship between response to NA therapy and risk of HCC is still unclear. Some previous papers have reported that low HBV DNA and normal ALT were associated with lower risk of development of HCC.[9,22–24] In this study, it was demonstrated that in CH patients who achieved normalization of ALT, loss of serum HBV DNA and HBeAg seroconversion, the risk of HCC was reduced remarkably, whereas the risk of HCC was not related to virological response to the therapy in LC patients. Hence, we should observe LC patients carefully for development of HCC during NA therapy, irrespective of a good virological response.

Because this was a multicenter study, HBsAg levels were determined by CLIA or CLEIA. These two methods

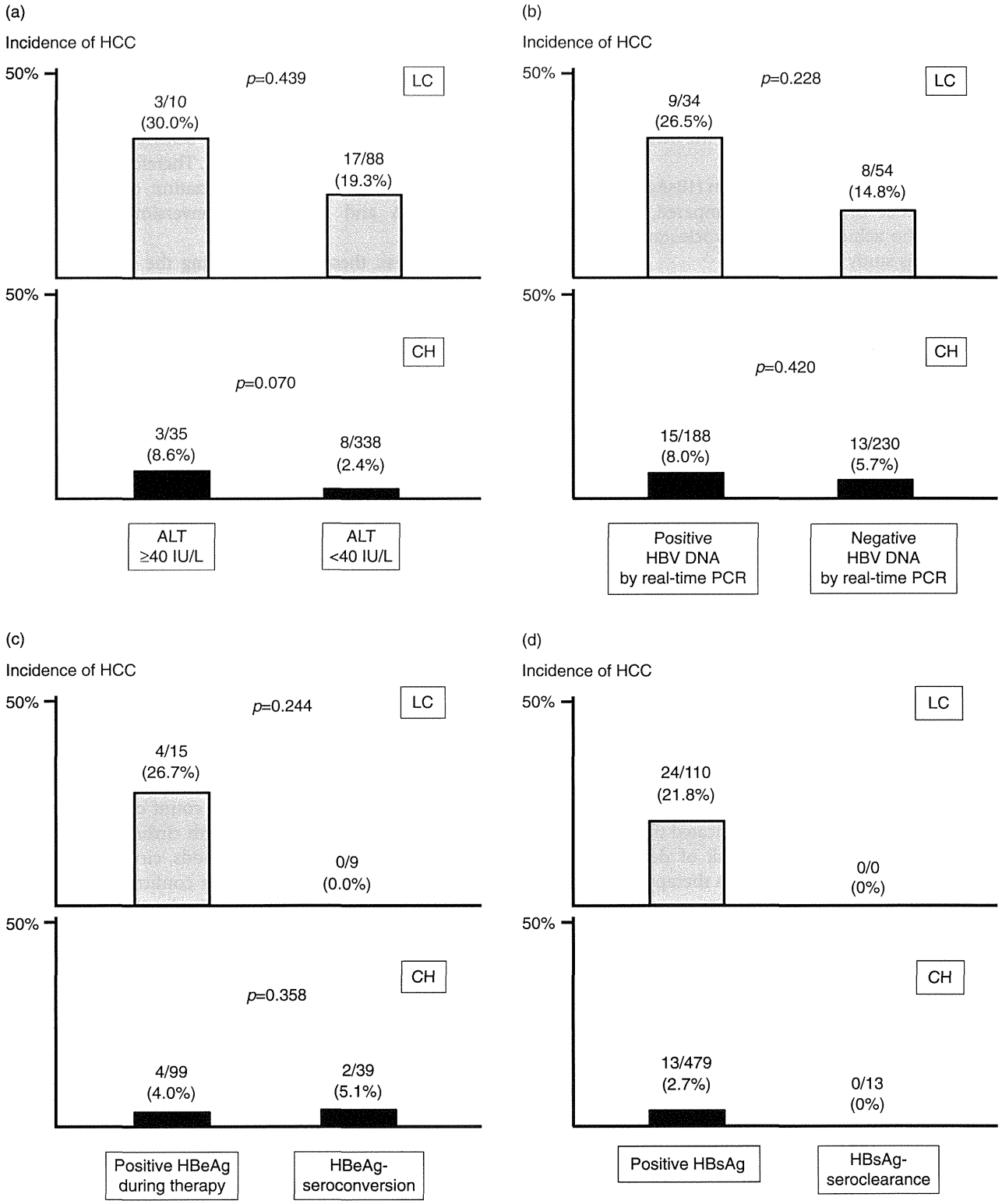

Figure 2 Relationship between incidence of hepatocellular carcinoma (HCC) and various virological responses. (a) Comparison between those with alanine aminotransferase (ALT) of ≥40 IU/L and <40 IU/L. (b) Comparison between positive and negative hepatitis B virus (HBV) DNA groups by real-time polymerase chain reaction (PCR). (c) Comparison between the positive hepatitis B e-antigen (HBeAg) group and the HBeAg seroconversion group. (d) Comparison between the positive hepatitis B surface antigen (HBsAg) group and HBsAg seroclearance group (HBsAg <0.05 IU/mL by chemiluminescent immunoassay or <0.03 IU/mL by chemiluminescence enzyme immunoassay). In these comparisons, in chronic hepatitis (CH) patients, a very low risk of HCC was observed, compared with liver cirrhosis (LC) patients, irrespective of conventional virological responses. However, none of the patients who achieved HBsAg seroclearance developed HCC during the therapy.

were demonstrated to be in good correlation with each other in previous studies.[25] In this study, HBsAg seroclearance was defined as less than 0.05 IU/mL by CLIA or less than 0.03 IU/mL by CLEIA. Only 13 out of 602 patients achieved HBsAg seroclearance in this study. While HBsAg seroclearance was not common, none of the 13 patients developed HCC during the therapy. Thus, HBsAg seroclearance was indicated to be the ultimate goal of the therapy.

In this study, a short duration of NA therapy, especially if less than 57 months, was revealed to carry a high risk of HCC in both CH and LC patients. In the early duration of therapy, inflammation in liver still may be active. It was supposed that a long enough duration of suppression of HBV and ALT by NA therapy was needed for suppression of development of HCC. However, because this study was retrospective, some selection bias of the patient data might not have been excluded. It was a concern that rather more patients who did not develop HCC during long-term therapy were collected in this study. However, careful observation for risk of development of HCC is necessary in the early stage of therapy.

In summary, we demonstrated that during NA therapy for chronic HBV infection, cirrhotic status was a significant risk factor of development of HCC. In such a scenario, careful observation is necessary irrespective of various virological responses. Finally, the ultimate goal of NA therapy, as well as other antiviral therapy, should be HBsAg seroclearance.

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EXPERT REVIEWS

Efficacy of daclatasvir in hepatitis C virus

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Daclatasvir is a novel NS5A inhibitor of hepatitis C virus (HCV). Daclatasvir combined with peginterferon α-2a and ribavirin in Japanese patients infected with genotype 1b HCV achieved sustained virological response (SVR) in 100% of treatment-naïve patients, due to high rates of favorable IL28B allele and genotype 1b. SVR 24 was achieved by asunaprevir and daclatasvir in 87.4% of intolerant and 80.5% of nonresponder patients. Baseline NS5A-resistant variants were detected and they failed to achieve SVR. Most patients with genotype 1a experienced virological breakthrough by dual oral treatment, and should be treated QUAD or replaced by all oral regimens that are more potent and have fewer side effects. IFN-free regimens including daclatasvir and asunaprevir for genotype 1 null responders should be tailored to subtype, and preexisting NS5A-resistant variants should be evaluated carefully before choosing the drugs. This regimen alone is unlikely to move forward without additional agents.

KEYWORDS: asunaprevir • chronic hepatitis C • DAA • daclatasvir • genotype 1

Hepatitis C virus (HCV) infection is one of the leading causes of liver cancer and end-stage liver disease, with 180 million people infected worldwide and a prevalence of approximately 2.8% [1]. Once patients are infected with HCV, more than half will progress to chronic hepatitis and 20% of these will progress to liver cirrhosis over 20–25 years [2]. Chronic hepatitis C and liver cirrhosis increase the risk of hepatocellular carcinoma (HCC) with advanced fibrosis of the liver. As a result, the annual incidence of HCC may be as high as 2–4% [3–5] in highly endemic areas such as Japan. The incidence of HCC decreases in patients who achieve a sustained virological response (SVR) to interferon (IFN) treatment [6,7]; therefore, the primary goal of anti-HCV treatment is to achieve SVR, defined as HCV RNA being undetectable in the blood, both at the end of treatment and at 24 weeks after cessation of treatment. Recently, SVR 12 is now considered the endpoint of therapeutic trials for chronic hepatitis C. The standard antiviral therapy used to be a combination of treatment with peginterferon (PEG IFN) α and ribavirin, and it has been reported that around 50% of patients who received PEG IFN and ribavirin combination therapy achieved SVR (>40% with genotype 1, >80% with genotype 2 or 3) [8,9].

The direct-acting antivirals (DAAs) have improved treatment response to therapy for patients persistently infected with HCV, and protease inhibitors such as telaprevir and boceprevir have achieved SVR rates from 73 to 89% [10,11]. However, the patients who failed to respond to a combination of a protease inhibitor and PEG IFN and ribavirin usually have a high risk of emergence of resistance mutations to protease inhibitors, especially those with previous null responses to PEG IFN and ribavirin [12]. For these patients, other DAAs with different mechanisms of action are necessary.

Daclatasvir is a first-in-class, NS5A replication complex inhibitor with potent pan-genotypic antiviral activity *in vitro* (HCV-genotypes 1–6) [13]. At doses of 1–100 mg daily, it was well tolerated, had a pharmacokinetics profile supportive of once-daily dosing and produced a rapid and substantial decrease in HCV RNA levels in patients chronically infected with HCV genotype 1 [14]. It is important to clarify clinically the effects of daclatasvir in combination with PEG IFN and ribavirin or with other DAAs.

Mechanism of action of antiviral agents

IFN injection induces IFN-stimulating genes (ISGs) and immune response in the host and

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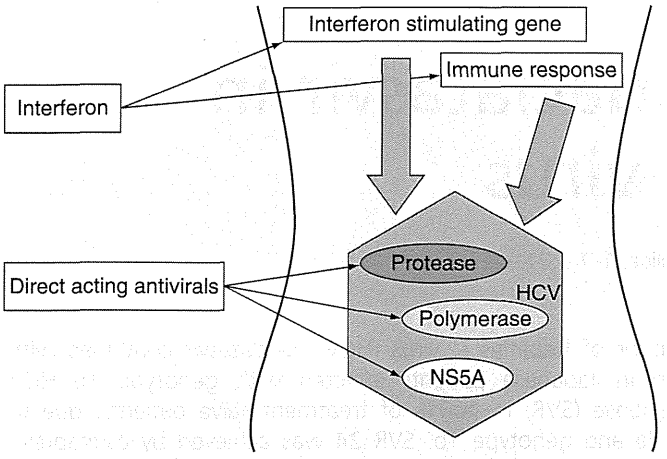

Figure 1. Mechanisms of action of antiviral agents.
Interferon injection induces interferon-stimulating genes and immune responses in the host, thus acting against HCV. The effects vary with the genotype of the host IL28B, but once the ISGs are induced by interferon in the host, these acts against the virus and the antiviral activity is not specific for HCV. On the other hand, direct-acting antivirals are effective for every patient but the effects of the direct-acting antivirals are diminished if the virus has the corresponding resistance mutations.
HCV: Hepatitis C virus.

thus acts against HCV [15]. The ISG response of the host varies according to a host single-nucleotide polymorphism (SNP) and is reduced in patients with IL28B minor alleles [16]. IL28B favorable allele is predominant in Asian patients and regimens including IFN are effective in Asia compared with non-Asian countries. However, once the ISGs have been induced by IFN in the host, they act against the virus and these antiviral activities are not specific for HCV. HCV is eliminated from the host even if it has a drug-resistance mutation.

On the other hand, DAAs are effective in every patient, and there is no difference in the effects between patients with IL28B major and minor alleles. However, the effects of DAAs are diminished if the virus has a resistance mutation to the agent. Resistance mutations are more likely to emerge in genotype 1a than 1b. Therefore, hepatologists should consider both the IFN responsiveness of the patients and resistance mutations in the virus before choosing the drugs for antiviral treatment (Figure 1).

Daclatasvir in combination with PEG IFN & ribavirin

Daclatasvir is a novel NS5A inhibitor of HCV with few adverse effects with once-daily dosing. Daclatasvir combined with PEG IFN α-2a and ribavirin has been used to treat Japanese patients infected with genotype 1 HCV; the SVR rate at week 24 post-treatment was 89 and 100% of treatment-naïve patients receiving 10 and 60 mg daclatasvir, respectively, versus 75% in PEG IFN and ribavirin plus placebo recipients. In the previous null responders, the SVR rate was as high as 78% for treatment with 60 mg daclatasvir once per day, in combination with PEG IFN α-2a plus ribavirin [17]. Adverse events occurred with a similar frequency among the treatment groups and were consistent with the adverse event profile of IFN α-2a plus ribavirin alone. When naïve patients with genotype 1b infection were treated with daclatasvir 10 or 60 mg in combination with PEG IFN α-2b plus ribavirin, the SVR rates were 66.7 and 90.0%, respectively [18]. Previous null responders had more frequent virological failure; 22.2 and 33.3% of 10 and 60 mg daclatasvir, respectively. From these results, regimens with greater antiviral potency are needed for previous nonresponders when they are to be treated with daclatasvir, PEG IFNα-2b and ribavirin. Virological escape was investigated in HCV-genotype 1-infected patients receiving daclatasvir plus PEG IFN α-2a or α-2b and was associated with enrichment of the NS5A-resistant variant L31V/M-Y93H. In previous nonresponders, emergent variants associated with failure also included NS5A-A92K and NS5A-ΔP32 [19]. From the resistance analysis of HCV genotype 1 null responders to prior treatment, NS5A-resistant variants persisted while NS3-resistant variants generally decayed, suggesting a higher relative fitness of NS5A variants [20].

The IL28B favorable allele is more frequent in Asian patients than Caucasians, and the overall response when treated by PEG IFN and ribavirin plus daclatasvir is higher than that in the non-Asian study. Additionally, genotype 1a which is more likely to obtain resistant variants than genotype 1b is more frequent in non-Asian patients. The differences in the IL28B allele and the genotype of HCV are responsible to be high response rate in Asia. In the non-Asian patients, genotype 1a is more frequent than 1b and PEG IFN and ribavirin plus daclatasvir is less effective. QUAD or sofosbuvir in combination with daclatasvir has been reported to achieve superior SVR rate in western countries [21,22].

It has demonstrated efficacy in genotype 2- and 3-infected patients in combination with PEG IFN α and ribavirin plus daclatasvir [23].

DAAs including daclatasvir without IFN

The dual oral DAAs asunaprevir and daclatasvir were investigated in 10 Japanese patients infected with HCV genotype 1b and with previous null response. All patients received 60 mg of daclatasvir once daily and 100 mg of asunaprevir twice daily for 24 weeks, without PEG IFN and ribavirin. Nine of the 10 patients achieved SVR 12 and there was no virological breakthrough. Diarrhea and headache, generally mild, were the most common adverse events. Transaminase elevations were reported in three patients, but did not result in discontinuation [24]. This is the first report of high viral clearance without IFN, and has become the promising regimen in the patients in whom IFN is ineligible or intolerant.

Furthermore, the same group reported the results of a Phase IIa study of Japanese patients with chronic HCV genotype 1b infection. Twenty-one null responders and 22 patients intolerant to or medically ineligible for PEG INF/ribavirin therapy received dual oral treatment for 24 weeks with 60 mg daclatasvir once daily and 200 mg of asunaprevir twice daily and SVR 24 was achieved in 90.5 and 63.6%,

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Table 1. Sustained virological response rate in Phase I, II and III Japanese studies and in clinical studies in Europe and the USA.

Clinical trials	Regimen	Patients (n)	Genotype	SVR rate (%)	Ref.
Phase I (Japan)	60 mg DCV q.d. + 200 mg ASV b.i.d.	10 (nonresponders)	1b	90	[24]
Phase II (Japan)	200 mg ASV b.i.d. + 60 mg DCV q.d.	21 (null responders) 22 (ineligible to interferon)	1b 1b	90.5 63.6	[25]
Phase III (Japan)	60 mg DCV q.d. + 200 mg ASV b.i.d.	133 (ineligible to interferon) 87 (null responders)	1b 1b	87.4 80.5	[27]
Phase IIa (Europe, USA)	60 mg DCV q.d. + 200 mg ASV b.i.d. 200 mg ASV q.d.	18 (null responders) 20 (null responders)	1b 1b	83 60	[21]
	60 mg DCV q.d. + 200 mg ASV b.i.d. + RBV	18 (null responders) 4 (null responders)	1a 1b	5.6 100	

ASV: Asunaprevir; b.i.d.: Two-times a day; DCV: Daclatasvir; q.d.: Once a daily.

respectively [25]. Clinical data at baseline and on-treatment virological escape were investigated in HCV genotype 1b patients treated with daclatasvir and asunaprevir. Karino *et al.* reported that baseline NS5A polymorphisms (L31 M, Y93H) associated with daclatasvir resistance (<25-fold) were detected in five null responders and six patients ineligible for IFN and they failed to achieve SVR. Furthermore, preexisting NS5A-Y93H polymorphisms persisted through 48 weeks post-treatment in patients with virological failure on daclatasvir/asunaprevir combination treatment [26]. Two patients who did not have resistance mutations to NS5A inhibitors before treatment by direct sequence did not achieve SVR, and emergence of resistance mutations to both the NS5A inhibitor and the protease inhibitor was observed. It is an important issue to examine the preexisting resistance mutation profiles to DAAs to choose the treatment drugs; however, it remains to be clarified whether checking resistance mutations using deep sequencing is necessary instead of direct sequencing or not.

The dual oral combination treatment with daclatasvir and asunaprevir was investigated in Europe and the USA, and in genotype 1 null responders, dual therapy with daclatasvir plus twice-daily asunaprevir was effective for most genotype 1b patients but the dual combination therapy was not effective for genotype 1a. Most genotype 1a patients experienced virological breakthrough. When the genotype 1a patients were treated by QUAD, that is, PEG IFN, ribavirin, daclatasvir and asunaprevir, the SVR 24 rate was reported as high as 95% [21]. IFN-free regimens for null responders, including daclatasvir and asunaprevir twice daily, should be tailored for the subtype (Table 1).

The dual oral combination therapy was further investigated in an open-label, Phase III study in Japan. One-hundred and thirty-three IFN-ineligible/intolerant and 87 nonresponder patients with chronic HCV genotype 1b infection were enrolled. The patients received 60 mg daclatasvir once daily plus 100 mg asunaprevir twice daily for 24 weeks. SVR 24 was achieved by 87.4% of the IFN-ineligible/intolerant patients and 80.5% of the nonresponder (null and partial response) patients (Figure 2). The SVR rate was similar between the patients with IL28B CC and non-CC genotypes. Increased alanine aminotransferase (ALT) and AST was observed in 15.8 and 12.6% of patients, respectively, and grade 3–4 ALT elevation was observed in 16 of 222 patients (7.2%) [27].

The effect of daclatasvir in combination with sofosbuvir was examined in previously treated or untreated chronic HCV genotype 1 infection, and 98% of 126 previously untreated patients and 98% of 41 who did not achieve SVR with HCV protease inhibitors had an SVR at week 12, after the end of

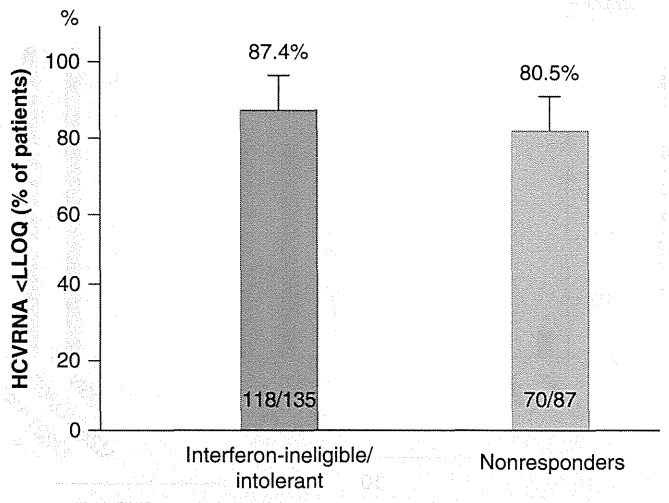

Figure 2. Changes in hepatitis C virus RNA levels during combination therapy with an NS5A inhibitor (daclatasvir) and a protease inhibitor (asunaprevir) in genotype 1b patients.
In the study conducted in Japan, HCV RNA levels decreased below the detectable threshold in virtually all cases 4 weeks after the start of treatment but increased again after 8 weeks and beyond in some subjects. This was presumably due to breakthrough following the virus acquiring resistance to the direct-acting antivirals. HCV : Hepatitis C virus; LLOQ: Lower limit of quantification. Reproduced with permission from [25].

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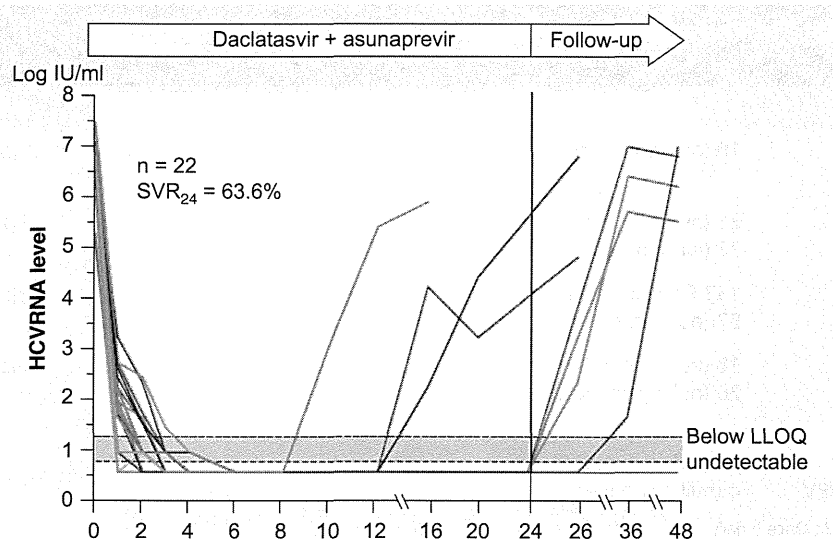

Figure 3. The resistance of emerging variants increasing daclatasvir treatment.
Concentrations in replicon variants with the indicated amino acid substitutions.
LLOQ: Lower limit of quantification.
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therapy [22]. A total of 92% of 26 patients with genotype 2 infection and 89% of 18 patients with genotype 3 infection had an SVR at week 12. This combination is promising; however, Phase III study has not been conducted.

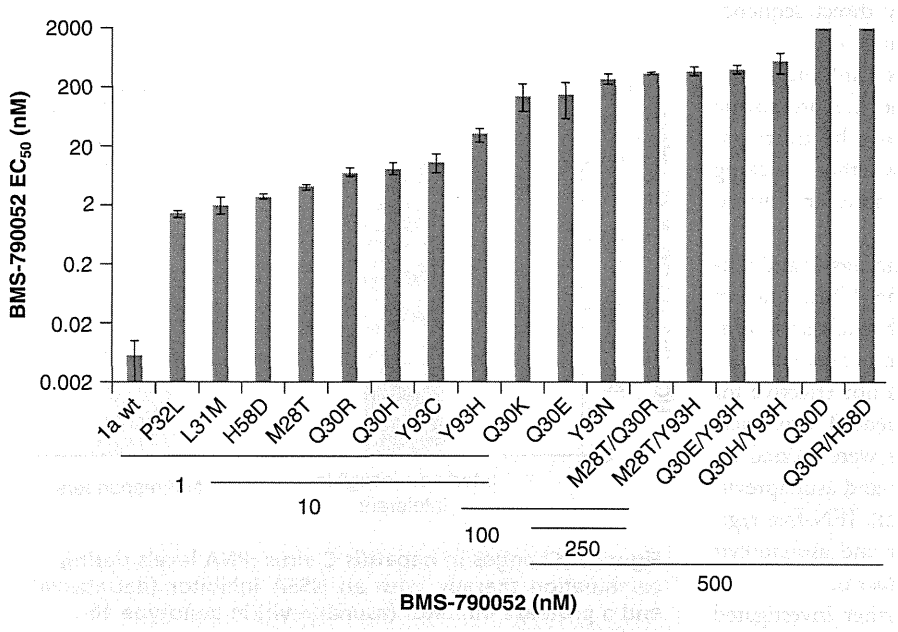

Figure 4. The sustained virological response (SVR) rate of a Phase III study of Japanese patients with chronic hepatitis C virus (HCV) genotype 1b infection.
One hundred and eighteen patients intolerant to or medically ineligible for peginterferon/ribavirin therapy and 87 nonresponders to previous treatment received dual oral treatment for 24 weeks with 60 mg daclatasvir once daily and 100 mg of asunaprevir twice daily and SVR 24 was achieved in 87.4 and 80.5%, respectively.
Reproduced with permission from [27].

Treatment strategy for HCV genotype 1b using daclatasvir

Among Japanese clinical studies, there have been reports of the efficacy of DAA-only therapy without IFN on HCV genotype 1b. The patients received two drugs orally, daclatasvir and asunaprevir, and 90.5% of previous nonresponder and 63.6% of the initially treated, IFN-intolerant patients achieved SVR. As daclatasvir and asunaprevir are administered orally once per day and once or twice per day, respectively, they are extremely convenient for the patient. Although a three-drug combination therapy with daclatasvir, PEG-IFN and ribavirin did not cause any adverse reactions attributable to daclatasvir [17,18]; adverse reactions such as increase in ALT and bilirubin levels were observed in the two-drug combination therapy cases. Furthermore, resistance mutations occurred when SVR was not achieved [25]. In clinical studies conducted in Japan, HCV RNA levels decreased below the detectable threshold 2 weeks after the start of treatment in virtually all cases, but increased again after 4 weeks and beyond in some subjects (Figure 3). This was presumably due to the breakthrough following the virus acquiring resistance to the DAAs [25].

In some cases where SVR was not achieved, NS5A resistance mutations were detected before treatment. The resistance profile has been extensively investigated in replicon cells treated with daclatasvir (Figure 4) [28]. Of the hepatitis C patients who were not treated with DAAs, 11.2% had HCV NS5A resistance-associated mutations, such as L31M and Y93H, while others had HCV with PI resistance-associated mutations, such as V36A, T54S, Q80R and D168E [29]. Furthermore, in cases where patients had HCV with resistance-associated mutations to NS5A inhibitors prior to treatment, seven cases were also observed to have resistance to NS3 PIs when these two drugs were administered orally (Table 2). Where the Y93H NS5A resistance-associated mutation was observed before treatment, SVR was achieved in 11/23 IFN-ineligible intolerant patients and 4/14 nonresponder patients treated with the two-drug combination therapy [27]. Thus, appropriate attention should be paid to resistance

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mutations. Other first-generation NS5A inhibitors such as ledipasvir and ABT-267, which are in Phase III clinical trials, have been shown to be less effective to the Y93H mutation. Since in Asian countries, sofosbuvir has not been used in clinical practice, null responders and ineligible patients to IFN should be carefully investigated whether they have resistant variants to NS5A inhibitors, and if they have resistant variants to NS5A inhibitors, they should wait treatment until more potent antiviral drugs.

To improve the SVR rate in genotype 1 infection, the all-oral, IFN-free, and ribavirin-free regimen of daclatasvir, asunaprevir and BMS-791325, a non-nucleoside NS5B inhibitor, achieved 92% SVR 12 [30]. To overcome preexisting resistance mutations to NS5A inhibitors, second-generation NS5A inhibitors with high potency are necessary. GS-5816, ACH-3102 and MK-8742 have displayed improvements in the genetic barrier while maintaining potency [31]. It is important to display continued viral suppression in the presence of multiple NS5A resistance mutation clinically, and if the second-generation NS5A inhibitors have no on-treatment viral breakthrough in genotype 1b patients, it must be promising.

For the future treatment of chronic hepatitis C, it is important to select medications appropriately by considering the possibility of drug-resistance mutations in addition to the patient's responsiveness to IFN. It is also necessary to prepare for the risks of a difficult-to-treat disease if HCV acquires multidrug-resistance mutations to orally administered DAAs, following unsuccessful treatment. Sofosbuvir has not been used in Asian countries, and before the approval of sofosbuvir, to choose the adequate drug is an important issue to prevent the emergence of resistance mutations to DAAs. According to AASLD and EASL guidelines for the treatment of HCV, where resistant variants of HCV before choosing the treatment drug is not recommended, except for testing Q80K in the case of treating genotype 1a patients by simeprevir. Although it is expected that new medications incorporating polymerase inhibitors and other NS5A inhibitors will become available in the future, the risks of hepatic carcinogenesis should be assessed carefully in each case to decide when to start the treatment.

Table 2. Resistance mutations to NS3 (protease) and NS5A inhibitors baseline and post viral breakthrough [25].

Patients		NS5A			NS3		
		L31	Q54	P58	Y93	Q80	D168
Viral breakthrough							
1	Baseline	L/M			Y/H		
	Post-VBT	M		A	H		A
2	Baseline		Y		Y/H	L	
	Post-VBT	M	Y		H		V
3	Baseline		Y		H		
	Post-VBT	M	Y		H		V
Posttreatment relapse							
4	Baseline			P/S	Y/H		
	Postrelapse	M			H		A
5	Baseline			L			
	Postrelapse	M		L	H		V/D
6	Baseline						
	Postrelapse	V			H		V
7	Baseline				H		
	Postrelapse	V/M			H		V

Seven patients had preexisting NS5A resistance mutations and both resistance mutations to NS5A and PIs were detected after treatment.
VBT: Viral breakthrough.

Expert commentary

Chronic hepatitis C has been treated by an IFN-based regimen for 20 years, but oral DAA combination provided a higher rate of SVR without IFN. If the patients with genotype 1 HCV infection are treated with the DAA combination, viral genotype and preexisting variants which shows resistance against DAA should be carefully evaluated before choosing treatment drugs. The dual oral combination of daclatasvir and asunaprevir was shown to be effective in genotype 1b without variants against NS5A and protease inhibitors, and these patients achieved viral elimination without IFN. However, since grade 3–4 ALT elevation was frequently observed in Phase II studies, adverse events should be carefully managed during the treatment.

DAAs with more potent effects to HCV are under clinical studies, and the viral elimination rate will be improved in combination with other DAAs such as sofosbuvir. To prevent the emergence of multidrug resistance mutations is the most important issue in the treatment of HCV infection, and it is an important issue to decide when the patient should be treated. The timing of treatment for the patients with HCV infection should be determined by evaluating the fibrosis of the liver and likelihood of developing HCC in each patient.

Five-year view

Daclatasvir in combination with a protease inhibitor provided viral elimination without IFN, but to prevent the emergence of multidrug resistance is an important issue in patients having preexisting variants against NS5A inhibitors. To overcome preexisting resistance mutations to NS5A inhibitors, second-generation NS5A inhibitors with high potency are necessary. GS-5816, ACH-3102 and MK-8742 have displayed improvements in the genetic barrier while maintaining potency. If the second-generation NS5A inhibitors have no on-treatment viral breakthrough in genotype 1b patients, it must be promising.

The addition of a non-nucleoside polymerase inhibitor may be helpful to improve the viral elimination rate, and all oral triple combination treatment should be examined if the incidence of severe adverse events is less frequent. If the preexisting variants against NS5A inhibitors are detected in the patients, they

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should be treated by a more potent combination of protease and nucleotide polymerase inhibitors. To choose the treatment for anti-HCV, genotype and preexisting variants should be carefully evaluated. It is another important issue that in the some patients, HCC develops even after achieving SVR. Male patients with advanced fibrosis before treatment should receive intermittent ultrasound or enhanced-CT scan to detect the development of HCC in early stage even after they achieve SVR.

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Key issues

- Daclatasvir is a first-in-class, NS5A replication complex inhibitor with potent pan-genotypic antiviral activity *in vitro*, and once-daily dosing of 1–100 mg produced a rapid and substantial decrease in hepatitis C virus (HCV)-RNA levels in patients chronically infected with HCV genotype 1.
- Overall response treated by pegIFN and ribavirin plus daclatasvir is higher in Asian studies than in non-Asian studies.
- The dual oral combination treatment with daclatasvir and asunaprevir showed a sustained virological response (SVR) 24 rate from 63.6 to 90.5% in a Phase II study in Japan, but most patients with preexisting NS5A-Y93H polymorphisms did not achieve SVR and emergence of resistance mutations to both the NS5A inhibitor and the protease inhibitor was observed. Preexisting viral variants should be carefully evaluated.
- Dual therapy with daclatasvir plus twice-daily asunaprevir is effective for most genotype 1b patients, but the dual combination therapy was not effective for genotype 1a in Europe and the USA. When genotype 1a patients were treated with QUAD, the SVR 24 rate was reported to be as high as 95%, and the dual oral regimen should be tailored for the subtype.
- Daclatasvir in combination with sofosbuvir provided an SVR 12 rate of 98%. If they have resistant variants to NS5A inhibitors, they should wait treatment until more potent antiviral drugs.

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Reduced Organic Anion Transporter Expression Is a Risk Factor for Hepatocellular Carcinoma in Chronic Hepatitis C Patients: A Propensity Score Matching Study

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Key Words
Hepatocellular carcinoma · SLC22A7 · Organic anion transporter 2 · Chronic hepatitis C · Hepatocarcinogenesis

Abstract
Objectives: Recent reports indicated that reduced SLC22A7 (a gene-encoding organic anion transporter 2) expression in noncancerous liver tissue predicts hepatocellular carcinoma (HCC) recurrence after curative resection. Our study aimed to elucidate the association between SLC22A7 expression and HCC development in chronic hepatitis C patients. ***Methods:*** HCC recurrence after local ablation therapy and SLC22A7 expression in noncancerous liver tissue were analyzed in 20 patients. Subsequently, the association between de novo HCC development and SLC22A7 expression was examined at baseline in 38 hepatitis C patients without HCC who subsequently developed HCC as well as in 76 hepatitis C patients who did not develop HCC and were matched for age, gender and stage of fibrosis. ***Results:*** In the patients whose HCC had been cured, reduced SLC22A7 expression in noncancerous liver tissue was significantly associated with a high incidence of multifocal HCC recurrence. In patients without HCC at baseline, cumulative incidence of de novo HCC development was significantly higher with a reduced SLC22A7 expression than with a normal expression ($p = 0.01$). This difference remained significant among patients without known risk factors for HCC like age and advanced fibrosis. ***Conclusion:*** Reduced SLC22A7 expression in the liver indicates a significant risk for HCC development in chronic hepatitis C, independently of other risk factors.

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Introduction

Hepatocellular carcinoma (HCC) is the third most common cancer worldwide [1] and the most frequent primary liver cancer [2]. Chronic hepatitis C virus (HCV) infection is a major risk factor for developing HCC [3], increasing the risk by 17-fold when compared with healthy individuals [4, 5]. Among HCV-positive patients, several risk factors for HCC have been well documented, including age, obesity, sex, serum platelet count and stage of liver fibrosis [6–10]. Advanced fibrosis, in particular, is the most significant risk factor for HCC in chronic HCV patients. The response to interferon therapy is also related to HCC risk [11, 12], mainly because the treatment attenuates hepatitis in responsive individuals. However, despite the absence of known risk factors, younger patients and those with nonadvanced fibrosis also develop HCC. Thus, surveillance is insufficient and additional risk analyses are required for those chronic HCV patients without known risk factors for HCC.

As for curatively treated HCC patients, tumor differentiation or progenitor-cell feature markers of cancerous tissue have been identified as predictors of recurrence [13, 14]. In contrast, only several reports have mentioned the importance of background noncancerous liver tissue and the microenvironment; these are predictive of HCC recurrences [15, 16]. Moreover, no specific features of noncancerous liver tissue have been clarified to be associated with de novo HCC development.

A recent prospective study showed that reduced SLC22A7 (organic anion transporter 2, OAT2) activity in noncancerous liver tissue is associated with multifocal recurrence after curative resection, independently of age and stage of fibrosis [17]. Furthermore, this study revealed that reduced SLC22A7 expression indicates a high risk for poor prognosis [18]. This observation indicates that the function of the transporter in noncancerous liver tissue is related to hepatic carcinogenesis, which may explain HCC development in patients who have no other known risk factors.

In this study, the use of SLC22A7 as a biomarker for HCC recurrence after curative local ablation therapy was assessed in order to validate and extend previously reported observations. Subsequently, the propensity score matching method was used to match patients with and without HCC development as well as to elucidate the association between SLC22A7 expression in hepatitis tissue and the risk of HCC development in chronic HCV patients.

Patients and Methods

Distant Recurrence after Radio Frequency Ablation Therapy for HCC

Patients

To reveal the relationship between multifocal HCC recurrence and SLC22A7 expression in noncancerous liver tissue, we conducted a retrospective study enrolling patients who received curative local ablation therapy. Twenty of the patients who enrolled in this cohort fulfilled the following criteria: (1) their HCC was treated curatively by radio frequency ablation (RFA); (2) they were infected with HCV and (3) they underwent liver biopsy at least 6 months after curative RFA. Written informed consent was obtained from all patients. The study was approved by the Ethical Committee of the Musashino Red Cross Hospital in accordance with the Declaration of Helsinki.

Data Collection and Histological Evaluation

Patient characteristics, treatment details and biochemical, hematological, virological and histological data were collected at enrollment.

Liver biopsy specimens were obtained using 13-gauge needles under laparoscopy or 15-gauge needles using an ultrasound guide. Liver biopsy specimens were scored by board-certified pathologists for stage of fibrosis and grade of inflammatory activity according to the classification by Desmet et al. [19].

Immunohistochemical Staining of SLC22A7

All liver biopsy specimens were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 4 μm and stained with anti-OAT2 (SLC22A) antibody (kindly provided by Dr. Anzai) at a 1:20 dilution. Immunohistochemical (IHC) staining was performed using an automated immunostainer (Ventana XT System; Ventana Medical Systems Inc., Tucson, Ariz., USA), with the same procedure as the previous study [17]. Cell staining was evaluated along the entire length of the biopsy core (>30 high-power fields). Staining was graded according to the following score: ≤25% = reduced staining of cells and >25% = normal staining of cells (fig. 1). Scoring of SLC22A7 staining was performed independently by two hepatologists (K.M. and A.K.) who were blinded to the clinical outcome, and average scores were used for analysis.

Surveillance for HCC

Patients were examined for HCC every 3–6 months by abdominal ultrasonography, dynamic computed tomography or magnetic resonance imaging. Serum alpha-fetoprotein levels were measured every 3 months. HCC diagnosis was confirmed from needle biopsies, surgical resection specimens or according to the typical radiological hallmarks of early enhancement and delayed washout. The start date of follow-up was the date of liver biopsy and the end date was HCC development or the latest medical attendance.

Relationship between SLC22A7 and de novo HCC Development in Chronic HCV without HCC at Baseline

Patients

To elucidate the relationship between SLC22A7 and de novo hepatic carcinogenesis, we conducted a study in an independent cohort. A consort diagram of this study is shown in figure 2. Since 1992, 1,512 chronic HCV patients provided liver biopsies prior to interferon therapy at Musashino Red Cross Hospital. A total of 1,003 of these patients did not achieve a sustained virological re-

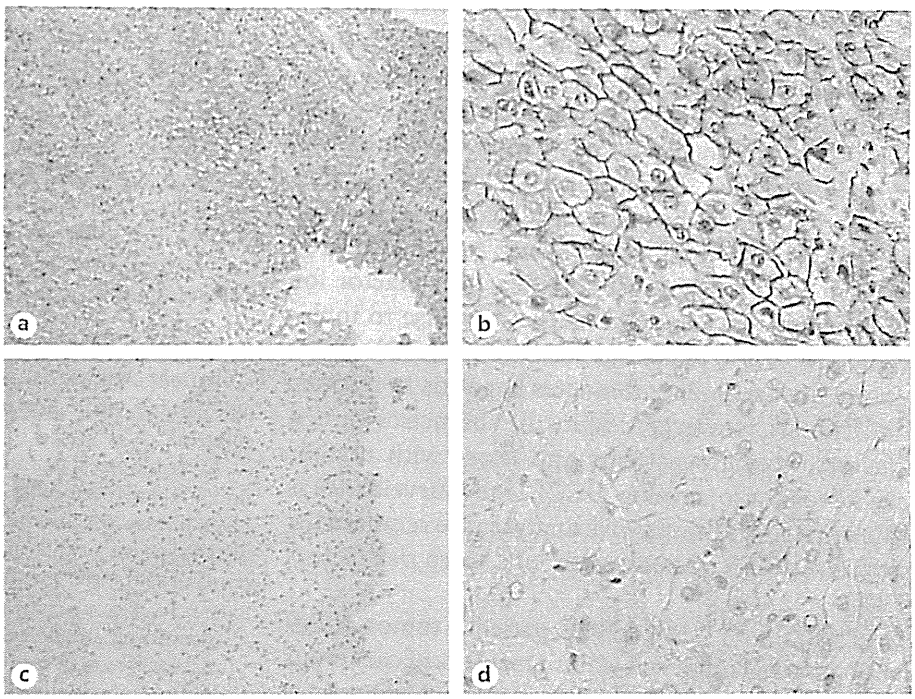

Fig. 1. IHC analysis of SLC22A7 in biopsy specimens. **a**, **b** Normal SLC22A7 expression (≥25% positive cells) **a** ×100. **b** ×400. **c**, **d** Reduced SLC22A7 expression (<25% positive cells). **c** ×100. **d** ×400.

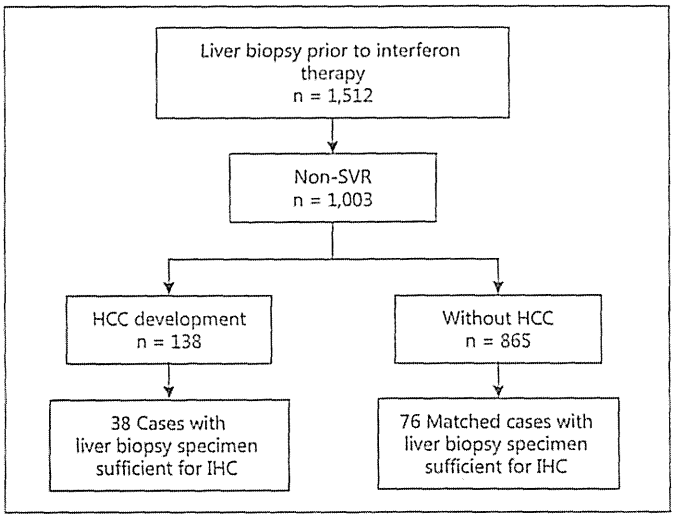

Fig. 2. Consort diagram of stratified analyses.

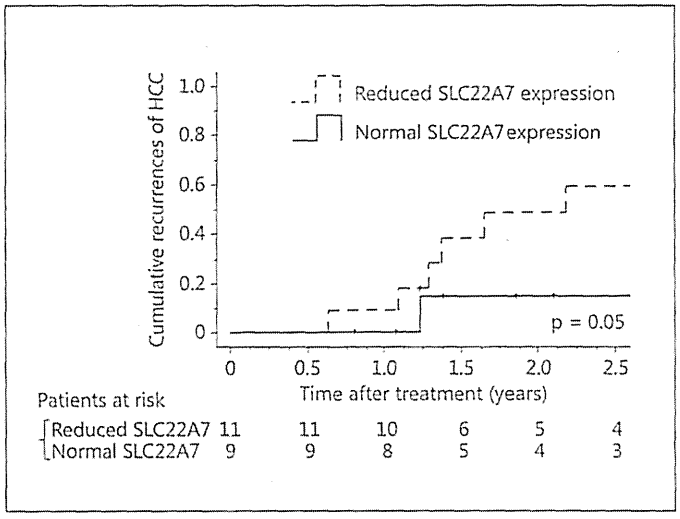

Fig. 3. Cumulative incidence of HCC recurrence after curative RFA was compared between patients with normal and reduced SLC22A7 expression.

sponse (SVR) to therapy and among these, 132 developed HCC. We enrolled 38 non-SVR patients who developed HCC and 76 matched non-SVR patients who did not develop HCC. Ninety-four patients who developed HCC were excluded because their liver biopsy specimens were of insufficient quality for IHC analyses. Matching was performed using a propensity score matching method. Histological evaluation, IHC staining and surveillance for HCC were performed as above. The average duration of follow-up was 6.6 years for all patients and 7.9 years for patients who did not develop HCC. As above, written informed consent was obtained from all patients and the study was approved by the Ethical Committee of Musashino Red Cross Hospital in accordance with the Declaration of Helsinki.

Propensity Score Matching

In multivariate analyses of 1,003 non-SVR patients, age, gender and stage of fibrosis were independent risk factors for HCC development. Using this multivariate logistic regression analysis, pro-

Table 1. Baseline characteristics of patients who underwent RFA

	Normal SLC22A7 expression (n = 9)	Reduced SLC22A7 expression (n = 11)	p value
Age, years	66.5±5.0	62.9±4.1	0.09
Gender (M/F)	4/5	3/8	0.64
Fibrosis (F0–2/F3–4)	5/4	4/7	0.65
Mean tumor size, mm	20.4±11.3	18.8±6.0	0.91
Albumin, g/dl	4.0±0.3	3.9±0.3	0.71
Bilirubin, mg/dl	0.7±0.2	0.9±0.4	0.09
AST, IU/l	82.0±47.1	74.2±30.6	0.84
ALT, IU/l	80.7±50.2	75.1±33.0	0.85
Glucose, mg/dl	100.3±11.6	123.5±38.7	0.25
Cholesterol, mg/dl	164.0±21.5	166.6±33.8	0.93
Alpha fetoprotein, ng/ml[a]	6.8 (3.7–106)	19.3 (5.9–87.3)	0.46
DCP, mAU/ml[a]	32 (14–129)	15 (14–26)	0.15

ALT = Alanine aminotransferase; DCP = des-gamma-carboxy prothrombin.
[a] Values are shown with median and range.

Table 2. Baseline characteristics of patients enrolled in study 2

	HCC cases (n = 38)	Non-HCC matching cases (n = 76)	p value
Age, years	64.6±7.1	64.6±6.4	0.98
Gender (M/F)	19/19	39/37	0.99
Fibrosis (F0–2/F3–4)	15/23	31/45	0.84
BMI	23.8±3.1	23.5±3.2	0.60
Albumin, g/dl	3.9±0.3	4.1±0.3	0.007
Bilirubin, mg/dl	0.7±0.3	0.7±0.3	0.42
AST, IU/l	83.5±39.2	66.2±37.7	0.07
ALT, IU/l	92.4±45.9	76.8±56.6	0.29
GGT, IU/l	74.6±59.0	63.2±54.0	0.42
Platelets, 10^4/μl	13.2±4.9	14.6±4.3	0.12
Glucose, mg/dl	116.8±20.9	112.4±24.1	0.16
Cholesterol, mg/dl	163.6±32.6	171.1±28.0	0.14

ALT = Alanine aminotransferase; BMI = body mass index; GGT = gamma-glutamyl transpeptidase.

pensity scores were calculated for each patient. These scores were used to match patients who developed HCC (HCC cases) with those who did not (non-HCC cases). Each HCC case was matched with 2 non-HCC cases whose propensity scores were similar to that of the HCC case (nearest-neighbor matching). Data analyses were performed using the Statistical Package for the Social Sciences software version 11.0 (SPSS, Chicago, Ill., USA).

Statistical Analysis

Continuous variables are reported as the mean and standard deviation (SD) or median and categorical variables are shown as counts and proportions. Statistical significance was assessed using the Student t test (mean), the Mann-Whitney U test (median) or the Fisher exact test. In all tests, 2-sided p values were calculated and differences were considered statistically significant when $p < 0.05$. Statistically significant differences identified in univariate analyses were further assessed in multivariate logistic regression analysis. The stepwise and multivariate Cox proportional hazard models were used to explore independent factors that could be used to predict HCC development. Statistical analyses were performed using the SPSS software version 11.0.

Results

SLC22A7 Expression and Distant Recurrence after Curative RFA

Baseline characteristics of patients who received RFA are shown in table 1. No significant differences were observed between patients with normal SLC22A7 expression and those with reduced SLC22A7 expression. Figure 3 shows the cumulative rates of distant recurrences

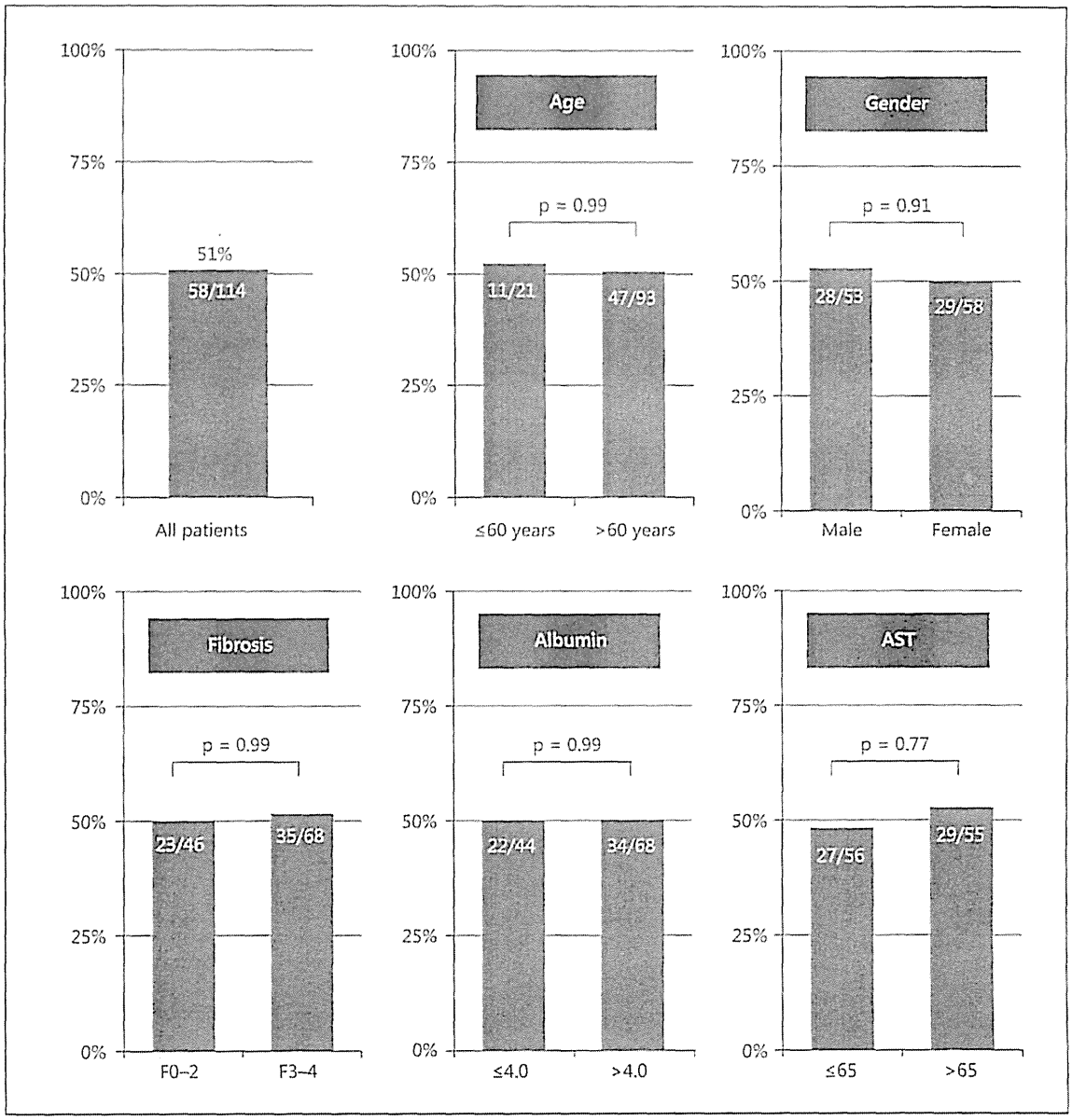

Fig. 4. Percentage of patients with normal SLC22A7 expression according to baseline clinical findings. No significant differences in the percentage of patients with normal SLC22A7 expression were observed after stratification by age, gender, fibrosis stage, albumin and/or AST.

after curative HCC treatment. Patients with reduced SLC22A7 expression had significantly higher rates of distant recurrence than those with normal SLC22A7 expression.

SLC22A7 Expression and de novo Hepatic Carcinogenesis in Chronic HCV Patients

Patient characteristics at the time of enrollment are shown in table 2. Age, gender and stage of liver fibrosis were matched using propensity scores. The distribution of serum albumin levels differed significantly between HCC cases and non-HCC cases. Serum aspartate aminotransferase (AST) levels were higher in patients with HCC than in those without HCC, although this was not statistically significant. Other factors, including body mass index, platelet count, serum glucose and serum cholesterol, which are known risk factors for HCC, were not significantly different between the patient groups.

Table 3. Factors associated with hepatic carcinogenesis according to the Cox proportional hazards model

Factors	Multivariable analysis	
	HR (95% CI)	p value
SLC22A7 (reduced expression)	3.49 (1.56–7.83)	0.002
Albumin (per 1 g/dl)	6.37 (1.56–25.6)	0.009

Normal SLC22A7 expression was found in 58 patients (51%) and reduced SLC22A7 expression was found in 56 patients. No significant differences in baseline characteristics were observed between these groups. When stratified by the matched risk factors age, gender and fibrosis stage, no significant differences were observed in the percentage of patients with normal SLC22A7 expression. Similarly, no significant differences were identified between the groups that were stratified by unmatched serum albumin and AST, which differed between HCC and non-HCC cases (fig. 4). In contrast, the percentage of patients with normal SLC22A7 expression was lower in HCC cases than in non-HCC cases (37 vs. 58%, respectively, p = 0.05). Furthermore, among patients aged <60 years, the percentage with normal SLC22A7 expression was significantly lower in HCC cases than in non-HCC cases (p = 0.02). This difference was observed in male patients (p = 0.001) and in patients with nonadvanced fibrosis (i.e. stages F0–2; p = 0.05; fig. 5). However, no significant differences were observed among patients aged >60 years, among female patients or among those with advanced fibrosis (i.e. stages F3–4).

The cumulative incidence of HCC was significantly higher in patients with reduced SLC22A7 expression than in those with normal SLC22A7 expression (33.9 vs. 13.8% after 5 years, respectively, p = 0.01). This difference remained significant in patients without a known risk of HCC development, such as older patients and those with advanced liver fibrosis (fig. 6). Importantly, in patients aged <60 years, the cumulative incidence of HCC after 5 years was 60 and 0% in those with reduced and normal SLC22A7 expression, respectively (p = 0.02). In patients with nonadvanced liver fibrosis, the cumulative incidence of HCC after 5 years was 31.3 and 12.0% in patients with reduced and normal SLC22A7 expression, respectively (p = 0.02). Because serum albumin levels differed between HCC and non-HCC cases, we assessed the cumulative incidence of HCC after stratification by this variable. Receiver operating characteristic analyses revealed that a level of 4.0 g/dl of serum albumin was the most appropriate cut-off for predicting HCC development. Therefore, we divided all cases into 2 groups with this cut-off. In patients with ≥4.0 g/dl of serum albumin, the cumulative incidence of HCC was significantly higher in patients with reduced SLC22A7 expression than in those with normal SLC22A7 expression (23.5 vs. 5.9% after 5 years, respectively, p = 0.03). In contrast, among patients with <4.0 g/dl of serum albumin, the cumulative incidence of HCC after 5 years was 50.0 and 22.7% in those with reduced and normal SLC22A7 expression, respectively (p = 0.06; fig. 6).

Multivariate analyses confirmed that serum albumin levels (odds ratio 3.1 and p = 0.003) and SLC22A7 expression (odds ratio 2.6 and p = 0.01) were independent risk factors for HCC in this cohort (table 3).

Discussion

This study demonstrates higher cumulative rates of multifocal HCC recurrence after curative treatment in patients with reduced SLC22A7 expression. Moreover, SLC22A7 expression in chronic HCV tissue specimens was a significant predictor for future development of HCC in chronic HCV patients. These analyses indicate the importance of SLC22A7 expression as a predictor of multifocal HCC, de novo and after curative treatment. In particular, among patients without known risk factors for HCC, the cumulative incidence of HCC was significantly higher in those with reduced SLC22A7 expression.

A recent study showed that reduced SLC22A7 expression is an independent risk factor for recurrence after HCC resection [17]. We hypothesized that SLC22A7 might be an IHC marker for the multifocal occurrence of HCC. Initially, we validated the previously reported utility of SLC22A7 as a biomarker for HCC recurrence after curative therapy in HCC patients treated with RFA instead of resection. Subsequently, we revealed a significant association between SLC22A7 expression in hepatitis tissue and the risk of future HCC in chronic HCV patients. Indeed, previous studies show several risk factors for HCC in these patients, including failure to achieve SVR, older age, male gender, obesity and advanced fibrosis and steatosis of the liver [20–22]. According to current data, assessments of transporter function in liver biopsies contribute an additional valuable predictor. This was further emphasized in patients who lacked known risk factors, such as older age and advanced fibrosis. Given the paucity of known risk factors for HCC among younger pa-

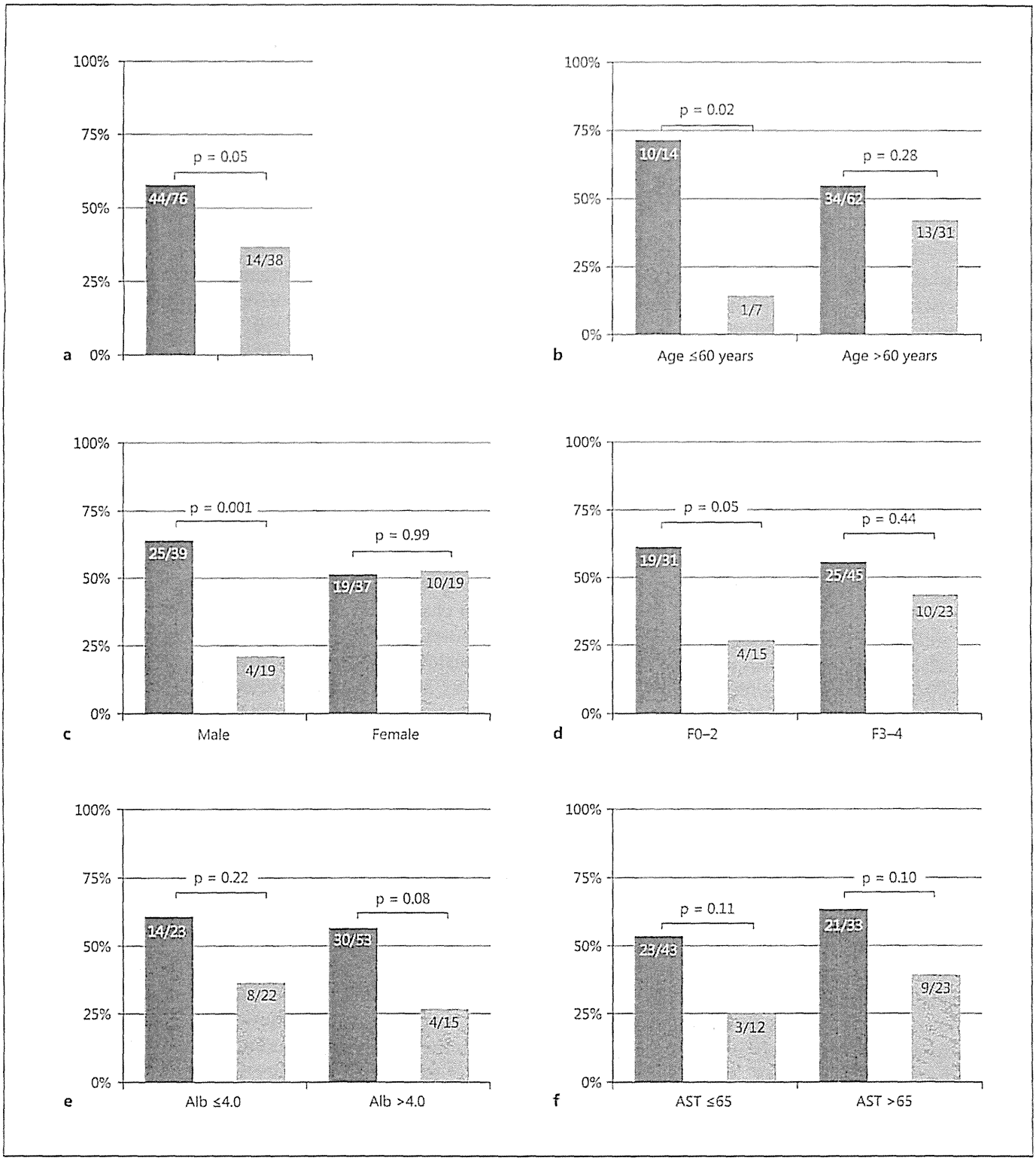

Fig. 5. Percentage of patients with normal SLC22A7 expression and HCC (**a**). SLC22A7 staining was compared between patients who did and did not develop HCC after stratification by age (**b**), gender (**c**), fibrosis stage (**d**), albumin (Alb, **e**) and AST levels (**f**). Light grey and dark grey bars represent patients with and without HCC, respectively.