

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表雑誌	巻	ページ	出版年
1) Mori K, (加藤)	Mechanism of action of ribavirin in a novel hepatitis C virus replication cell system.	Virus Research	15 1	61-70	2011
2) Ariumi Y, (加藤)	Hepatitis C virus hijacks P-body and stress granule components around lipid droplets.	Journal of Virology	85	6882-6892	2011
3) Ueda Y, (加藤)	Plural assay systems derived from different cell lines and hepatitis C virus strains are required for the objective evaluation of anti-hepatitis C virus reagents.	Biochemical and Biophysical Research Communication	40 9	663-668	2011
4) Takeshita S, (加藤)	Geranylgeranylacetone has anti-hepatitis C virus activity via activation of mTOR in human hepatoma cells.	Journal of Gastroenterology		DOI 10.1007/s00535-011-0481-z	2011
5) Mori K, (加藤)	Development of a drug assay system with hepatitis C virus genome derived from a patient with acute hepatitis C.	Virus Genes		DOI 10.1007/s11262-012-0712-2	2012
6) Iikura M, (加藤)	ENT1, a ribavirin transporter, plays a pivotal role in antiviral efficacy of ribavirin in a hepatitis C virus replication cell system.	Antimicrobial Agents and Chemotherapy		DOI 10.1128//AAC.05762-11	2012

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Miyamura T, Kanda T, Nakamoto S, Wu S, Fujiwara K, Imazeki F, <u>Yokosuka O.</u>	Hepatic STAT1- nuclear translocation and interleukin 28B polymorphisms predict treatment outcomes in hepatitis C virus genotype 1-infected patients.	PLoS One	6(12)	e28617	2011
Murayama A, Kato T, Akazawa D, Sugiyama N, Date T, Masaki T, Nakamoto S, Tanaka Y, Mizokami M, <u>Yokosuka O.</u> , Nomoto A, Wakita T.	Production of Infectious Chimeric Hepatitis C Virus Genotype 2b Harboring Minimal Regions of JFH-1.	J Virol	86(4)	2143-52	2012
Miyamura T, Ishii K, Kanda T, Tawada A, Sekimoto T, Wu S, Nakamoto S, Arai M, Fujiwara K, Imazeki F, Kiyohara T, Wakita T, <u>Yokosuka O.</u>	Possible widespread presence of hepatitis A virus subgenotype IIIA in Japan: Recent trend of hepatitis A causing acute liver failure.	Hepatol Res			2011 Dec 13. [Epub ahead of print]

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Togo S, Arai M, Tawada A, Chiba T, Kanda T, Fujiwara K, Imazeki F, <u>Yokosuka O.</u>	Clinical importance of serum hepatitis B surface antigen levels in chronic hepatitis B.	J Viral Hepat	18(10)	e508-15	2011
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Nakamoto S, Kanda T, Imazeki F, Wu S, Arai M, Fujiwara K, <u>Yokosuka O.</u>	Simple assay based on restriction fragment length polymorphism associated with IL28B in chronic hepatitis C patients.	Scand J Gastroenterol	46(7-8)	955-61	2011

IV. 研究成果の刊行物・別冊



Correlation between Pretreatment Viral Sequences and the Emergence of Lamivudine Resistance in Hepatitis B Virus Infection.

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Correlation between Pretreatment Viral Sequences and the Emergence of Lamivudine Resistance in Hepatitis B Virus Infection.

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Key words: HBV, lamivudine resistance, pre-S substitution, ORF sequence analysis

Running title: Prediction of HBV lamivudine resistance

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5 The emergence of amino acid or nucleotide substitutions leads to lamivudine resistance in
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7 Hepatitis B virus (HBV) infected patients. The aim of this study was to investigate whether viral
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9 sequences help predict the emergence of lamivudine resistance. The study subjects comprised 59
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11 consecutive patients infected with HBV treated with daily therapy of 100 mg lamivudine. Among
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13 those, 32 patients with adequate pretreatment serum preservation were investigated for the correlation
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15 between viral amino acid substitutions and the appearance of lamivudine resistance with consideration
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17 of clinical background by determining dominant HBV full open reading frames. Viral resistance to
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19 lamivudine emerged in 28 of 59 patients (47%) in a median period of 2.45 years. Sequence
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21 comparisons of HBV genomes between patients who later developed lamivudine resistance and
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23 patients who did not revealed the existence of significant differences between the two groups in the
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25 pre-S1 84 ($P=0.042$), pre-S2 1 ($P=0.017$) and 22 ($P=0.015$), and polymerase tp 95 ($P=0.046$), judged
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27 by a log-rank test. Viral sequence analyses revealed the presence of amino acid substitutions in HBV
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29 pre-S1 and pre-S2 that may be associated with the emergence of lamivudine resistance during chronic
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31 HBV infection.
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INTRODUCTION

Hepatitis B virus (HBV) infects persistently more than 350 million people worldwide [Liang, 2009], and increases their risk of developing liver cirrhosis, hepatic decompensation, and hepatocellular carcinoma (HCC) over the typically long disease course. High serum virus titers have been shown recently to promote significantly disease progression, as well as the development of HCC [Chen et al., 2006b; Iloeje et al., 2006]. Therefore, effective suppression of the serum viral load by an antiviral agent might inhibit disease progression [Lim et al., 2009].

Lamivudine was introduced clinically as one of the first-generation nucleoside analogs to inhibit HBV replication [Liaw et al., 2004]. Lamivudine is safe [Lok et al., 2003], effectively decreases serum viral load, improves alanine aminotransferase (ALT) levels and liver fibrosis [Leung, 2000; Villeneuve et al., 2000], and enhances hepatitis B e antigen (HBeAg) seroconversion rates [Chen et al., 2006a; Leung et al., 2001; Liaw et al., 2000], which lead to the suppression of HCC development [Liaw et al., 2004]. In contrast, prolonged use of lamivudine may lead to the emergence of drug-resistant HBV mutants in a substantial percentage of patients. When resistance emerges, patients should be treated with a different nucleoside analog, which does not show cross-resistance, alone or in combination with lamivudine [Carey and Harrison, 2009; Chen et al., 2009; Rizzetto et al., 2005]. Newly introduced second-generation nucleoside analogs, such as entecavir and tenofovir, have been shown to be superior in suppressing viral load and preventing the emergence of drug-resistant viruses. However, because of its high economical efficacy compared to other, newer-generation nucleoside analogs, the appropriate selection of patients suitable for lamivudine therapy by accurate prediction of the emergence of resistance would benefit economically-challenged patients worldwide. On the other hand, prediction of the eventual emergence of resistance to lamivudine has been difficult.

Many previous studies have shown a correlation between lamivudine resistance and the HBV mutations that appear with viral acquisition of lamivudine resistance. These mutations lead to amino acid mutations in the HBV polymerase, including rt M204I/V in the C domain and rt V173L and rt L180M in the B domain [Ghany and Doo, 2009; Ling et al., 1996; Tipples et al., 1996]. However, it is not known whether any specific sequences of viral genomes not exposed to lamivudine might predict

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3 the development of resistance following the commencement of lamivudine treatment. Typically, the
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5 emergence of lamivudine resistance has been predicted by pretreatment or in-treatment clinical
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7 variables, such as HBeAg positivity, higher baseline HBV DNA levels, female sex, lower ALT levels
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9 and a poor early viral response to lamivudine [Andersson and Chung, 2009; Zhou et al., 2009].
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11 The present study was conducted to clarify and characterize pretreatment HBV sequences
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13 associated with the subsequent emergence of lamivudine resistance by determining the complete
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15 sequences of HBV ORFs by direct nucleotide sequencing, using patients' sera as the source of HBV
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PATIENTS AND METHODS

Patients

Fifty-nine patients with chronic hepatitis or liver cirrhosis, infected with HBV and who underwent lamivudine therapy at Yamanashi University Hospital from May 2001 to June 2010 were enrolled initially in the study. All patients received lamivudine orally, initially at a dosage of 100 mg per day. Although all 59 patients responded initially to lamivudine therapy and HBV DNA became undetectable, lamivudine resistance was diagnosed in 28 patients (47%) because HBV DNA reappeared during the observation period, while in the other 31 patients it did not (Fig. 1). Because pretreatment serum from 32 of the patients had been preserved adequately for determination of the complete HBV nucleotide sequence, the final analysis was based on these 32 patients. All patients included were positive for hepatitis B surface antigen (HBsAg) and were tested for HBV DNA by the Quantiplex HBV DNA assay (Bayer Diagnostics, Emeryville, CA, USA), transcription-mediated amplification assay (Chugai Diagnostics Science Co., Ltd., Tokyo, Japan), or COBAS[®] Amplicor HBV Monitor Test v2.0 (Roche Diagnostics, Indianapolis, IN, USA). Patients with co-existing autoimmune hepatitis, alcoholic liver disease, drug-induced liver injury, chronic hepatitis C, or human immunodeficiency virus infection were excluded from the study. For patients with emerging drug resistance, adefovir dipivoxil was started at a dosage of 10 mg per day, in addition to lamivudine, according to the guideline established by a hepatitis study group of the Ministry of Health, Labour, and Welfare, Japan. A signed consent was obtained for the study protocol that had been approved by Human Ethics Review Committee of Yamanashi University Hospital.

DNA extraction, PCR, and direct sequencing

Full-length HBV DNA was amplified by two-step PCR from patients' sera and sequenced directly as described elsewhere [Sugauchi et al., 2001]. Sequence reads were assembled using Vector NTI software (Invitrogen, Tokyo, Japan) and base-calling errors were corrected following inspection of the chromatogram. For ambiguous reads, only the dominant base was assigned after evaluation of all overlapping fragments. Full-length HBV genome sequences were assembled using this information

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3 and translated *in silico* and the ORFs of drug-resistant and sensitive genomes were compared.
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7 **Statistical analysis**

9 Statistical differences in the parameters, including all available demographic, biochemical,
10 hematological, and virological statuses, were determined for the different patient groups by Student's t
11 test for numerical variables and Fisher's exact probability test for categorical variables. The odds ratio
12 and 95% confidence intervals were calculated. *P* values of <0.05 by the two-tailed test were
13 considered to indicate statistical significance. In order to evaluate the contribution of pretreatment
14 viral amino acid sequences to the development of lamivudine resistance, Kaplan-Meier analysis and
15 Cox proportional hazards model was performed.
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RESULTS

Patients' clinical parameters

The pretreatment clinical and virological characteristics of the 32 patients, prior to starting lamivudine therapy, are shown in Table I, sorted according to the subsequent emergence of lamivudine resistance. Although HBV DNA became undetectable initially after the commencement of lamivudine therapy, drug resistance was diagnosed in 14 patients because of reappearance of HBV DNA during the observation period. No statistical difference was observed in age, sex, ALT, total bilirubin, choline esterase, total cholesterol, prothrombin time, platelets, alpha-fetoprotein, HBeAg/anti-HBe positivity, viral genotypes, liver disease (chronic hepatitis or liver cirrhosis), or pretreatment HBV DNA level. Genotype C was most prevalent in both groups (16/18 in the non-resistant group and 13/14 in the resistant group). In contrast, the time for HBV DNA to become undetectable was longer in this group, compared to that in the susceptible group ($P=0.024$). Figure 1 shows the length of therapy for all 59 patients; "x" denotes the time of lamivudine resistance onset. Lamivudine resistance was diagnosed in 28 (47%) of 59 patients during a median observation period of 2.45 years.

Comparison of the HBV ORFs of the lamivudine resistant and non-resistant groups

Full-length HBV genomic sequences from the 32 patients were determined by direct nucleotide sequencing. Conceptual *in silico* translation of the dominant pretreatment HBV DNA sequences allowed correlation of the amino acid substitution numbers in each viral ORF with the drug resistance of the virus. Table II shows that the number of amino acid changes in each viral ORF did not differ significantly between the two groups. However, although not significant, there was a tendency that amino acid substitutions in the pre-S2 region were more frequent in patients with eventual development of lamivudine resistance (the median numbers of non-synonymous mutations were 0 and 2 in the sensitive and resistant groups, respectively; $P=0.06$).

Next, the amino acid residues differing between the two groups at each position in each viral protein were compared. The vertical line representing the P value for each HBV ORF (Figs. 2a–d) indicates the difference between the two groups. Comparison of the two groups revealed amino acid

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3 differences at the residues indicated as follows: pre-S1 56, 84, pre-S2 1 and 22, S 130 (Fig. 2a), and
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5 polymerase rt 138, tp 95, spacer 37, 59, 84, and 87 (Fig. 2c). The polymerase was numbered according
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7 to the standardized numbering system [Stuyver et al., 2001]. The most significant difference was
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9 observed at polymerase tp 95 in the (Fig. 2c). In contrast, only a slight difference was observed in the
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11 precore and core and X (Figs. 2b and d). In particular, the changes at pre-S1 84 and polymerase spacer
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13 87 were seen to be coexistent because the pre-S1 and polymerase ORFs overlap. In contrast, the
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15 coding changes at polymerase rt M204I/V, rt L180M, rt 173L, rt A181V, and rt N236T, and at S I195M,
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17 S W196L, and S W196 (stop), previously reported to result from mutations associated with viral
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19 acquisition of resistance to lamivudine or adefovir, were not observed prior to lamivudine therapy in
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21 any patients in this study.
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24 Thereafter, Kaplan-Meier curves were constructed to understand better the potential
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26 influence of the amino acid changes, as revealed above, on the emergence of lamivudine resistance
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28 (Fig. 3, log-rank test). When the time of emergence of resistance was considered, a significant
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30 difference was observed with respect to the substitutions of pre-S1 84 ($P=0.042$), pre-S2 1 ($P=0.017$)
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32 and 22 ($P = 0.015$), and polymerase tp 95 ($P=0.046$). Figure 4 shows a multiple alignment of amino
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34 acid sequences within the pre-S1, pre-S2, and polymerase ORFs.
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38 **Patient characteristics related to HBV ORF substitutions**

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40 As shown in Table III, patients with isoleucine, threonine, leucine or valine at pre-S1 84 had
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42 significantly lower HBV DNA levels, which became undetectable earlier than in patients with alanine
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44 at pre-S1 84. There were no evident differences between the characteristics of patients with and
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46 without substitutions at pre-S2 1 of. Patients with substitutions at pre-S2 22 were older ($P=0.003$,
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48 Table IV). On the other hand, patients with substitutions in the polymerase tp 95 had increased total
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50 bilirubin ($P=0.049$), ALT values ($P=0.495$) and alpha-fetoprotein values ($P=0.034$, Table V).
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56 **Multivariate analysis to reveal independent factors predicting lamivudine resistance**

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58 In an attempt to define independent factors that might predict the emergence of lamivudine
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Sueki et al.

resistance, a multivariate analysis using the Cox proportional hazards model was performed. As shown in Table VI, the duration of lamivudine treatment until HBV DNA became undetectable, serum albumin level, pre-S1 84 substitutions or pre-S2 1 and 22 substitutions, and polymerase tp 95 substitution were entered into the analysis. As a result, the pre-S1 84 and pre-S2 1 substitution could be identified as independent variables.

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DISCUSSION

In this study, the correlation between pretreatment HBV genomic sequences and the emergence of resistance in patients administered lamivudine to treat chronic HBV infection were investigated. Investigation was focused on determining whether a correlation exists between the viral genome diversity and emergence of lamivudine resistance. This was accomplished by determining the complete nucleotide sequences of HBV genomes amplified from the patients' pretreatment sera. Sequence comparisons revealed that substitutions in the pre-S1 and pre-S2 ORFs serve as predictors of emergence of lamivudine resistance.

In previous studies reporting the correlation between drug resistance and mutations in the HBV genome, the focus was confined to the HBV polymerase [Ghany and Doo, 2009]. Because the polymerase protein is the direct target of nucleoside analogues, amino acid residue changes in the HBV polymerase are considered to result from selective and mutational pressure exerted by those agents. Therefore, prominent amino acid sequence changes are generally considered to appear during therapy [Kobayashi et al., 2009]. However, the emergence of resistance obviously cannot be predicted by these mutations, and the emergence of resistance usually is predicted by studying clinical factors. Among these conventional pretreatment and in-treatment predictors of lamivudine resistance, it was observed that longer periods of HBV persistence (determined by DNA detection) after commencing lamivudine therapy correlated with the appearance of resistance, an observation that was consistent with most previous studies.[Andersson and Chung, 2009; Zhou et al., 2009]. This demonstrates that studied patients did not represent outliers from random populations studied previously.

Here, amino acid differences between patients were compared, according to their responses to lamivudine treatment, at each position in each viral ORF, and showed that patients who developed resistance accumulated more substitutions within specific regions of the pre-S1, pre-S2, and polymerase ORFs. Thereafter, a statistical analysis was conducted to investigate whether these substitutions correlated with the emergence of drug resistance. It was found that preexisting substitutions in pre-S1 84 and pre-S2 1 correlated significantly and independently with lamivudine resistance. Because the HBV polymerase genes evaluated all encoded rt 204V/I mutations at the time

Sueki et al.

of appearance of lamivudine-resistance, it is considered that the preexisting substitutions in those pre-S regions enabled the later mutation of rt 204V/I in the polymerase gene. On the other hand, although regions of the polymerase gene overlapping with pre-S1 84 and pre-S2 1, 22 genes were evaluated for their association with lamivudine resistance, the corresponding amino acid changes in the polymerase gene did not correlate with lamivudine resistance according to Kaplan-Meier analysis, demonstrating the importance of the pre-S regions in the development of resistance (data not shown). Interestingly, patients with a substitution in pre-S1 84 exhibited high viral loads and displayed longer times until HBV DNA became undetectable compared to patients without this substitution. In contrast, a substitution in pre-S2 22 correlated with increased age, and the substitution in polymerase tp 95 with advanced disease.

Although the study was focused on the viral amino acid substitutions, viral nucleotide differences also were compared between patients, according to their responses to lamivudine treatment (data not shown). In this analysis, pretreatment substitutions at nucleotide position 53 in the polymerase/pre-S1 region and at nucleotide position 2151 in the core region correlated significantly with the later appearance of lamivudine resistance. In fact, nucleotide position 53 corresponds to the pre-S1 84, and its substitution causes an amino acid change at pre-S1 84. On the other hand, the substitution at nucleotide position 2152 in the core region is synonymous and the role of this substitution should be investigated in a further study.

The pre-S1/pre-S2/S region encodes the small surface (S), middle (M), and large (L) proteins using alternative codons for the initiation of translation [Gao et al., 2007]. These proteins are considered to have crucial functional roles in the life cycle of HBV [Cooper et al., 2003; De Meyer et al., 1997; Kay and Zoulim, 2007; Lian et al., 2008; Ni et al., ; Watanabe et al., 2007]. Apart from the HBV life cycle, recent studies have shown that pre-S sequences significantly impact on the pathogenesis of liver disease [Fang et al., 2008; Sugauchi et al., 2003; Zhang et al., 2007]. The pre-S1 and pre-S2 regions serve as immune targets for T and B cells accumulating in the liver [Bauer et al., 2002], while mutant HBV pre-S epitopes stimulated a lower T cell response than wild-type HBV. HBV with pre-S substitutions leads to cellular retention of viral proteins and a dramatic reduction of virion production [Ni et al.]. The appearance of pre-S substitutions inhibits apoptosis of infected hepatocytes

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3 [Ni et al.]. Patients with progressive liver disease or HCC experience a higher frequency of pre-S
4 substitutions or deletions than patients with stable disease [Chaudhuri et al., 2004]. In association with
5 nucleoside analog therapy, Ohkawa et al. showed the possibility that pre-S2 substitutions might
6 support the replication capacity of lamivudine-resistant HBV [Ohkawa et al., 2008].
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11 On the other hand, there have been no previous studies reporting the correlation between
12 pretreatment pre-S substitutions and the development of lamivudine resistance to date. While the
13 mechanisms need further clarification, it is possible to hypothesize a model explaining the correlation,
14 considering these previous findings. Because those previous reports indicate that HBVs with pre-S
15 substitutions function as immune escape mutants, it is possible that HBVs with pre-S substitutions are
16 advantageous for viral survival and replication in hepatocytes, despite that virion production is
17 reduced. In addition, those infected cells are themselves protected from apoptosis. In those
18 circumstances of persistent viral replication, the chances of acquiring the essential substitutions in the
19 polymerase gene conferring lamivudine resistance might increase.
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29 Before these findings can be applied confidently in clinical settings, some caveats must be
30 considered. First, the number of patients analyzed in the study was quite small, and therefore the
31 potential role of the substitutions detected in drug susceptibility must be evaluated by studies of larger
32 populations. Second, because HBV sequences were determined directly, as opposed to first cloning
33 multiple genomes, the dynamics of minor HBV populations and their contribution to the appearance of
34 resistance are not known. Subcloning analysis or deep sequencing might help further to establish the
35 clinical importance and role of these substitutions in drug resistance. The utility of these viral
36 substitutions for designing HBV therapies with the second-generation nucleoside analogs requires
37 additional research. As for the stability of these predictive viral regions during the treatment period,
38 five patients were available for the analysis of the complete HBV genome sequence after the
39 acquisition of lamivudine resistance. Interestingly, the predictive positions of Pre-S1 84 and Pre-S2 1
40 changed after the acquisition of lamivudine resistance in some patients. However, the role of those
41 changes needs to be further clarified by larger sample sizes.
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55 In conclusion, it was demonstrated that the presence of pre-S1 and pre-S2 substitutions in the
56 HBV genome prior to treatment might play an important role in the subsequent evolution of
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