B型慢性肝炎のマネジメント

改訂版

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Chapter 2

2．B型肝炎ウイルスの遺伝子変異と病態

はじめに

B型肝炎の原因となるB型肝炎ウイルス（hepatitis B virus：HBV）は，一過性感染（急性肝炎）から慢性肝炎，肝硬変症，肝細胞癌まで種々の病態を引き起こす。このHBVの遺伝子は，約3,200塩基対から成る一部一本鎖の二本鎖DNAである。HBVは，自身の有するDNAポリメラーゼの働きにより，スーパーコイル型DNAから（－）鎖DNAを鋳型として，2種類のmajorな転写産物（3.5 kbと2.1 kb）と2種類のminorな転写産物（2.4 kbと0.7 kb）が転写される。これらの転写物（mRNA：messenger ribonucleic acid）より，7種類のウイルス蛋白が支配されている（表1）。HBVはDNAをコピーする際に逆転写を用いるため，種々の変異が発生しやすく，B型肝炎の病態にも影響を及ぼす。HBVによる支配蛋白のうち，HBs抗原，HBe抗原の発現に関係する遺伝子変異と臨床病態について記述するとともに，核酸アナログ製剤を使用中に出現するポリメラーゼの変異についても述べる。

1．プレコア変異（precore変異）

precore領域の開始コドン（ATG）を含む3.5 kbのmRNAから翻訳されるPreC/core蛋白（HBe抗原の前駆体）は，細胞内で小胞体へ誘導され，N末端のアミノ酸が切断される。さらに，Golgi体でC末端のアミノ酸が切断除去され，HBe抗原蛋白として血中へ分泌される。このprecore領域に変異が生じると，precore領域からcore遺伝子への連続的翻訳により産生されるHBe抗原蛋白の産生が阻害される。変異としては，点突然変異によりprecore領域内の83番目に終止コドン（TAG）を形成する例が最も多い（図1）。この変異によってHBe抗原の産生が停止し，HBe抗原価の低下が起こる。

表1 HBV遺伝子と支配蛋白

遺伝子	支配蛋白	アミノ酸数
S	Small HBs	226
PreS2 + S	Middle HBs	281
PreS1 + PreS2 + S	Large HBs	400
PreC + C	HBe Ag	157-170
C	HBc Ag	183
X	HBx Ag	154
P	DNAポリメラーゼ	843

7種類の蛋白が産生される。

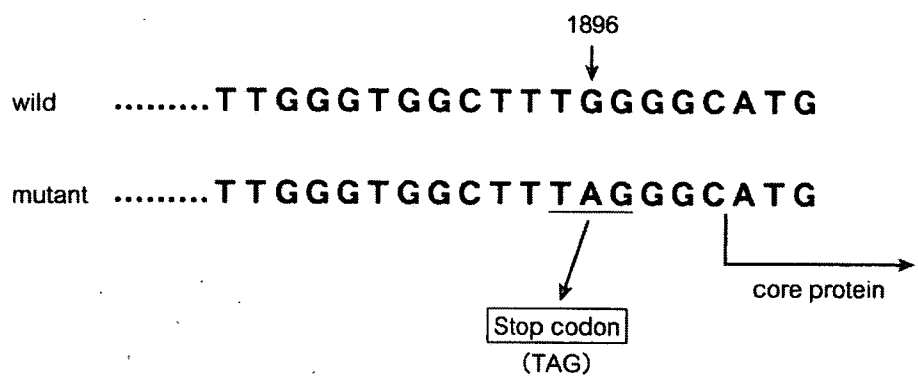

図1 precore mutation (nt1896)

HBVの1986番目の塩基がGからAにかわることによって，HBe抗原の産生が抑制される。

このprecore変異（G1896A；1896番目のヌクレオチドがGからAに変異する）の頻度を，虎の門病院にて慢性肝炎症例でHBV genotype別に検討した。初診時の測定結果でprecore変異の頻度は，genotype Aで0％（0例/11例），genotype Bで48％（12例/25例），genotype Cで22％（37例/167例）であった。その後，自然経過や抗ウイルス療法を施行した後の最終観察時点でのprecore変異の頻度は，genotype Aで0％（0例/11例），genotype Bで80％（20例/25例），genotype Cで53％（89例/167例）であっ

た。このようにgenotype Aでは，precore変異が起こりにくく，genotype Bでは高率に変異が起こりやすいことがわかる（genotype Aでは，precore領域のencapsidation signal〔ε〕のステムループ構造において1896番目の塩基と相対する塩基である1858番目がCであるため，変異しにくいものと考えられている）。

次に治療によるprecore領域の変異について述べる。HBe抗原陽性例でインターフェロン治療の効果が認められた症例では，precore wild（1896G）からmutant（1896A）に変化し，HBeAgのseroconversionが起こる場合とwildのままの症例があると報告されている[1]。また，治療前のprecore変異の有無がインターフェロン治療の効果に関係するという報告[2, 3]と，関係ないという報告[4]があり，この領域のデータだけでは治療効果を予測することは難しい。

次に核酸アナログ製剤であるラミブジン使用時のprecore変異の推移について述べる。ラミブジンを使用した場合，precore wildよりもprecore mutantの方がラミブジン感受性が高いことが報告されている[5, 6]。さらに，ラミブジン耐性ウイルスによる肝炎に対してアデフォビルを投与した場合も，precore mutantの方がアデフォビルに感受性が高いことが報告されている[7]。このように，核酸アナログ製剤は，precore mutantにより有効に作用しているものと考えられる。

2．コアプロモーター変異（core promoter変異）

3.5 kb mRNAの転写開始点の上流に，プロモーター活性を成す領域が同定されている。コアプロモーター領域（core promoter）は，1742から1849番目の塩基と考えられているが，このうち1762番目と1764番目の変異が高頻度に認められる（図2）[8]。このcore promoter変異は実験的には，precore mRNAの転写を抑制し，結果的にはHBe抗原の産生低下に繋がると報告されている[9]。

このcore promoter変異（A1762T，G1764A）の頻度を慢性肝炎症例でHBV genotype別に検討した。初診時の測定結果でprecore変異の頻度は，genotype Aで64%（7例/11例），genotype Bで24%（6例/25例），genotype Cで69%（116例/167例）であっ

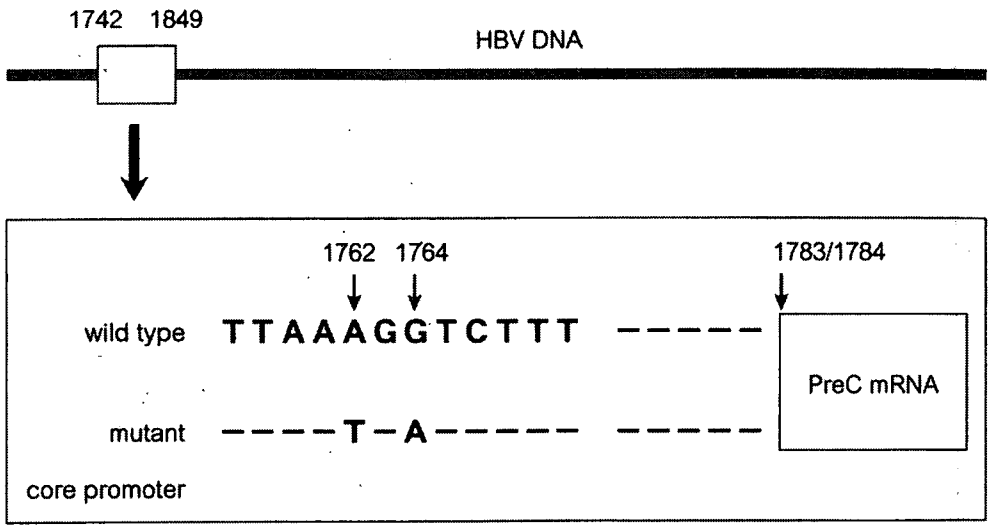

図2 core promoter (nt1762, 1764) の変異
HBV の 1762 番目と 1764 番目の変異を示す。

た。その後，自然経過や抗ウイルス療法を施行した後の最終観察時点での core promoter 変異の頻度は，genotype A で 73%（8 例/11 例），genotype B で 40%（10 例/25 例），genotype C で 70%（117 例/167 例）であった。

このように genotype A と C では，core promoter 変異が起こりやすく，genotype B では起こりにくい。precore 領域の変異と同様に，core promoter 領域でも genotype 間で変異の出現率に差を認めることが，臨床経過や治療への反応性に少なからず影響していると考えられている。

次に核酸アナログ製剤であるラミブジン使用時の core promoter 変異（A1762T，G1764A）について述べる。ラミブジンを使用した場合，core promoter wild よりも mutant の方がラミブジン感受性が高いことが報告されている[5, 6]。一方，ラミブジン耐性ウイルスによる肝炎に対してアデフォビルを投与した場合は，core promoter mutant への作用は少ない[7]。したがって，core promoter に対する核酸アナログ製剤の効果は，precore mutant に対するものよりも弱い作用である。

3．HB ワクチンエスケープミュータント

HBV キャリアの母親からの出産時に，出生児に対して HB ワクチンと高力価 HBs 抗体含有免疫グロブリン（anti-HBs immunoglobulin：HBIG）を投与することによって，ほとんどの出生児で感染防御が可能である。しかし，経過中に HBs 抗原が陽性化した症例が報告され，“vaccine-induced escape mutant”として報告された[10]。これは HBV のＳ遺伝子のうち，共通抗原決定基’a’の領域に変異が起こることによって HBs 抗原の抗原性が変化し，HB ワクチンや HBIG による HBs 抗体からの免疫学的な圧力から逃避してウイルスが増殖したものである。共通抗原決定基’a’のなかで，126 番目と 145 番目のアミノ酸変異の報告が多いが（図 3），141 番目や 144 番目の変異も報告され，その他複数個のアミノ酸

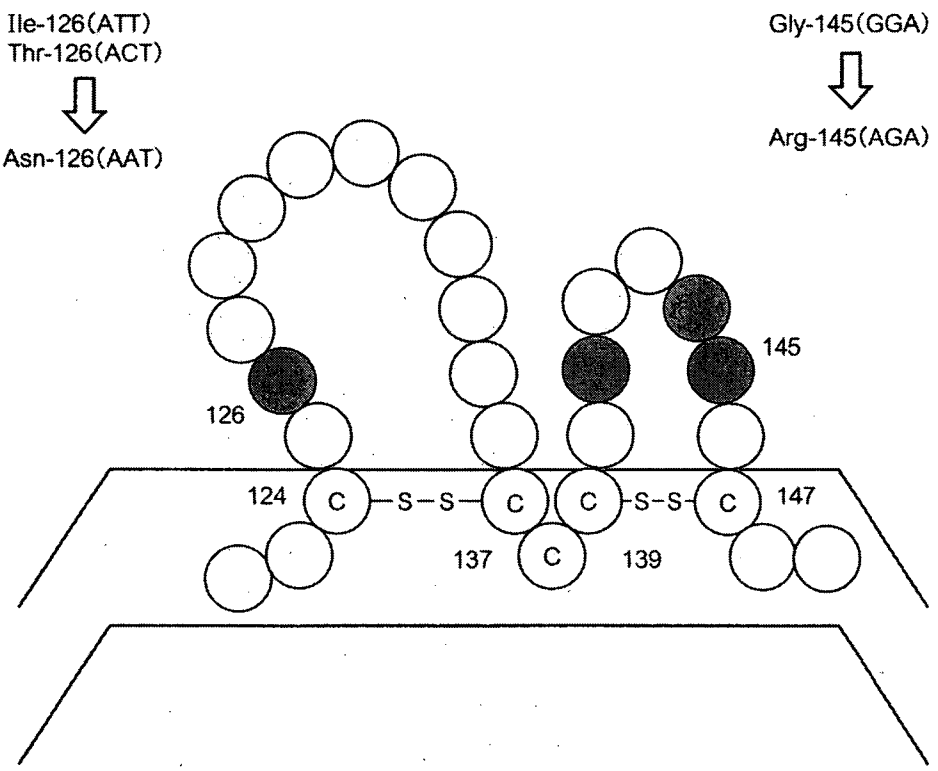

図 3　HBs 抗原遺伝子内の抗原決定基’a’の構造

抗原決定基’a’内の 126 番目や 145 番目のアミノ酸が変異することによって HBs 抗原価が低下する。

残基が挿入された変異も報告されている。

これらの変異は当初，HBワクチンやHBIGの投与に関連したものと考えられていたが，自然経過中のHBV持続感染者からも同様の変異が報告されている[11, 12]。このような症例の場合，HBV持続感染症例でありながら，HBs抗原陰性でHBs抗体陽性になっている。実際には，このような症例はまれではあるものの存在し，現在のHBs抗原，HBs抗体系の測定のみではHBV感染症例を見逃してしまう可能性がある。

4．ラミブジン投与中に出現するYMDD motif mutation

ラミブジンはHBVが複製される際に，RNAを鋳型にしてDNAを合成する過程（逆転写の過程）においてchain terminatorとして作用し，DNA合成を終焉させる。わが国においてラミブジンは2000年より保険適応になり，B型慢性肝炎に投与が認められている（現在は代償性肝硬変症にも保険適応になっている）。わが国におけるラミブジンの治療成績も欧米やアジア諸国のデータと同様に，従来の治療と比較して良好である。特に，HBe抗原陰性症例に対するラミブジンの効果は高い[13]。

しかし，ラミブジン治療の大きな問題点は，投与中止により肝炎の再燃を起こす可能性が高いことと，薬剤耐性株（HBVポリメラーゼ領域内のYMDD〔tyrosine-metionine-aspartic acid-aspartic acid〕motifの変異を伴うウイルス）の出現である。中止後肝炎再燃率が高率であるため，現在は長期投与を施行することが多い[14]。一方，ラミブジン耐性ウイルスは一般的には6～9カ月の投与後に出現し始め，治療の長期化とともに増加する。

虎の門病院の3年以上ラミブジンを投与した290例の解析においても，2年目，4年目，6年目の耐性ウイルスの出現率は，それぞれ35%，55%，62%であった。また，耐性ウイルスはHBe抗原陽性例において陰性例よりも有意に高率に出現していた。一般的には，耐性ウイルスの出現時は無症候性であるが，その後3～4カ月後からHBV DNAの上昇（breakthrough）とALT（alanine aminotransferase）値の上昇（breakthrough hepatitis）が多くの症例で認められる。この場合，耐性ウイルスによる肝炎の程度は軽いといわれている。しかし，一部症例では耐性ウイルスによる肝炎においても，重症の肝炎を発症することが報告されている[15, 16]。

このような臨床的背景があるが，ラミブジン耐性に最も関係するYMDD motifの変異について述べる。ラミブジンは内服後吸収され，肝細胞内に取り込まれるとリン酸化を受け，活性型の三リン酸化体となる。この三リン酸誘導体はdCTP（deoxycytidine triphosphate）と似た構造をもち，dCTPと競合的にDNA鎖に取り込まれDNA伸長を停止させる。ラミブジン耐性は，ポリメラーゼのreverse transcriptase(rt)領域内のYMDD motifの変異によって生じる（**図4**）。

このYMDD motifは，rt領域内の保存された領域の1つであるDomain Cのなかに存在している。耐性ウイルスの変異のパターンには，rtの204番目がメチオニンからバリンに変化するタイプ（rtM204V）と，イソロイシン（rtM204I）に変化するタイプが認められる（**図5**）。実験的にもこれらの変異はラミブジン耐性であることが報告されている[17]。実際の臨床症例でも，この変異が耐性

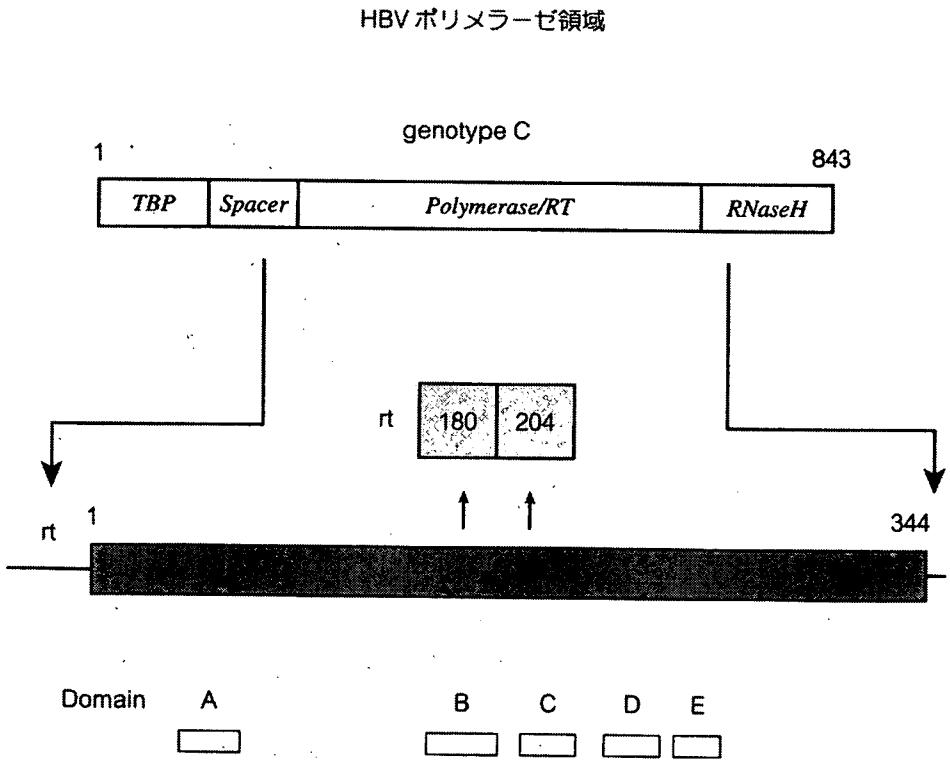

図4 HBVポリメラーゼ内のreverse transcriptase領域内のラミブジン耐性に関係する変異
Domain C内の変異によってラミブジン耐性になる。

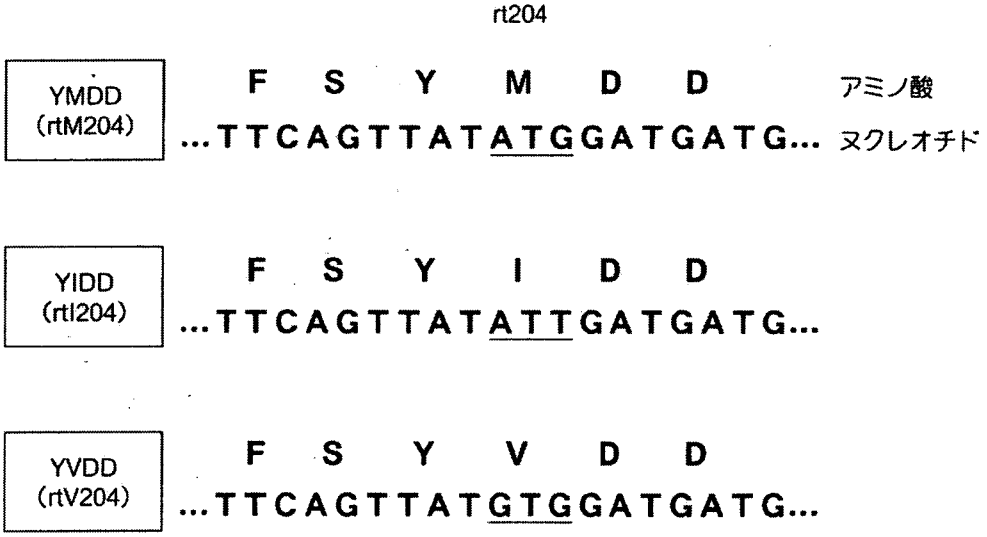

図5 ラミブジン耐性ウイルス（rtM204I/v）

YMDD motif内の変異のパターンには2種類あり，YIDD, YVDD変異株といわれている。

YIDD：tyrosine-isoleucine-aspartic acid-aspartic acid

YVDD：tyrosine-valine-aspartic acid-aspartic acid

ウイルスとして増加し肝炎を引き起こすことが明らかである。

また，YVDD変異のタイプでは，Domain Bのなかに含まれるrt180番目のアミノ酸がロイシンからメチオニンに変化する変異（rtL180M）が高率に認められる。YMDD motifの変異がラミブジン開始後出現した場合には，厳重な経過観察が必要である。

5．アデフォビル・ディピボキシル投与時の耐性ウイルス

アデフォビルはアデニンのアナログであり，米国では2002年9月にHBVに対して承認された経口薬である。アデフォビルは*in vitro*の実験系で，HBVの野生株に対してラミブジンと同等の抗ウイルス効果を有するのみならず，ラミブジン耐性株にも有効であることが示された。実際の患者においても，ラミブジン耐性ウイルスによる肝炎再燃例に対しても有効性が確認されている。欧米でのラミブジン耐性ウイルスに対するアデフォビルの成績では，HBV DNA量を$10^2 - 10^4$分の1に減少させ，ALT値の改善を認めている。わが国においても，2004年12月にラミブジン耐性ウイルスによる肝炎に対してアデフォビル1日10 mgの投与が保険適応となっている。この場合，ラミブジンはアデフォビルの

投与と併用投与する必要がある。

虎の門病院におけるラミブジン耐性ウイルスによるbreakthrough hepatitisに対するアデフォビルの投与成績を述べる。2002年11月から2004年12月までにアデフォビルを開始し，6カ月以上の投与期間のある87例を対象とした。いずれの症例もラミブジンは中止せず，1日10 mgのアデフォビルとの併用投与を施行している。アデフォビル開始後，HBV DNAのamplicor法による陰性化（2.6 log copies/mL未満）率は，6カ月目55%，12カ月目65%，18カ月目84%であった。また，ALT値の正常化率は6カ月目78%，12カ月目89%，18カ月目94%であった。このように，アデフォビルのラミブジン耐性ウイルスに対する効果は高く，肝炎の改善率も高率であった。また，開始時HBe抗原陽性症例52例のHBe抗原の陰性化率は1年目22%，2年目44%であった。

さらに，これらHBe抗原が陰性化した症例を検討すると，アデフォビル開始時のALT値が高い症例ほどHBe抗原の陰性化率が高い。このことより，HBe抗原陽性症例では，アデフォビルの投与はALT値の高い時期に開始することが望ましい。しかし，肝病変の進行した症例ではbreakthrough hepatitisによる重症化の危険性もあり，注意深い経過観察と患者ごとの臨床背景を考慮した治療を行っていく必要がある。また，アデフォビルの副作用としては，クレアチニンの上昇が認められることがある。このような症例では，アデフォビルの投与を10 mg隔日投与に減量することによって，クレアチニンの改善を認めている。

しかし，アデフォビルにも耐性ウイルスが出現する。アデフォビル耐性ウイルスの変異は，rt236番目のアミノ酸がアスパラギンからスレオニン（rtN236T）へ変異タイプと，rt181番目のアミノ酸がアラニンからバリンまたはスレオニンに変異（rtA181V/T）するタイプが報告されている[18]。これらの耐性ウイルスにはラミブジンが有効である。

6．エンテカビル投与時の耐性ウイルス

エンテカビルはグアニンのアナログであり，*in vitro*ではHBVに対してラミブジンよりも強力な抗ウイルス作用を示す。最近報告されたエンテカビルの海外での成績を提示する。HBe抗原陽性B

型慢性肝炎患者に1日0.01 mg（54例），0.1 mg（36例）あるいは0.5 mg（46例）のエンテカビルを24週間投与し，100 mgのラミブジン投与例（41例）と比較した。ラミブジン100 mgの投与と比較して，エンテカビル1日0.1 mgあるいは0.5 mg投与群では，それぞれ1.0 logまたは1.3 log，ラミブジンよりもHBV DNAを低下させた。HBV DNA量（branched DNA assay法にて）は，ラミブジン投与群で57.5%が陰性化したのに対して，エンテカビル0.5 mg投与群では83.7%が陰性化していた。ALT値の正常化率は，ラミブジン投与群で59.1%，エンテカビル投与群では0.1 mg投与群で83.3%，0.5 mg投与群で69%であり，いずれのデータもラミブジン投与群を上回っていた。副作用についても重篤な副作用は認められなかった。

また，エンテカビルはラミブジン耐性に対しても効果があることが確認されている。海外でのラミブジン耐性ウイルスに対するrandomized, double-blindの成績であるが，B型慢性肝炎患者に1日0.1 mg（47例），0.5 mg（47例）あるいは1.0 mg（42例）のエンテカビルを76週間以上投与し，100 mgのラミブジン投与例（45例）と比較した。治療開始後24週の時点でのHBV DNA量（branched DNA assay法にて）の陰性化は，エンテカビル1日1.0 mgあるいは0.5 mg投与群ではそれぞれ79%, 51%であったが，ラミブジン投与群では13%であった。また，エンテカビル1.0 mg投与群は0.5 mg投与群よりもより効果的であった。ALT値の正常化率も，ラミブジン投与群で6％であるのに対して，エンテカビル投与群では0.1 mg投与群で47%，0.5 mg投与群で59%，1.0 mg投与群で68%であり，いずれのデータもラミブジン投与群を上回っていた。また，別のエンテカビル1年間投与のstudyでは，HBe抗原陽性例でのHBe抗原陰性化率は，開始時ラミブジン非投与例で27%，ラミブジン耐性出現例では10%であると報告されている。

しかし，エンテカビルにおいても耐性ウイルスの報告がなされている。エンテカビル耐性は，ラミブジン耐性であるrtL180MとrtM204Vの変異の上に，rtT184番目，rtS202番目またはrtM250番目のアミノ酸の変異が追加されたものである[19]。アミノ酸の変異パターンはいくつか認められる。当初はラミブジン耐性ウイルスに対してエンテカビルを使用した際に出現すると考えられてい

た[20]が，最近核酸アナログ未使用の症例からも認められ，注意が必要である[21]。

おわりに

HBVの遺伝子変異と病態，治療との関係について記載した。HBVはその自然経過でも種々の遺伝子変異を起こす。しかし，最近は核酸アナログ製剤の使用によって新たな遺伝子変異が出現し，治療を行う上で非常に重要な問題点となっている。このような点を考慮して治療を行っていく必要がある。

（鈴木　文孝）

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Amino Acid Substitutions in the Hepatitis C Virus Core Region are the Important Predictor of Hepatocarcinogenesis

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We showed previously that amino acid (aa) substitutions in hepatitis C virus core region (HCV-CR) are negative predictors of virologic response to pegylated interferon (IFN) plus ribavirin therapy. HCV-CR induces hepatocellular carcinoma in transgenic mice, but the clinical impact is still unclear. To evaluate the impact of aa substitutions in HCV-CR on hepatocarcinogenesis, we performed a follow-up study on 313 noncirrhotic consecutive naïve patients infected with HCV genotype 1b who received IFN monotherapy. The median follow-up was 14.7 years. A sustained virologic response (SVR) after the first IFN was achieved by 65 patients (20.8%) (group A). Of 248 patients (79.2%) of non-SVR after first IFN, 112 (35.8%) did not receive additional IFN (group B), and the remaining 136 (43.5%) received multicourse IFN monotherapy (group C). As a whole, cumulative hepatocarcinogenesis rates in double wild-type (arginine at aa 70/ leucine at aa 91) of HCV-CR were significantly lower than those in nondouble wild-type. Multivariate analyses identified 3 parameters (fibrosis stage 3, nondouble wild-type of HCV-CR, and group B) that tended to or significantly influenced hepatocarcinogenesis independently. With regard to hepatocarcinogenesis rates in group C according to HCV-CR and the mean alanine aminotransferase (ALT) during IFN-free period, significantly higher rates were noted in patients of nondouble wild-type with ALT levels of more than 1.5 times the upper limit of normal (25.7%) compared with the others (2.4%). *Conclusion:* Amino acid substitutions in the HCV-CR are the important predictor of hepatocarcinogenesis. In multicourse IFN therapy to nondouble wild-type, we emphasize the importance of reducing the risk of hepatocarcinogenesis by mean ALT during an IFN-free period below 1.5 times the upper limit of normal. (HEPATOLOGY 2007;46: 1357-1364.)

Hepatitis C virus usually causes chronic infection, which can result in chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC).[1-5] In patients with chronic HCV, treatment with IFN can induce viral clearance and marked biochemical and histological improvement.[6,7]

For chronic HCV infection, peginterferon (PEG-IFN) plus ribavirin (RBV) combination therapy is an expensive treatment modality that is accompanied by severe side effects and high sustained virological response (SVR). Patients who do not achieve SVR need to be identified before the start of combination therapy to avoid unnecessary side effects and high costs. Thus, safer IFN monotherapy should be considered to reduce the risk of hepatocarcinogenesis in patients unsuitable for PEG-IFN plus RBV therapy. We studied previous determinants of response to PEG-IFN plus RBV in patients with high titers of HCV genotype 1b (≥100 KIU/mL), which is dominant in Japan. Our results identified substitution of amino acids (aa) 70 and/or 91 in the HCV core region (HCV-CR) as an independent and significant negative predictor associated with virological response.[8-10] Furthermore, we reported that multicourse IFN monotherapy reduces the risk of hepatocarcinogenesis and increases survival even if patients fail to achieve SVR after a single-course IFN, and

Abbreviations: aa, amino acid(s); HCV-CR, hepatitis C virus core region; MU, million units; PEG-IFN, peginterferon; RBV, ribavirin; SVR, sustained virologic response.

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that low ALT levels during an IFN-free period is associated with lower rates of hepatocarcinogenesis.[11] Hence, multicourse IFN monotherapy might be expected to reduce the risk of hepatocarcinogenesis in patients who have negative predictors for PEG-IFN plus RBV.

Despite numerous lines of epidemiological evidence connecting HCV infection and the development of HCC, it remains controversial whether HCV itself plays a direct or indirect role in the pathogenesis of HCC.[12] It has become evident that HCV-CR has oncogenic potential through the use of transgenic mice, but the clinical impact of HCV-CR on hepatocarcinogenesis is still unclear.[13] Whether substitution of aa 70 and/or 91 in HCV-CR as a predictor of virological response for PEG-IFN plus RBV therapy also affects hepatocarcinogenesis awaits further investigation.

The present study included 313 consecutive naïve cases infected with HCV genotype 1b in whom 15 years had elapsed since induction of IFN monotherapy. The aims of the study were: (1) to evaluate the clinical impact of aa substitutions in the HCV-CR on hepatocarcinogenesis; (2) to analyze the predictive factors associated with hepatocarcinogenesis in patients who received IFN monotherapy; and (3) to evaluate the long-term efficacy of multicourse IFN monotherapy on hepatocarcinogenesis as examined through analysis of the outcomes of single and multicourses of IFN.

Patients and Methods

Patients. Among 573 consecutive HCV-infected patients in whom IFN monotherapy was induced between February 1987 and August 1992 at Toranomon Hospital, 313 were selected in the present study based on the following criteria: (1) patients naïve to IFN monotherapy; (2) patients infected with HCV genotype 1b alone; (3) patients with chronic hepatitis, without cirrhosis or HCC, as confirmed via biopsy examination within 6 months of enrollment; (4) patients not treated with IFN plus RBV combination therapy during follow-up time; (5) patients negative for hepatitis B surface antigen (radioimmunoassay, Dainabot, Tokyo, Japan), positive for anti-HCV (third-generation enzyme immunoassay, Chiron Corp., Emeryville, CA), and positive for HCV RNA qualitative analysis with PCR (nested polymerase chain reaction or Amplicor, Roche Diagnostic Systems, CA); (6) patients free of coinfection with human immunodeficiency virus; (7) patients not treated with antiviral or immunosuppressive agents within 6 months of enrollment; (8) lifetime cumulative alcohol intake <500 kg (mild to moderate alcohol intake); (9) patients free of other types of hepatitis, including hemochromatosis, Wilson's disease, primary biliary cirrhosis, alcoholic liver disease, and autoimmune liver disease; (10) patients without or with well-controlled diabetes; and (11) patients who consented to the study.

With regard to the clinical features of 313 patients at the start of the first course of IFN monotherapy, there were 223 men and 90 women aged 15-66 years with a median age of 47 years. The numbers of patients with fibrosis stages 1, 2, and 3 were 179, 107, and 27, respectively. The median ALT level was 138 IU/L (range, 24-636 IU/L), and the median platelet count was $17.4 \times 10^4/\mu L$ (range, 8.9×10^4-$39.2 \times 10^4/\mu L$). The median viremia level was 4.0 Meq/mL (range, <0.5-67.0 Meq/mL). The median follow-up time was 14.7 years (range, 0.1-20.1 years).

Furthermore, at the first course of IFN monotherapy, 222 patients (70.9%) received IFN-α alone; 83 patients (26.5%) received IFN-β alone; and the remaining 8 patients (2.6%) received a combination of IFN-α and IFN-β. A median IFN dose per day of 6 million units (MU) (range, 1-10 MU) was administered. As a whole, a median total dose of IFN of 525 MU (range, 22-3,696 MU) was administered during a median period of 23.9 weeks (range, 0.6 to 205.4 weeks). Patients mainly received IFN monotherapy, including initial aggressive induction therapy (every day within 8 weeks, followed by 3 times per week).

The study protocol was approved by the Human Ethics Review Committee of Toranomon Hospital.

Methods. The primary measure of efficacy of treatment was sustained virological response (SVR), defined as negative HCV RNA via qualitative analysis with PCR at 24 weeks after cessation of IFN therapy. Patients who achieved SVR after the first course of IFN monotherapy were classified as group A. Patients who did not achieve SVR after the first course of IFN monotherapy were classified into 2 groups based on whether they received other courses of IFN monotherapy. Patients who did not receive further courses of IFN monotherapy based on concerns about adverse effects, lack of time for treatment, physician recommendation based on the appearance of depression, and cardiopulmonary disease during and after the first course of IFN, or the lower levels of ALT, were classified as group B. Patients who received 2 or more courses of IFN monotherapy were classified as group C.

Laboratory Investigations. Blood samples were frozen at −80°C within 4 hours of collection and were not thawed until used for testing. HCV genotype was determined via PCR using a mixed primer set derived from nucleotide sequences of the NS5 region.[14] In all cases, HCV-RNA viremia level was measured by branched DNA assay version 2.0 (Chiron Corp.) at commence-

ment of therapy using frozen samples, and the results were expressed as 10^6 genomic equivalents per milliliter (Meq/mL). The lower limit of the assay was 0.5 Meq/mL. Samples with undetectable levels using this quantitative assay (<0.5 Meq/mL) were also evaluated via HCV-RNA qualitative analysis with PCR (nested PCR or Amplicor) during and after therapy especially, and the results were expressed as positive or negative. The lower limit of the assay was 100 copies/mL.

Detection of Amino Acid Substitutions in Core Region. We developed a simple and low-cost PCR method for detecting substitutions of aa 70 or aa 91 in HCV-CR of genotype 1b using mutation-specific primer as an alternative to the direct sequencing method. The major protein type was determined based on the relative intensity of the bands for wild (aa 70, arginine; aa 91, leucine) and mutant (aa 70, glutamine/histidine; aa 91, methionine) in agarose gel electrophoresis. If the intensities of the bands were similar, the case was regarded as competitive. The detection rate was 94.4%, the sensitivity was 10 KIU/mL using quantitative assay with PCR (Cobas Amplicor HCV monitor version 2.0 using the 10-fold dilution method), the reproducibility was high, and consistency with direct sequencing was 97.1% in positive cases.[15] In this study, the pattern of arginine (wild) at aa 70 and leucine (wild) at aa 91 was evaluated as double wild-type, while the other patterns were nondouble wild-type. The mutation in this study refers to substitution from consensus sequence. In previous studies, HCV-J was considered as a prototype, and the aa substitution was evaluated by comparison with the consensus sequence prepared from 50 clinical trial samples.[8,16] In this study, the PCR genotyping could be performed in 232 patients; the remaining 81 patients could not be analyzed due to the lack of adequate serum samples obtained before treatment.

Liver Histopathological Examination. Liver biopsy specimens were obtained percutaneously or at peritoneoscopy using a modified Vim Silverman needle with an internal diameter of 2 mm (Tohoku University style, Kakinuma Factory, Tokyo), fixed in 10% formalin, and stained with hematoxylin-eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. All specimens for examination contained 6 or more portal areas. Histopathological diagnosis was made by an experienced liver pathologist (H. K.) who was blinded to the clinical data. Chronic hepatitis was diagnosed based on histopathological assessment according to the scoring system of Desmet et al.[17]

Follow-Up. Clinical and laboratory assessments were performed at least once every month before, during, and after treatment. Adverse effects were monitored clinically by careful interviews and medical examination at least once every month. Patient compliance with treatment was evaluated with a questionnaire. Blood samples were also obtained at least once every month before, during, and after treatment, and were also analyzed for ALT levels and HCV-RNA levels at various time points.

Follow-up time represented the time from the start of the first course of IFN treatment until death or until the last visit.

Diagnosis of HCC. Patients were examined for HCC via abdominal ultrasonography every 3-6 months. If HCC was suspected based on ultrasonographic results, additional procedures such as CT, magnetic resonance imaging, abdominal angiography, and ultrasonography-guided tumor biopsy (if necessary), were used to confirm the diagnosis.

Statistical Analysis. The χ^2 test, Fisher exact probability test, and Mann-Whitney U test were used to compare background characteristics between groups. Multiple comparisons were examined by the Bonferroni test. Cumulative hepatocarcinogeneses were calculated using the Kaplan-Meier technique; differences between survival curves were tested using the log-rank test. Statistical analyses of hepatocarcinogenesis according to groups were calculated using the period from start of the first course of IFN monotherapy. Stepwise Cox regression analysis was used to determine independent predictive factors that were associated with hepatocarcinogenesis. We also calculated the OR and 95% CI. Potential predictive factors associated with hepatocarcinogenesis included the following 11 variables: age, sex, histological stage, viremia level, serum AST, serum ALT, platelet count, aa substitutions in HCV-CR, total IFN dose, total IFN duration, and group of treatment. Each variable was transformed into categorical data consisting of 2 simple ordinal numbers for univariate and multivariate analyses. Variables that achieved statistical significance ($P < 0.05$) or marginal significance ($P < 0.10$) on univariate analysis were tested using the multivariate Cox proportional hazard model to identify significant independent factors. Statistical comparisons were performed using the SPSS software (SPSS Inc., Chicago, IL). All P values of less than 0.05 by the 2-tailed test were considered significant.

Results

Efficacy of IFN Monotherapy. 65 patients (20.8%) achieved SVR after the first course of IFN monotherapy (group A). Of 248 (79.2%) non-SVR patients after the first course of IFN, 112 (35.8%) did not receive a second course of IFN monotherapy (group B), while the remain-

Table 1. Patient Characteristics at Start of First Course of IFN Monotherapy

	Group A (n = 65)	Group B (n = 112)	Group C (n = 136)
Sex (male/female)	45/20	75/37	103/33
Age (years)*	44 (15-64)†	51 (23-66)	45 (22-63)‡
Viremia level (Meq/mL)*	0.6 (<0.5-45.0)	5.9 (<0.5-67.0)§	5.3 (<0.5-57.0)‖
Fibrosis stage (F1/F2/F3)	49/14/2	54/50/8¶	76/43/17#
AST (IU/L)*	83 (16-198)	74 (22-398)	75 (24-400)
ALT (IU/L)*	153 (24-416)	120 (38-636)	138 (50-594)
Platelet count ($\times 10^4/\mu L$)*	18.7 (9.7-31.0)	17.1 (9.7-39.2)	17.0 (8.9-31.2)
Core region (double wild/nondouble wild/ND)*	10/15/5	31/44/7	41/71/8

*Median †$P = 0.009$, ‡$P = 0.007$ compared with group B via Bonferroni test. §$P < 0.0001$, ‖$P < 0.0001$, ¶$P = 0.006$, #$P = 0.009$, compared with group A via Bonferroni test.

** Amino acid substitutions were evaluated in pretreatment serum samples of 232 patients via PCR with mutation-specific primers. Two patterns of mutant and competitive were labeled as nonwild. Wild at aa 70 and wild at aa 91 were evaluated as double-wild-type, while the other patterns were considered nondouble wild-type.

Abbreviation: ND, not determined.

ing 136 (43.5%) received 2 or more courses of IFN monotherapy (group C). Of 136 patients in group C, 80 patients received 2 courses of IFN (21 of whom achieved SVR), 44 patients received 3 courses (6 of whom achieved SVR), 11 patients received 4 courses (2 of whom achieved SVR), and 1 patient received 6 courses (and did not achieve SVR). Thus, 29 patients in group C achieved SVR after multiple courses of IFN monotherapy.

In groups A and B, the median total duration of IFN was 24.1 weeks (range, 4.0-205.4 weeks) and 23.7 weeks (range, 2.9-75.1 weeks). The median total dose of IFN was 528 MU (range, 43-3,696 MU) and 498 MU (range, 72-870 MU). In the first, second, third, fourth, fifth, and sixth courses of IFN monotherapy in group C, the median total durations of IFN were 23.9 weeks (range, 0.6-136.4 weeks), 24.0 weeks (range, 1.3-313.7 weeks), 25.3 weeks (range, 3.1-198.1 weeks), 40.4 weeks (range, 21.0-86.3 weeks), 23.6 weeks, and 67.9 weeks, respectively. In the first, second, third, fourth, fifth, and sixth courses of IFN monotherapy in group C, the median total doses of IFN were 525 MU (range, 22-2,312 MU), 558 MU (range, 57-4005 MU), 522 MU (range, 28-3,477 MU), 565 MU (range, 363-1,080 MU), 708 MU, and 1,200 MU, respectively. The median cumulative total durations and cumulative total doses, which represented the cumulative total duration and total dose of every course of every patient of group C, were 65.6 weeks (range, 8.4-474.4 weeks) and 1,388 MU (range, 354-4,805 MU), respectively. The median periods free of IFN in group C were 3.6 years (range, 0.1-7.3 years). In conclusion, the median dose of IFN per week in group A, B, and C were 21.8 MU/week (range, 6.7-42.0 MU/week), 22.0 MU/week (range, 4.5-42.0 MU/week), and 21.9 MU/week (range, 3.7-43.9 MU/week), respectively.

Clinical Features of Patients and Cumulative Hepatocarcinogenesis Rates According to Study Groups. The clinical features of patients in groups A, B, and C, at the start of the first IFN monotherapy are summarized in Table 1. The age of patients of group B was significantly higher than those of group A ($P = 0.009$; Bonferroni test) and group C ($P = 0.007$; Bonferroni test). Viremia levels in group A were significantly lower than those in group B ($P < 0.001$; Bonferroni test) and group C ($P < 0.001$; Bonferroni test). Fibrosis stage of group A was significantly milder than those of group B ($P = 0.006$; Bonferroni test) and group C ($P = 0.009$; Bonferroni test). There were no other significant differences in clinical features at the start of IFN therapy among the 3 groups.

During follow-up, 1 (1.5%), 17 (15.2%), and 15 (11.0%) patients developed HCC in groups A, B, and C, respectively. In groups A, B, and C, the cumulative hepatocarcinogenesis rates were 2.3%, 11.5%, and 0.8%, respectively, at the end of 5 years; 2.3%, 25.3%, and 7.2%, respectively, at the end of 10 years; and 2.3%, 33.0%, and 25.6%, respectively, at the end of 15 years. The rates were significantly different among the 3 groups ($P < 0.001$; Log-rank test) (Figure 1). In particular, the rates in group B were significantly higher than in group C ($P < 0.001$; Log-rank test) and group A ($P < 0.001$; Log-rank test), and the rates in group C were significantly higher than group A ($P = 0.037$; Log-rank test).

Hepatocarcinogenesis Rates According to aa Substitutions of HCV-CR. During follow-up, 5 of 82 patients (6.1%) and 18 of 130 patients (13.8%) developed HCC in double wild-type and nondouble wild-type, respectively. In double wild-type and nondouble wild-type, the cumulative hepatocarcinogenesis rates were, respectively, 1.6% and 2.6% at the end of 5 years; 3.4% and 12.3% at the end of 10 years; and 11.3% and 23.5% at the end of 15 years. The rates in double wild-type of HCV-CR were significantly lower than those in nondouble wild-type ($P = 0.036$; log-rank test) (Fig. 2).

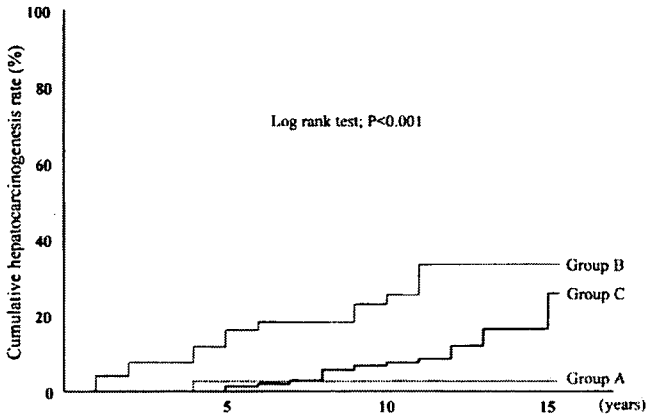

Fig. 1. Cumulative hepatocarcinogenesis rates were significantly different among the 3 study groups ($P < 0.001$; Log-rank test). In particular, the rates in group B were significantly higher than in group C ($P < 0.001$; Log-rank test) and group A ($P < 0.001$; log-rank test), and the rates in group C were significantly higher than in group A ($P = 0.037$; log-rank test).

Predictive Factors Associated with Hepatocarcinogenesis via Multivariate Analysis. We then analyzed the data for the whole population sample to determine those factors that could predict hepatocarcinogenesis. Univariate analysis identified 6 parameters that tended to or significantly correlated with carcinogenesis: age ($P < 0.001$), fibrosis stage ($P < 0.001$), platelet count ($P < 0.001$), group ($P < 0.001$), viremia level ($P = 0.018$), and aa substitution in HCV-CR ($P = 0.036$). These factors were entered into multivariate analysis, which identified 3 parameters that tended to or significantly influenced carcinogenesis independently: fibrosis stage ($P < 0.001$), aa substitutions in HCV-CR ($P = 0.008$), and group ($P = 0.056$) (Table 2).

We also analyzed the data for 219 patients, except for 94 patients who achieved SVR, to determine those factors that could predict hepatocarcinogenesis. Univariate analysis identified 5 parameters that tended to or significantly correlated with carcinogenesis: fibrosis stage ($P < 0.001$), platelet count ($P < 0.001$), age ($P = 0.001$), group ($P = 0.008$), and aa substitution in HCV-CR ($P = 0.028$). These factors were entered into multivariate analysis, which identified 2 parameters that significantly influenced carcinogenesis independently: fibrosis stage ($P < 0.001$) and aa substitution in HCV-CR ($P = 0.017$) (Table 3).

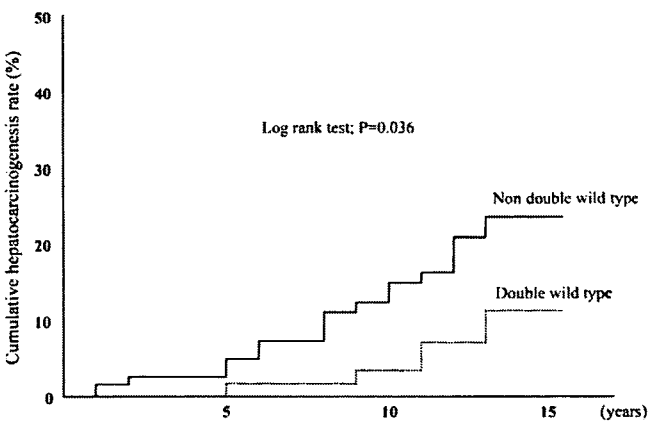

Fig. 2. Cumulative hepatocarcinogenesis rates according to aa substitutions of HCV-CR. The rates in double wild-type (arginine at aa 70/leucine at aa 91) of HCV-CR were significantly lower than those in nondouble wild-type ($P = 0.036$; log-rank test).

Table 2. Factors Associated With Hepatocareinogenesis in 313 Patients Infected with HCV Genotype 1b, Identified via Multivariate Analysis

Factors	Category	Odds Ratio (95% CI)	P Value
Fibrosis stage	1: F1, F2	1	
	2: F3	10.2 (3.65-28.5)	<0.001
Amino acid substitutions in the core region	1: double-wild	1	
	2: nondouble-wild	5.92 (1.58-22.2)	0.008
Group	1: A, C	1	
	2: B	2.75 (0.98-7.76)	0.056

NOTE. Cox proportional hazard model.

Hepatocarcinogenesis Rates in Group C According to HCV-CR and ALT Levels. In group C, the hepatocarcinogenesis rates were evaluated according to the ALT levels at the start of IFN. For this purpose, we selected 112 patients (82.4%) from group C in whom HCV-CR could be evaluated. In double wild-type, the hepatocarcinogenesis rates in patients with ALT levels below 1.5 (<75 IU/L) and above 1.5 (>75 IU/L) times the upper limit of normal (6-50 IU/L) were 0% (0/6 patients) and 8.6% (3/35 patients), respectively. In nondouble wild-type, the hepatocarcinogenesis rates in patients with ALT levels below 1.5 and above 1.5 times the upper limit of normal was 0% (0/7 patients), and 15.6% (10/64 patients), respectively (Table 4). In conclusion, regardless of whether aa substitutions in HCV-CR are present or not, lower hepatocarcinogenesis rates were noted in patients with ALT levels below 1.5 the upper limit of normal (0%) than in other patients (13.1%), but they did not achieve statistical significance on univariate analysis.

Table 3. Factors Associated with Hepatocarcinogenesis in 219 Patients of Non-SVR Infected with HCV Genotype 1b, Identified via Multivariate Analysis

Factors	Category	Odds Ratio (95% CI)	P Value
Fibrosis stage	1: F1, F2	1	
	2: F3	6.50 (2.39-17.6)	<0.001
Amino acid substitutions in the core region	1: double-wild type	1	
	2: nondouble wild-type	4.65 (1.32-16.4)	0.017

NOTE. Cox proportional hazard model.

Table 4. Hepatocarcinogenesis Rates in Group C According HCV Core Region and ALT Levels at the Start of IFN

	ALT Level (IU/L)*			
	<75	75-100	100-200	>200
Nondouble wild-type	0% (0/7)	14.3% (2/14)	13.3% (4/30)	20.0% (4/20)
Double wild-type	0% (0/6)	16.7% (1/6)	5.6% (1/18)	9.1% (1/11)

* Normal level of ALT: 6-50 IU/L.

In group C, the hepatocarcinogenesis rates were also evaluated according to the mean ALT levels at the IFN-free period. For this purpose, we selected 76 consecutive patients (55.9%) from group C in whom ALT levels were closely monitored. In double wild-type, the hepatocarcinogenesis rates in patients with ALT levels below 4 (<200 IU/L) and above 4 (>200 IU/L) times the upper limit of normal were 0% (0/26 patients) and 50% (1/2 patients), respectively. In nondouble wild-type, the hepatocarcinogenesis rates in patients with ALT levels below 1.5 (<75 IU/L), from 1.5 to 2 (75-100 IU/L), from 2 to 4 (100-200 IU/L), and above 4 (>200 IU/L) times the upper limit of normal were 0% (0/13 patients), 33.3% (3/9 patients), 22.7% (5/22 patients), and 25.0% (1/4 patients), respectively (Table 5). In conclusion, regardless of whether aa substitutions in HCV-CR are present or not, significantly lower hepatocarcinogenesis rates were noted in patients with ALT levels below 1.5 times the upper limit of normal (0%) than in other patients (18.9%) ($P = 0.027$). In particular, significantly higher hepatocarcinogenesis rates were noted in patients of nondouble–wild-type with ALT levels above 1.5 times the upper limit of normal (25.7%) than in other patients (2.4%) ($P = 0.004$).

Discussion

Despite numerous lines of epidemiological evidence connecting HCV infection and the development of HCC, it remains controversial whether HCV itself plays a direct or indirect role in the pathogenesis of HCC.[12] It is evident that the HCV-CR has oncogenic potential through the use of transgenic mice,[13] but its clinical impact on hepatocarcinogenesis is still unclear. Our study identified that cumulative hepatocarcinogenesis rates of double wild-type HCV-CR, as a predictor of virological response for PEG-IFN plus RBV therapy, were significantly lower than those of nondouble wild-type. We speculate that the resistant cases for treatment might reasonably lead to HCC. To our knowledge, this is the first report to support the findings of oncogenic potential via HCV-CR from the clinical aspect. Previous reports identified PA28γ-dependent pathway as one of the mechanisms of HCV-associated hepatocarcinogenesis. Morishi and colleagues showed that a knockout of the PA28γ gene induces the accumulation of HCV core protein in the nucleus of hepatocytes of HCV core gene transgenic mice and disrupts development of both hepatic steatosis and HCC.[18,19] Furthermore, HCV core protein also enhanced the binding of liver X receptor α/retinoid X receptor α to liver X receptor response element in the presence of PA28γ.[19] Thus, it is reported that PA28γ plays a crucial role in the development of HCV-associated steatogenesis and hepatocarcinogenesis. Further studies should be performed to connect evidence from animal model studies and the clinical impact of aa substitution in HCV-CR on hepatocarcinogenesis.

Viral factors associated with hepatocarcinogenesis in patients infected with HCV are still incompletely investigated. Ogata et al. reported that HCV genotype 1b strains might be associated with HCC on the basis of the secondary structure of an amino-terminal portion of the HCV NS3 protein.[20] Giménez-Barcons et al. reported that high aa variability within the NS5A of HCV might be associated with HCC in patients with HCV-1b–related cirrhosis.[21] In the present study, we could not investigate the clinical impact of the other region on hepatocarcinogenesis, except for the HCV-CR. Further studies should be performed to investigate the clinical impact of the other region of HCV on hepatocarcinogenesis.

Patients who fail to achieve SVR after single-course IFN should receive multicourse IFN at the time of ALT relapse at certain intervals. Based on previous reports showing increased incidence of HCC in 5 years or more

Table 5. Hepatocarcinogenesis Rates in Group C According HCV Core Region and ALT Levels at the IFN-Free Period

	ALT level (IU/L)*			
	<75	75-100	100-200	>200
Nondouble wild-type	0% (0/13)	33.3% (3/9)	22.7% (5/22)	25.0% (1/4)
Double wild-type	0% (0/10)	0% (0/4)	0% (0/12)	50.0% (1/2)

* Normal level of ALT: 6-50 IU/L.