3. Fried, M.W. *et al.* Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N. Engl. J. Med.* **347**, 975–982 (2002).
4. Hadziyannis, S.J. *et al.* Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. *Ann. Intern. Med.* **140**, 346–355 (2004).
5. Bruno, S. *et al.* Peginterferon alfa-2b plus ribavirin for naive patients with genotype 1 chronic hepatitis C: a randomized controlled trial. *J. Hepatol.* **41**, 474–481 (2004).
6. Sezaki, H. *et al.* Poor response to pegylated interferon and ribavirin in older women infected with hepatitis C virus of genotype 1b in high viral loads. *Dig. Dis. Sci.* **54**, 1317–1324 (2009).
7. Fried, M.W. Side effects of therapy of hepatitis C and their management. *Hepatology* **36**, S237–S244 (2002).
8. Pascu, M. *et al.* Sustained virological response in hepatitis C virus type 1b infected patients is predicted by the number of mutations within the NS5A-ISDR: a meta-analysis focused on geographical differences. *Gut* **53**, 1345–1351 (2004).
9. Shirakawa, H. *et al.* Pretreatment prediction of virological response to peginterferon plus ribavirin therapy in chronic hepatitis C patients using viral and host factors. *Hepatology* **48**, 1753–1760 (2008).
10. Akuta, N. *et al.* Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: amino acid substitutions in the core region and low-density lipoprotein cholesterol levels. *J. Hepatol.* **46**, 403–410 (2007).
11. Walsh, M.J. *et al.* Non-response to antiviral therapy is associated with obesity and increased hepatic expression of suppressor of cytokine signalling 3 (SOCS-3) in patients with chronic hepatitis C, viral genotype 1. *Gut* **55**, 529–535 (2006).
12. Gao, B., Hong, F. & Radaeva, S. Host factors and failure of interferon-alpha treatment in hepatitis C virus. *Hepatology* **39**, 880–890 (2004).
13. Matsuyama, N. *et al.* The dinucleotide microsatellite polymorphism of the IFNAR1 gene promoter correlates with responsiveness of hepatitis C patients to interferon. *Hepatol. Res.* **25**, 221–225 (2003).
14. Tsukada, H. *et al.* A polymorphism in MAPKAPK3 affects response to interferon therapy for chronic hepatitis C. *Gastroenterology* **136**, 1796–1805 (2009).
15. Nishida, N. *et al.* Evaluating the performance of Affymetrix SNP Array 6.0 platform with 400 Japanese individuals. *BMC Genomics* **9**, 431 (2008).
16. Kotenko, S.V. *et al.* IFN-λs mediate antiviral protection through a distinct class II cytokine receptor complex. *Nat. Immunol.* **4**, 69–77 (2003).
17. Sheppard, P. *et al.* IL-28, IL-29 and their class II cytokine receptor IL-28R. *Nat. Immunol.* **4**, 63–68 (2003).
18. Ank, N. *et al.* An important role for type III interferon (IFN-lambda/IL-28) in TLR-induced antiviral activity. *J. Immunol.* **180**, 2474–2485 (2008).
19. Marcello, T. *et al.* Interferons alpha and lambda inhibit hepatitis C virus replication with distinct signal transduction and gene regulation kinetics. *Gastroenterology* **131**, 1887–1898 (2006).
20. Desmet, V.J., Gerber, M., Hoofnagle, J.H., Manns, M. & Scheuer, P.J. Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology* **19**, 1513–1520 (1994).

IL28B：C型肝炎治療効果を規定する遺伝子多型 C型肝炎に対するテーラーメイド治療の確立を目指したゲノムワイド関連解析

田中靖人* 徳永勝士** 溝上雅史***

索引用語：IL28B，SNPs，IFNラムダ，GWAS，HCV

1 背景

わが国のC型肝炎ウイルス(HCV)感染者は約200万人存在するとされ，わが国における最大の感染症である．HCVは一旦感染すると6～8割が慢性肝炎に移行し，自然に治ることはほとんどなく，多くは肝硬変・肝癌へと進展し，本邦では年間約2万5千人が肝がんで死亡しているのが現状である．

そのHCVの根治治療で，現時点で最強治療であるペグインターフェロン＋リバビリン併用療法で根治させることができるようになったが，日本人に最も多いGenotype 1型高ウイルス量の症例では50％程度の根治しか得られず，約20％はペグインターフェロン＋リバビリン併用療法が全く効かないのが現状である．しかも，全治療期間が長期にわたるため，高齢者ではさまざまな副作用により減量・中断を余儀なくされる治療法である．したがって，治療前の効果予測が極めて重要と考えられる．その治療効果予測因子として，HCVゲノタイプ，ウイルス量，コア領域やNS5A領域のアミノ酸変異などのウイルス側因子に加えて，ペグインターフェロン，リバビリンのアドヒアランス(薬剤因子)，年齢，性差，肝線維化進展度，インシュリン抵抗性などの宿主側因子の重要性が多数報告されているが，それらの因子を総動員して解析しても治療前効果予測は約50％程度に留まる．

一方，ヒトゲノム計画の成功により，ヒト遺伝子は個人差として約300個に1個の遺伝子変異(SNP)が存在し，このSNPが個々の薬剤反応性や副作用に大きく関与することが続々と明らかとなってきている．近年，ゲノムワイドに均一に配置された約90万箇所(日

Yasuhito TANAKA et al: IL28B/genetic variation associated with response to pegylated interferon-alfa plus ribavirin therapy for chronic hepatitis C

*名古屋市立大学大学院医学研究科病態医科学［〒467-8601 名古屋市瑞穂区瑞穂町字川澄1］

東京大学大学院人類遺伝学，*国立国際医療センター国府台病院 肝炎・免疫研究センター

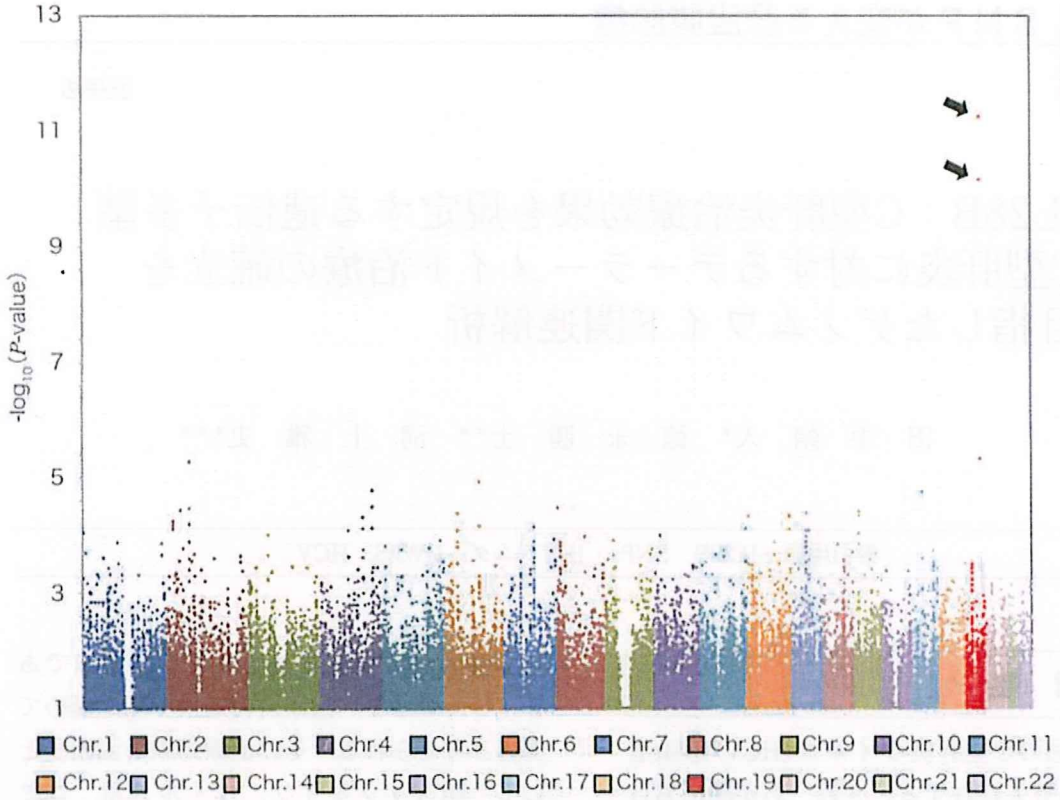


図1　ゲノムワイド関連解析：90万SNPs
Genome-wide association study（GWAS）
⇒19番染色体に有意な遺伝子多型（SNPs）

表1　治療無効に関連する因子

因子	Odds Ratio	95% Conf. Interval	P-values
rs8099917（マイナーアリル）	37.68	16.71-83.85	<0.0001
年齢	1.02	0.98-1.07	0.292
性別（女性）	3.32	1.49-7.39	0.003
再治療例	1.12	0.55-2.33	0.750
血小板数	0.93	0.87-1.01	0.080
Aminotransferase	1.00	0.99-1.00	0.735
Fibrosis stage	1.10	0.73-1.66	0.658
HCV-RNA量	1.01	0.99-1.02	0.139

本人では62万箇所）のSNPsを一括タイピングすることが可能になり，病態進展に多因子が関与すると想定されてきたⅡ型糖尿病，クローン病，B型慢性肝炎などにおいて疾患感受性遺伝子の同定が矢継ぎ早に報告されている[1~8]．

2　IL28B遺伝子多型と治療効果

われわれは，ペグインターフェロン＋リバビリン併用療法の有効性に関連するSNPsを

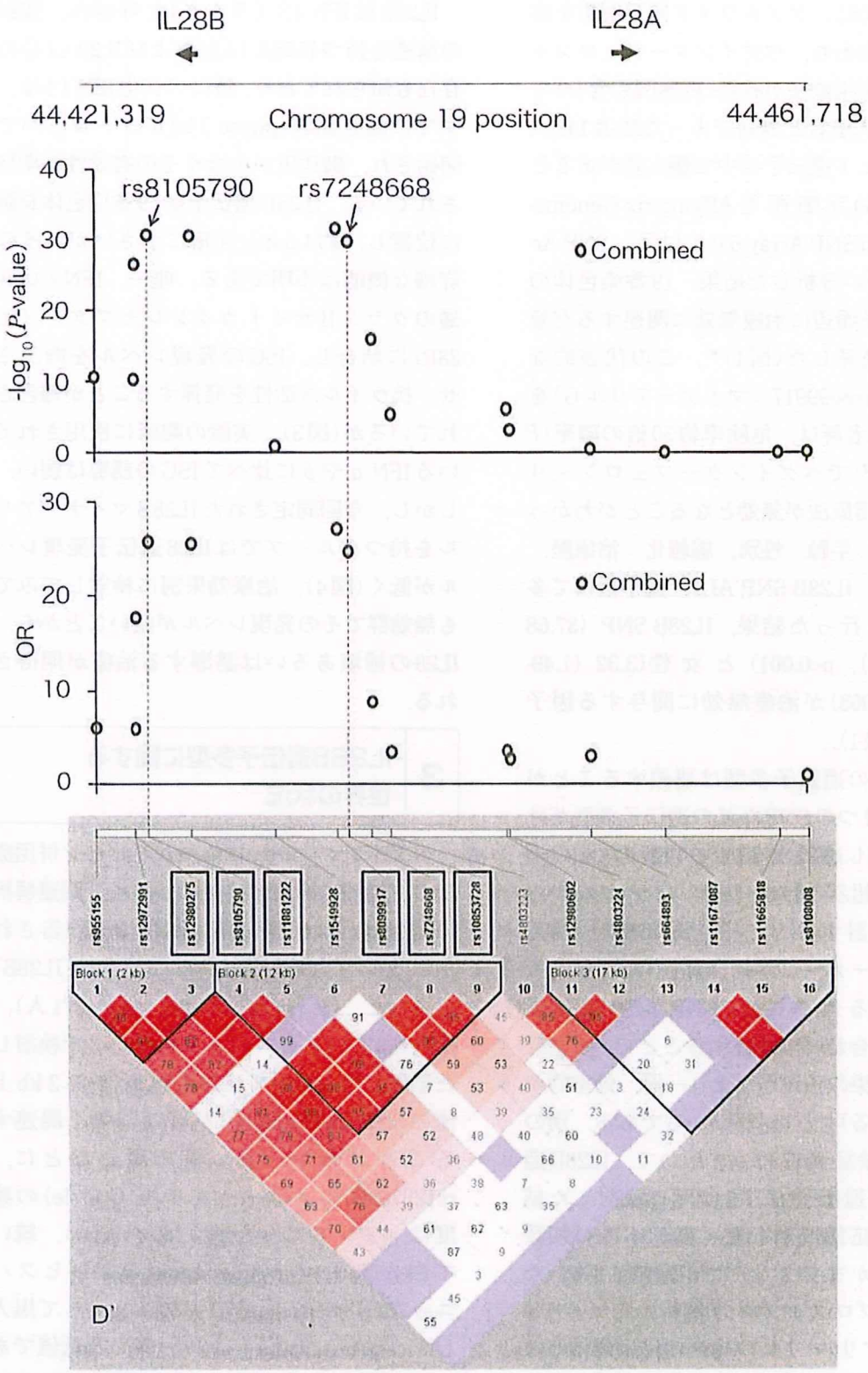


図2　IL28B，IL28A周辺のLD map．D'は連鎖不平衡係数のことで，連鎖不平衡の強さを示している．

同定するために，ゲノムワイド関連解析を実施した．すなわち，ペグインターフェロン＋リバビリン併用療法が有効(再燃例も含む)であった日本人患者と無効であった患者142人に関して，ヒト遺伝子の中で個人差があるとされる約90万箇所をAffymetrix Genome-Wide Human SNP Array 6.0（以下，SNP Array 6.0)を用い分析した結果，19番染色体のIL28B遺伝子周辺に治療無効に関連する有意なSNPsを発見した(図1)[9]．この代表的なSNPであるrs8099917（マイナーアリルG)を持つHCV患者群は，危険率約30倍の確率($P = 2.68 \times 10^{-32}$)でペグインターフェロン＋リバビリン併用療法が無効となることがわかった．さらに，年齢，性別，線維化，治療歴，ウイルス量，IL28B SNP, ALT，血小板にて多変量解析を行った結果，IL28B SNP（37.68 (16.71-83.85)，p<0.001）と女性(3.32（1.49-7.39)．p=0.003)が治療無効に関与する因子であった(表1)．

SNPなどの遺伝子多型は連鎖することが多いため，1つのSNPなどの遺伝子多型だけでなく近接し連鎖する複数のSNPなどの遺伝子多型を組み合わせ，ハプロタイプを作った上で，検討することが重要である．例えば，このマーカー（遺伝子)内のハプロタイプを表現するタグ(代表）SNPなどの遺伝子多型を組み合わせて検査することで，C型肝炎の治療効果の予測が，より一層，効率的かつ確実となる可能性が高いからである．別のコホート(検証群172人)を用いて，IL28B遺伝子および遺伝子周辺を詳細に検討した結果，治療反応性に強く関与するSNPsは複数存在し，しかもすべてが連鎖不平衡であった(図2)．ハプロタイプ解析からマイナーアリル(リスクアリル)を持つ場合の治療無効となるオッズ比は11.1 ($P=1.35 \times 10^{-25}$)であった．

IL28BはIFNλ3（ラムダ)と呼ばれ，類似の構造を持つIL28A (λ2)およびIL29 (λ1)の存在も知られており，特にペグ化IFNλ1は，すでに臨床試験(phase Ⅰb)がヨーロッパで開始され，副作用が少なくその有効性が期待されている．IL28B遺伝子は19番染色体長腕に位置し，約1.5 kと非常に小さいが，その詳細な機能は不明である．唯一，IFNλは共通のクラスⅡサイトカインレセプター（IL-28R)に結合し，ISGの発現レベルを向上させ，抗ウイルス活性を発揮することが報告されているが(図3)，実際の臨床に使用されているIFN αやβに比べてISGの誘導は弱い．しかし，今回同定されたIL28Bマイナーアリルを持つグループではIL28遺伝子発現レベルが低く(図4)，治療効果別に検討してみても無効群でその発現レベルが低いことから，IL28の補填あるいは誘導する治療が期待される．

3 IL28B遺伝子多型に関する世界の状況

ペグインターフェロン＋リバビリン併用療法の有効性に関連するゲノムワイド関連解析の結果は，ほぼ同時期に欧米でも報告された[10,11]．いずれの報告もキーワードは"IL28B"であった．Geらの報告では，白人(871人)，黒人(191人)，ヒスパニック(75人)で検討した結果，白人おいてIL28B遺伝子から3 kb上流のSNP (rs12979860)が著効に強く関連することがわかった．興味深いことに，rs12979860のメジャーアリル(C-allele)の頻度は，アジアで最も多く(80～90%)，続いて白人(European-Americans)およびヒスパニック(Hispanics)が70～80%，そして黒人(African-Americans）は30～50%と低値であり，この頻度が著効率と正に相関しているこ

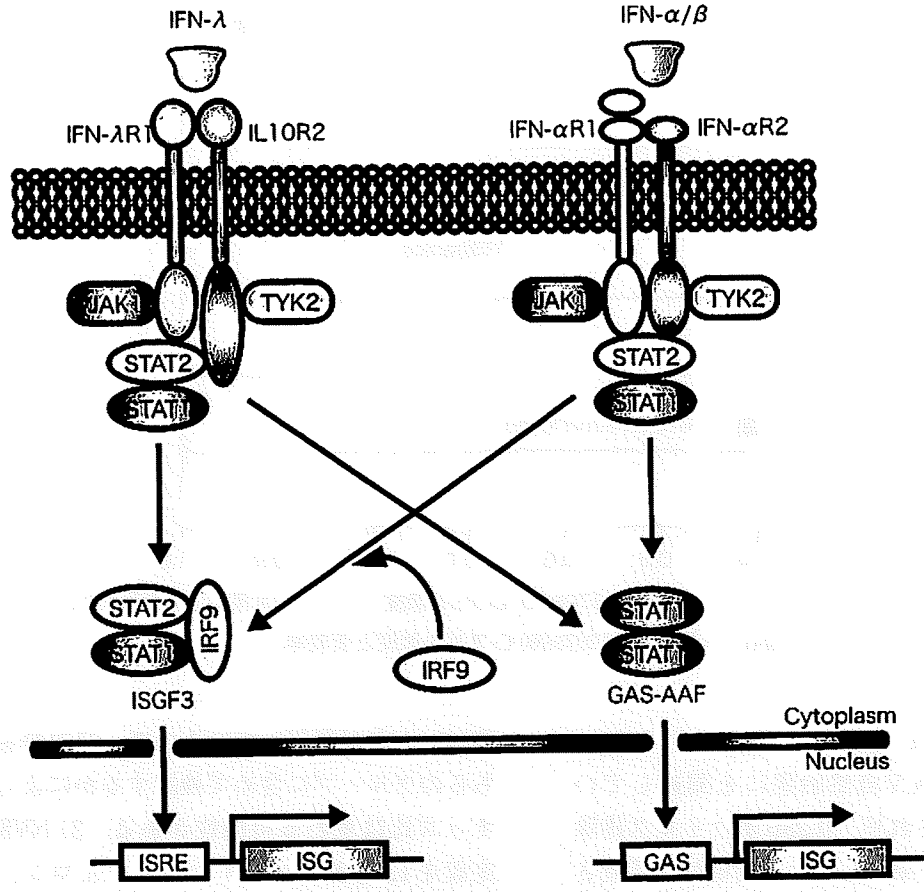


図3　IFNシグナル伝達系

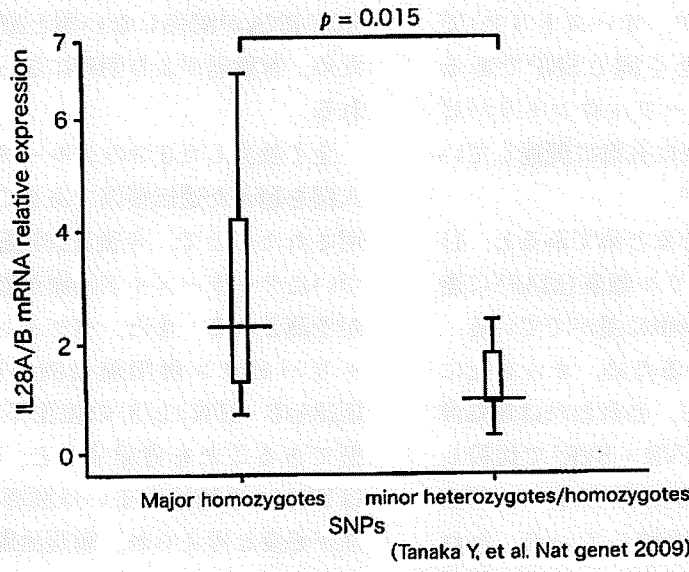


図4　アリル別のIL28A/B遺伝子発現レベル

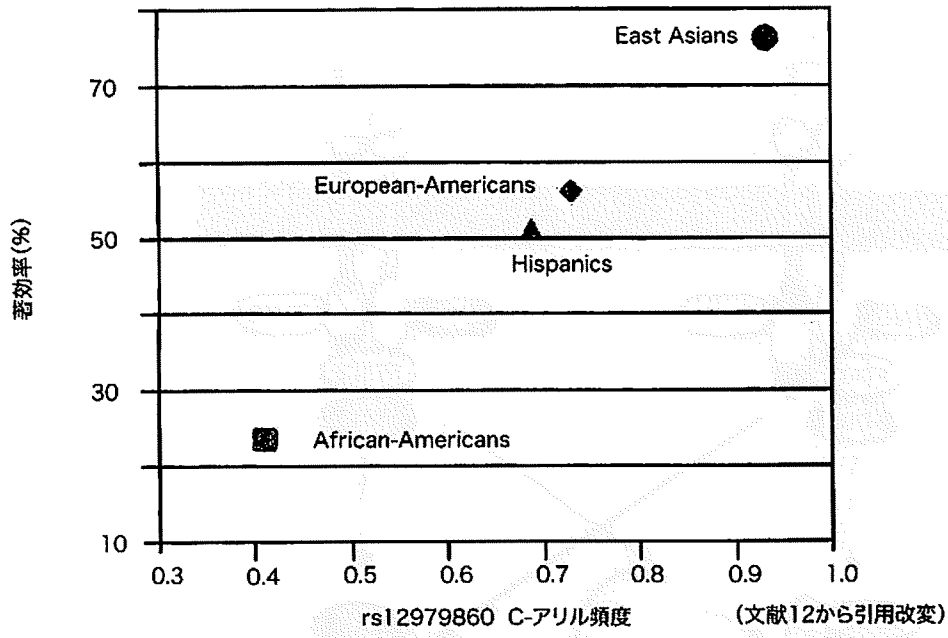


図5　人種別のrs12979860 C-アリル頻度と著効率

とが述べられている(図5)．さらに，rs12979860はHCV自然治癒にも関連していることが報告され[12]，IL28Bがウイルス排除に重要な役割を果たしている可能性が示唆された．Suppiah Vらは，オーストラリア(北ヨーロッパ起源)およびヨーロッパ諸国(イギリス，ドイツ，イタリア，オーストリア)で検討し，われわれと全く同じSNPであるrs8099917がペグインターフェロン＋リバビリン併用療法の治療効果に有意に関連していることを報告している[11]．

これらの論文の特徴をまとめてみると，1) 人種によるマイナーアリル頻度(MAF)に差があり，これにより著効率に差がでている．2) Caseの取り方が異なるため，オッズ比に差がでている．すなわち，われわれは無効群(NVR) vs. 無効群以外(TVR＋SVR)で比較しているが，他の2報は著効群(SVR) vs. 著効群以外(TVR＋NVR)で比較している．われわれのデータからはSVR群とTVR群(再燃群)のMAFが類似しているので，TVR群をどちらのグループに含めて検討するかによってオッズ比は変わる可能性がある．3) NVRの定義を12週までペグインターフェロン，リバビリンの両剤が80％以上の投与量にも関わらず，12週で2 log減少がなく24週でHCV-RNAが消失しない例と厳密に設定したため，有意差がより明確になったものと思われる．

全く独立した3つのグループから，しかも人種を超えた遺伝要因であることが一気に証明されたことで，今後IL28B遺伝子多型に基づいたテーラーメイド治療が展開されることが予測される．また，ペグインターフェロン＋リバビリン併用無効例のほとんどは，IL28A/B (IFNλ2/3)の産生不十分な遺伝子型であることを考慮すると，IFNλ2あるいはIFNλ3を補填あるいは誘導する方法の確立が必要と考えられ，新規治療薬の開発が期待される．

4 おわりに

実際の臨床において，ペグインターフェロン＋リバビリン併用療法の前にこの遺伝子多型(SNPs)を測定することで，根治の見込める患者群を高い確率(的中率 約80％)で選別できるし，効かない人たちからは無用な苦痛や出費から免れることができる(的中率 約80％)．すなわち，C型肝炎診療の中で，治療前にこの遺伝子多型を調べることで高い確率で治療効果の予測が可能となり，テーラーメード医療として期待される．このIL28B遺伝子は通常C型肝炎の治療に使用されているIFN-αやβとは異なるIFN-λの1種でその下流に存在するIFN誘導遺伝子群を誘導して抗ウイルス効果をもたらすので，今後このIL28Bを増強する新規薬剤を開発することで，現在ペグインターフェロン＋リバビリン併用療法で効かない人達や効果の不十分な人達も根治が望める可能性がある．現在，肝炎治療の効果的促進(経済的負担軽減)をはかるため2008年4月1日より「B型・C型肝炎患者医療給付事業」がスタートしているが，これらの公費助成も効率的運用が図れることを意味する．

謝　辞

「テーラーメイド治療を目指した肝炎ウイルスデータベース構築に関する研究」の一環として行われる．H19～21：厚労省科研費肝炎等克服緊急対策研究事業

文　献

1) Barrett JC, Hansoul S, Nicolae DL et al : Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. Nat Genet 40 : 955–962, 2008
2) Saxena R, Voight BF, Lyssenko V et al : Genome-wide association analysis identifies loci for type 2 diabetes and triglyceride levels. Science 316 : 1331–1336, 2007.
3) Sladek R, Rocheleau G, Rung J et al : A genome-wide association study identifies novel risk loci for type 2 diabetes. Nature 445 : 881–885, 2007
4) Easton DF, Pooley KA, Dunning AM et al : Genome-wide association study identifies novel breast cancer susceptibility loci. Nature 447 : 1087–1093, 2007
5) Hakonarson H, Grant SF, Bradfield JP et al : A genome-wide association study identifies KIAA0350 as a type 1 diabetes gene. Nature 448 : 591–594, 2007
6) Shi J, Levinson DF, Duan J et al : Common variants on chromosome 6p22.1 are associated with schizophrenia. Nature 460 : 753–757, 2009
7) Wang K, Zhang H, Ma D et al : Common genetic variants on 5p14.1 associate with autism spectrum disorders. Nature 459 : 528–533, 2009
8) Weiss LA, Arking DE, Daly MJ et al : A genome-wide linkage and association scan reveals novel loci for autism. Nature 461 : 802–808, 2009
9) Tanaka Y, Nishida N, Sugiyama M et al : Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet 41 : 1105–1109, 2009
10) Ge D, Fellay J, Thompson AJ et al : Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 461 : 399–401, 2009
11) Suppiah V, Moldovan M, Ahlenstiel G et al : IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet 41 : 1100–1104, 2009
12) Thomas DL, Thio CL, Martin MP et al : Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. Nature 461 : 798–801, 2009

*　　*　　*

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 2010, p. 882–889 Vol. 54, No. 2
0066-4804/10/$12.00 doi:10.1128/AAC.01061-09

Mechanism of Entecavir Resistance of Hepatitis B Virus with Viral Breakthrough as Determined by Long-Term Clinical Assessment and Molecular Docking Simulation▽†

Motokazu Mukaide,[1,2] Yasuhito Tanaka,[1]* Tadasu Shin-I,[1] Man-Fung Yuen,[3] Fuat Kurbanov,[1] Osamu Yokosuka,[4] Michio Sata,[5] Yoshiyasu Karino,[6] Gotaro Yamada,[7] Kohsaku Sakaguchi,[8] Etsuro Orito,[9] Manami Inoue,[10] Sumbella Baqai,[11] Ching-Lung Lai,[3] and Masashi Mizokami[12]

Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan[1]; SRL, Inc., Tokyo, Japan[2]; Department of Medicine, University of Hong Kong, Queen Mary Hospital, Hong Kong[3]; Department of Medicine and Clinical Oncology, K1, Graduate School of Medicine, Chiba University, Chiba, Japan[4]; Department of Internal Medicine, Kurume University School of Medicine, Kurume, Japan[5]; Department of Gastroenterology, Sapporo Kosei Hospital, Sapporo, Japan[6]; Department of Internal Medicine, Center for Liver Diseases, Kawasaki Hospital, Kawasaki Medical School, Okayama, Japan[7]; Department of Gastroenterology and Hepatology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan[8]; Department of Gastroenterology, Nagoya Daini Red Cross Hospital, Nagoya, Japan[9]; Epidemiology and Prevention Division, Research Center for Cancer Prevention and Screening, National Cancer Center, Tokyo, Japan[10]; Department of Internal Medicine, Alameda County Medical Center, Oakland, California[11]; and Research Center for Hepatitis and Immunology, International Medical Center of Japan Konodai Hospital, Ichikawa, Japan[12]

Received 21 July 2009/Returned for modification 13 August 2009/Accepted 18 November 2009

The mechanism by which entecavir resistance (ETVr) substitutions of hepatitis B virus (HBV) can induce breakthrough (BT) during ETV therapy is largely unknown. We conducted a cross-sectional study of 49 lamivudine (LVD)-refractory patients and 59 naïve patients with chronic hepatitis B. BT was observed in 26.8% of the LVD-refractory group during weeks 60 to 144 of ETV therapy. A line probe assay revealed ETVr substitutions only in the LVD-refractory group, i.e., in 4.9% of patients at baseline, increasing to 14.6%, 24.4%, and 44.8% at weeks 48, 96, and 144, respectively. Multivariate logistic regression analysis adjusted for age, gender, HBV DNA levels, and LVD resistance (LVDr) (L180M and M204V, but not M204I) indicated that T184 substitutions and S202G (not S202C) were a significant factor for BT (adjusted odds ratio [OR], 141.12, and 95% confidence interval [CI], 6.94 to 2,870.20; OR, 201.25, and 95% CI, 11.22 to 3608.65, respectively). Modeling of HBV reverse transcriptase (RT) by docking simulation indicated that a combination of LVDr and ETVr (T184L or S202G) was characterized by a change in the direction of the D205 residue and steric conflict in the binding pocket of ETV triphosphate (ETV-TP), by significantly longer minimal distances (2.2 Å and 2.1 Å), and by higher potential energy (−117 and −99.8 Kcal/mol) for ETV-TP compared with the wild type (1.3 Å; −178 Kcal/mol) and LVDr substitutions (1.5 Å; −141 Kcal/mol). Our data suggest that the low binding affinity of ETV-TP for the HBV RT, involving conformational change of the binding pocket of HBV RT by L180M, M204V plus T184L, and S202G, could induce BT.

Infection with hepatitis B virus (HBV) is extremely widespread and affects more than 350 million people worldwide. Chronic HBV infection leads to the development of complications, such as liver cirrhosis (LC) and hepatocellular carcinoma (HCC) (12). HBV has been classified into 8 geographically, genetically, and clinically diverse genotypes, designated alphabetically from A to H according to their order of discovery (14). Genotypes B and C are prevalent in Asia, and genotype C is associated with more serious liver disease, including LC and HCC, and a poorer response to interferon therapy than genotype B (5). The ultimate therapeutic goal when treating chronic HBV infection is to prevent the development of LC and HCC by eliminating or producing sustained suppression of HBV replication. However, lamivudine resistance (LVDr) was reported to occur in 24% of patients treated for 1 year and in 74% of those treated for 5 years (16, 26). The rate of adefovir resistance (ADVr) in nucleoside-naive hepatitis B e antigen (HBeAg)-negative patients has been reported to be 0% after 1 year, but after 5 years of treatment, the rate increases to 28% to 42% (13). Entecavir (ETV) has been shown to be more potent *in vitro* than either LVD or ADV. Results from clinical studies showed that the efficacy of ETV was superior to that of the direct comparator, LVD, in both nucleoside-naive and LVD-refractory patients (6, 11, 15, 18).

* Corresponding author. Mailing address: Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Kawasumi, Mizuho, Nagoya 467-8601, Japan. Phone: 81-52-853-8191. Fax: 81-52-842-0021. E-mail: ytanaka@med.nagoya-cu.ac.jp.

† Supplemental material for this article may be found at http://aac.asm.org/.

▽ Published ahead of print on 23 November 2009.

TABLE 1. Patient characteristics of naïve and LVD-refractory patients

Characteristic	Value	
	Naïve (n = 59)[a]	LVD refractory (n = 41)[a]
Male/female no.	41/18	34/7
Mean age (yr)	46.5 ± 8.4	48.6 ± 8.3
HBeAg positive (%)	33 (55.9)	23 (56.1)
Mean ALT (U/liter)	118.9 ± 108.6	119.8 ± 99.0
Mean HBV DNA ($\log_{10}$ copies/ml)	6.7 ± 1.8	6.8 ± 1.0
Genotypes (no. A/B/C/D/E)	3/11/43/1/1	5/7/28/1/0

[a] Values are means ± standard deviations.

The persistence of LVDr substitutions in patients switched to ETV is worrisome, because LVDr was shown to enhance the risk of developing ETVr and treatment failure, defined as viral breakthrough (BT) (an increase in serum HBV DNA of at least 1 $\log_{10}$ copy/ml compared with the nadir value as observed during ETV therapy) (20). A recent *in vitro* study showed that LVDr (L180M and M204V) substitutions confer an ~8-fold reduction in susceptibility to ETV and that additional substitutions at residues T184, S202, and M250 are needed to confer high levels of ETVr and BT (2, 3).

These analyses, however, used a limited number of patient isolates and/or laboratory HBV clones, and there has been a paucity of community-based data derived from long-term trials regarding the clinical outcomes of ETVr variants in naïve or LVD-refractory patients. Therefore, the aim of this study was to evaluate the incidence of ETVr and BT by comparing outcomes following 3-year ETV treatment in treatment-naïve patients and LVD-refractory patients. ETVr was assessed by using a recently reported line probe assay (HBV DR v.3) (7). Importantly, as the mechanism by which ETVr substitutions can induce BT during ETV therapy is largely unknown, changes in the conformation of HBV reverse transcriptase (RT) arising from LVDr and ETVr substitutions were modeled by using 3-dimensional (3D) docking simulation.

MATERIALS AND METHODS

Study design. We conducted a cross-sectional study of 100 patients (Tables 1 and 2), 45 of whom were from Japan, 25 from the United States, and 30 from Hong Kong. The patients were subdivided into two groups; treatment-naïve (n = 59) and LVD-refractory (n = 41) patients, whose gender, age, HBeAg status, and mean HBV DNA levels are summarized in Table 1. The patients received 0.5 mg or 1.0 mg ETV. The 1.0-mg ETV once-daily (QD) dosage has been approved for use in LVD-refractory patients, and only patients treated with 1.0 mg per day were included in resistance assessments. The study protocol conformed to the 1975 Declaration of Helsinki and was approved by the Ethics Committees of the institutions, and written informed consent was obtained from each participant.

Screening for drug-resistant substitutions. Simultaneous detection of wild-type HBV and drug-induced substitutions was performed using HBV DR v.3 and v.2 (Innogenetics, Ghent, Belgium) according to the manufacture's protocol. HBV DR v.3 and v.2 were developed for detection of ETVr-specific substitutions (T184SCGA/ILFM, S202G/C/I, and M250V/I/L), TDFr-specific substitutions (A194T), and newly reported ADVr (I233V) substitutions, as well as LVDr (L80V/I, V/G173L, L180M, and M204V/I) and ADVr (A181T/V and N236T) substitutions. The HBV DR assay consistently detected ETVr-specific substitutions present in ≥5% of the virus population when the HBV DNA concentration was ≥4 $\log_{10}$ copies/ml (7). The AUTOLiPA (Innogenetics, Ghent, Belgium) was used for the automated test procedure. An 867-bp-long fragment of the polymerase gene (domains A to F) was amplified using biotinylated PCR primers (HBV DR v.3 and v.2). PCR products were directly sequenced.

Statistical analyses. The statistical significance of observed differences was assessed using the chi-square test and the Mann-Whitney U test, where appropriate. In the 67 patients (38 naïve and 29 LVD refractory) with 3 years of ETV treatment (Fig. 1), the logistic regression model was used to assess the factors associated with BT. STATA 10 (Statacorp LP, TX) and the Statistical Program for Social Sciences (SPSS 12.0 for Windows; SPSS Inc., Chicago, IL) were used for all analyses.

HBV polymerase sequencing. HBV DNA was extracted from serum samples using a Qiagen QIAamp DNA blood minikit (Qiagen GmbH, Germany), and an 867-bp-long fragment of the polymerase gene (domains A to F) was amplified using biotinylated PCR primers (INNO-LiPA). PCR products were directly sequenced. Nucleotide mixtures were reliably detected when they were mixed at a ratio of approximately 25% or greater.

Three-dimensional-structure-based docking simulation methods. The amino acid sequence of HBV RT was retrieved from GenBank (gene Pol product of accession no. X75665), and the 323rd to 697th residues, which correspond to the finger, palm, and thumb domains, were extracted. The sequence and that of HIV RT, retrieved from the Protein Data Bank (accession no. 1RTD), were aligned using BLASTP (1), and then the resulting alignment was modified manually to obtain a match of the RT-specific motifs in both sequences. The main-chain structure of HBV RT was built from the alignment and the 3D structure of HIV RT (accession no. 1RTD) (8) by the use of the "nest" module (17) in the JACKAL package (19), where global energy minimization was done to find the most stable backbone structure. The loop and secondary-structure regions were then refined (24), after which the side chain structure was refined by the use of the "scap" module in the package (23). The 3D structures of HBV RT containing three sets of substitutions, L180M plus M204V, L180M plus S202G plus M204V, and L180M plus T184L plus M204V, were also designed in the same manner.

The binding site of ETV was searched on the wild-type HBV RT molecule by docking simulation. First, the structure of ETV triphosphate (ETV-TP) was designed by a small-molecule-editing function in the SYBYL 8.0 package (Tripos Inc., St. Louis, MO). Then, the possible binding sites of the ligand were searched from the surface of the protein by the use of the "Surflex-Dock" (9) module in the package. Here, the docking candidate area was restricted to the surfaces of the residues that were within 3 Å from L180, T184, S202, Y203, M204, D205, or D206. The binding potential was estimated from the GOLD score calculated by

TABLE 2. Three-year assessment (HBeAg loss, ALT normalization, and HBV-DNA 2.6) of naïve and LVD-refractory patients

Parameter	Value for follow-up week:					
	Naïve (n = 59)[a]			LVD refractory (n = 41)[a]		
	48	96	144	48	96	144
Follow-up [n (%)]	59 (100)	39 (66.1)	38 (64.4)[b]	41 (100)	40 (97.6)[c]	26 (63.4)[d]
HBeAg loss [n (%)]	5 (15.2)	7 (24.1)	9 (32.1)	4 (17.4)	7 (31.8)	4 (22.2)
ALT normalization [n (%)]	24 (40.7)	25 (64.1)	27 (71.1)	16 (39.0)	20 (50.0)	13 (50.0)
HBV DNA loss [n (%)]	24 (40.7)	28 (71.8)	28 (73.7)	15 (36.6)	19 (47.5)	12 (46.2)

[a] Values are means ± standard deviations.
[b] One naïve patient (J44) stopped ETV therapy at week 80 (ALT, 119 U/liter, and HBV DNA, 7.6 $\log_{10}$ copies/ml at baseline; ALT, 17, and HBV DNA, <2.6 at week 80) due to severe headache during therapy. Twenty patients in Hong Kong stopped ETV therapy between weeks 48 and 72.
[c] One LVD-refractory patient (J37) switched from ETV therapy to LVD plus adefovir due to BTH with ETVr before week 96.
[d] Two patients (J33 and J40) switched from ETV therapy to LVD plus adefovir due to BTH with ETVr before week 144. Twelve patients in the United States were treated with ETV for <120 weeks.

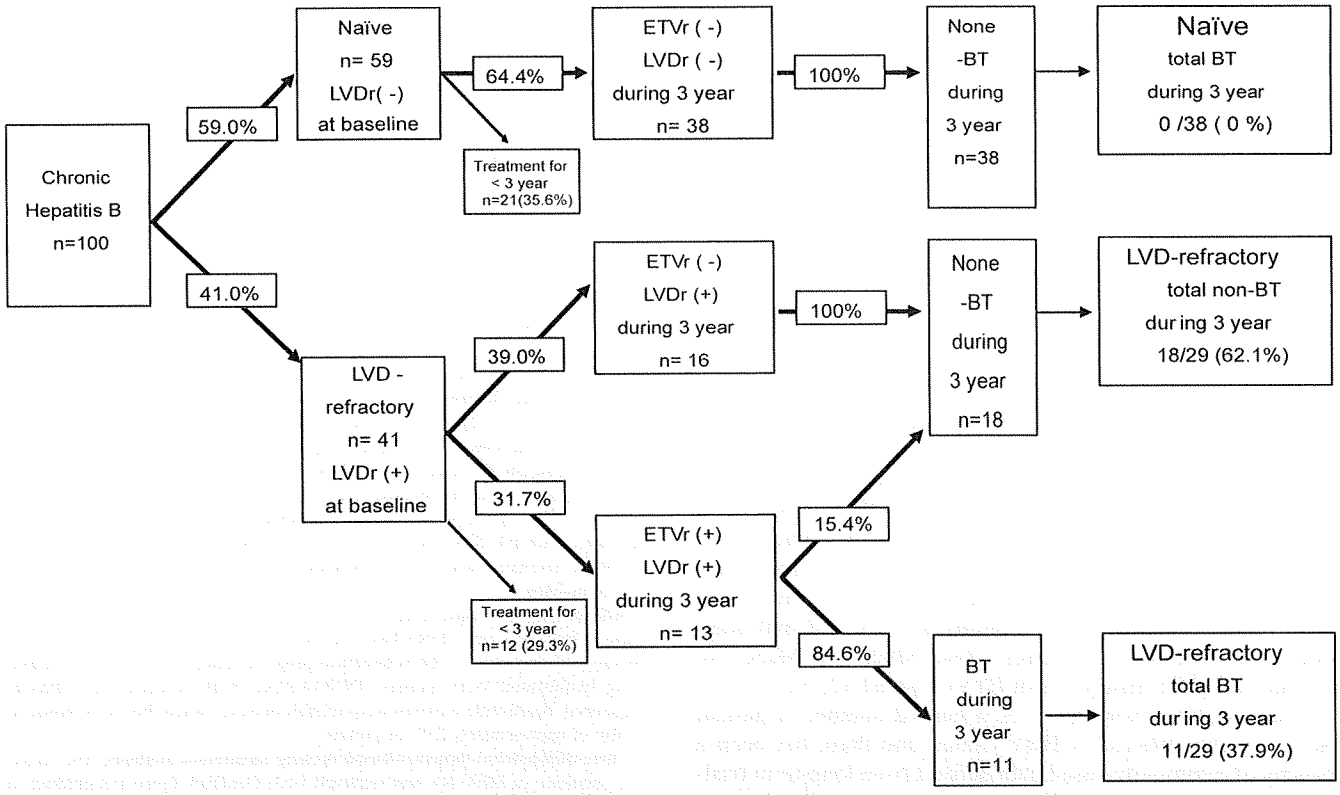


FIG. 1. Flowchart of 100 naïve/LVD-refractory patients during ETV therapy.

the "CScore" module (4) in the package. The score was evaluated based on hydrogen bond energy, the internal energy of molecules, and complex energy between ligand and protein. The minimal distance between their molecular surfaces was also calculated.

RESULTS

Clinical efficacy. The clinical backgrounds and the percentages of LVD-naïve and LVD-refractory patients who achieved HBeAg loss, alanine aminotransferase (ALT) normalization, and non-PCR-detectable HBV DNA levels (<2.6 $\log_{10}$ copies/ml) during the ETV treatment course are summarized in Table 2. There were no significant differences in clinical data at entry between the 2 groups. The rates of HBeAg loss, ALT normalization, and HBV DNA loss were significantly higher in naïve patients than in LVD-refractory patients.

Detection of substitutions responsible for ETV resistance in naive and LVD-refractory patients during treatment for 144 weeks. The characteristics of patients who had ETVr substitutions detected by HBV DR v.3 are summarized in Table 3. The percentage of the typical LVDr (L180M, M204V, and M204I) or ETVr observed in naïve patients was 0% (0/38) during the 144-week treatment period.

Among the patients examined at entry prior to treatment with ETV, in 41 LVD-refractory patients, M204V (30/41; 72.4%), M204I (24/41; 58.5%), L180M (38/41; 92.7%), L80V (6/41; 14.6%), L80I (18/41; 43.9%), and V173L (4/41; 9.76%) substitutions were detected. In the 41 LVD-refractory patients, the cumulative ETVr substitutions were detected in 2/41 (4.9%) at baseline and increased to 6/41 (14.6%), 10/41 (24.4%), and 13/29 (44.8%) at weeks 48, 96, and 144, respectively (Fig. 2). In the 29 patients treated with ETV for 3 years, T184SCGA, T184ILMF, S202G, and S202C were found in 5 (17.2%), 4 (13.8%), 9 (31.0%), and 1 (3.4%), respectively. Neither S202I nor M250V/I/L substitutions were detected in this population.

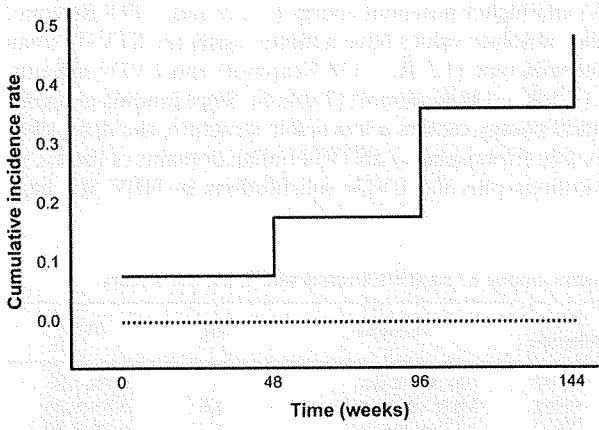
A comparative summary of the ETVr substitutions, detected by HBV DR v.3 and direct sequencing, during week −8 (8 weeks before the start of treatment) and week 144 is presented in Table 4. HBV DR v.3 revealed ETVr substitutions earlier (up to 48 weeks) than did direct sequencing. In addition, HBV DR v.3 allowed the detection of mixed quasispecies containing different substitutions.

Viral BT during the 144 weeks on treatment. The rates of BT among 59 naive and 41 LVD-refractory patients treated with ETV for 144 weeks are summarized in Fig. 1. There were no cases of BT in the LVD-naïve group during the 144-week treatment period, whereas in the LVD-refractory group treated with 1.0 mg ETV, 11 of 13 patients with genotypic ETVr had evidence of BT after 60 to 144 weeks of treatment, followed by 7 breakthrough hepatitis (BTH) (defined as a flare up of ALT) patients (median interval, 11.4 weeks after BT). The LVDr substitutions (L180M and M204V/I) were detected in all of the BT patients in specimens obtained at baseline (Table 3). Among the 11 patients with BT, 8 (72.7%) had an additional S202G substitution and 7 (63.6%) had a T184SCGA or T184ILMF substitution, indicating that the T184 and/or S202 substitution emerged before BT during ETV treatment (Table 3 and Fig. 2). Seven patients with BTH had LVDr

TABLE 3. Characteristics of ETVr detected by HBV DR v3 among 13 patients at week 0, 24, 48, 72, 96, and 144

Case	Age (yr)	Sex	HBV genotype	ALT (IU/liter)			HBV DNA (copies/ml)			Wk			No. of wks ETVr to BT	LVDr at baseline	ETVr at baseline	ETVr emerged during therapy
				Baseline	Nadir	Peak	Baseline	Nadir	Peak	ETVr	BT	BTH				
J20	34	M	C	82	22	131	7.6	4.5	7.4	<92[a]	92	100	<22	M180, V204	ND[f]	ILMF184, G202
J27	61	M	C	79	38	150	7.4	3.9	7	<128[b]	128	144	<32	M180, V204	ND	SCGA184
J30	43	M	C	95	21	108	7.6	4.6	6.8	48	76	92	28	I80, M180, V/I204	ND	G202
J33	43	M	C	69	21	199	7.6	5.1	7.1	96	116	128	18	I80, L173, M180, VI204	ND	(ILFM184),[c] G202
J37[d]	48	M	C	465	43	76	7.6	4.7	6	48	60	60	12	I80, M180, V204, I204	ND	G202
J39	59	M	C	35	22	82	7	4.6	8	72	108	128	36	M180, V204	ND	SCGA/ILFM184, G202
J40	28	M	C	149	15	398	5.3	3.6	6.6	−8	64	72	72	M180, V204	(G+C) 202	G202
J22	44	M	C	240	17	24	7.6	3.5	5.6	<140[e]	144	NO	<44	M180, I204	ND	G202
J28	45	M	B	43	15	23	6.9	5.5	6.5	0	144	NO	144	M180, V204	SCGA184, V233	SCGA184, V233
U72	47	F	C	29	25	44	7.5	3.8	5	<144	144	NO	<48	L + V80, M180, V204	ND	SCGA184, (S+G)202
H55	47	F	C	102	19	20	6.1	3	4.2	48	88	NO	40	V + I80, M180, V204	ND	ILFM184
J19	57	M	B	233	29	43	7.4	2.8	3.3	24	NO	NO	NO	V + 180, M180, I204	ND	G202
U42	52	M	A	135	36	50	>7.6	5.3	5.8	<80	NO	NO	NO	M180, V204	ND	SCGA184

[a] Not tested during weeks 72 and 92.
[b] Not tested during weeks 96 and 128.
[c] ILFM184 was detected at week 144.
[d] Switch to ADV/LVD at week 60.
[e] Not tested during weeks 96 and 140.
[f] ND, not detected.



LVD-refractory patients

Weeks	0	48	96	144
No.	41	41	41	29*
ETVr	2 (4.9%)	6 (14.6%)	10 (24.4%)	13 (44.8%)
T184SCGA	1	1	3	5
T184ILFM	0	1	4	4
S202G	1	4	7	9
S202C	1	1	1	1
S202I	0	0	0	0
M250V/L	0	0	0	0

*Twelve patients in the US have ETV treatment for <120 weeks.

FIG. 2. Kaplan-Meier plot and tabulated data for time to ETVr and cumulative ETVr patterns over 144 weeks. Pretreatment variables (solid line, LVD refractory; broken line, naïve) were analyzed in relation to the occurrence of ETVr. A previous LVD treatment was associated with a more rapid occurrence of ETVr (Breslow analysis; $P < 0.001$). Among the patients examined at entry prior to treatment with ETV, for the 41 LVD-refractory patients, the cumulative ETVr substitutions were detected in 2/41 (4.9%) at baseline and increased to 6/41 (14.6%), 10/41 (24.4%), and 13/29 (44.8%) at weeks 48, 96, and 144, respectively. Neither the S202I nor the M250V/I/L substitution was detected in this population.

(100% for both M180 and V204) at baseline and ETVr substitutions (S202G, 85.7%; T184SCGA/ILFM, 57.1%) during 3-year ETV treatment. Representative cases with BTH during ETV therapy are shown in the supplemental material.

Pretreatment status (LVD refractory or naïve) was analyzed in relation to the occurrence of ETVr, BT, and BTH during the 144-week course of ETV therapy. The log rank analysis of pretreatment variables showed that prior refractoriness to LVD was associated with more rapid occurrence of ETVr (Fig. 2), BT, and BTH ($P \leq 0.001$, $P < 0.001$, and $P = 0.0039$, respectively). Additionally, among the 67 patients receiving 3-year ETV treatment, BT occurred in 10 of 52 (19.2%) patients with HBV genotype C and 1 of 8 (12.5%) with genotype B, whereas no BT was observed in patients with genotypes A, D, and E. No significant association between BT and HBV genotypes was found.

Baseline characteristics and factors associated with viral breakthrough during 3-year ETV therapy. When non-BT and BT groups within the 67 patients treated with ETV for 144 weeks were compared, no significant baseline differences were observed in mean age, gender, serum ALT levels, HBV DNA, or HBeAg status (Table 1), while 2-$\log_{10}$-unit reductions in HBV DNA levels or undetectable (<2.6) HBV DNA levels at the end of year 1 were significantly higher in the non-BT group (Table 5). Interestingly, the proportion of patients refractory to LVD with both the L180M and M204V substitutions at baseline ($P < 0.001$) and the incidence of S202G or T184SCGA/ILMF substitutions during the 3-year ETV treatment ($P < 0.001$) were significantly higher in the BT group.

None of the BT cases reached undetectable HBV DNA levels at the end of the first year of ETV treatment (BT, 0%, versus non-BT, 58.9%), but all were refractory to LVD (BT, 100%, versus non-BT, 32.1%) and had the L180M substitution

TABLE 4. Detection of ETVr mutations by HBV DR v3 and direct sequencing at weeks 0, 24, 48, 72, 96, and 144

Case	INNO-LiPA detection		Direct-sequencing detection	
	Wk	ETVr	Wk	ETVr
J20	96	ILFM184 + G202	96	L184 (L184 and G202 detected at wk 144)
J27	144	SCGA184 + T184	144	S184 + T184
J30	48	G202	96	G202 (ND[b] at wk 48)
J39	72	SCGA/ILFM184 + G202	96	I184, G202 (ND at wk 72)
J40	−8	G202	0	G202 (ND at wk −8)
J33	96	G202	144	G202 (ND at wk 96)
J19	24	G202 + C202	48	G202 (ND at wk 24)
J22	144	S202 + G202	144	S202
J28	0	SCGA184, V233	0	A184, V233
J37[a]	48	S202 + G202	48	S202
U42	80	SCGA184 + T184	80	T184 + A184
U72	144	T184 + SCGA184, S202 + G202	144	T184 + A184, S202
H55	48	T184 + ILFM184	48	T184

[a] Switched from ETV to LVD plus ADV therapy at week 60.
[b] ND, ETVr was not detected.

at baseline (BT, 100%, versus non-BT, 28.6%) (Table 5). For other factors, additional analysis showed that ETVr substitutions (i.e., S202G, T184SCGA/ILFM, and M204V) were strongly associated with BT during the 3-year ETV treatment (OR, 146.67 [95% CI, 13.55 to 1,587.24], 96.25 [95% CI, 9.38 to 987.41], and 10.91 [95% CI, 4.72 to 354.28]) (Table 5).

After adjustment for age, gender, baseline HBV DNA, and reduction in HBV DNA, we found that ETVr substitutions (i.e., T184SCGA/ILFM and S202G) significantly increased the risk of BT among patients with LVDr (OR, 141.12 [95% CI, 6.94 to 2,870.20] and 201.25 [95% CI, 11.22 to 3,608.65], respectively).

Mechanism of ETVr assessed by 3D docking simulation. Modeling of the DNA binding cleft of HBV RT by docking simulation indicated that ETVr substitutions (T184L and S202G), which are located in the palm, were found to change the direction of the D205 residue (YMDD domain) and to narrow the binding pocket in comparison with the wild type and LVDr substitutions (M204V and L180M) (Fig. 3). The results of docking simulation showed that ETVr substitutions (T184L and S202G) plus LVDr substitutions (M204V and L180M) have significantly longer minimal distances between the molecular surfaces of the protein and the drug (2.2 Å and 2.1 Å) and higher potential energy (−118 and −99.8 Kcal/mol [smaller absolute values have a minus sign]) for ETV-TP than for the wild type (1.3 Å; −178 Kcal/mol) and LVDr substitutions (1.5 Å; −141 Kcal/mol) (Table 6). Since binding at higher potential energy creates a less stable structure, the deoxyribonucleotide triphosphate (dNTP)-binding domains of the ETVr substitutions plus the LVDr substitutions in HBV RT have

TABLE 5. ORs and 95% CIs of BT according to baseline characteristics among 67 patients treated with ETV for 3 years

Characteristic	Non-BT (n = 56)[a]	BT (n = 11)[a]	P for difference	Contrast	OR	95% CI
Age (mean)	45.4 ± 8.1	45.4 ± 9.4	0.932	1-yr increase	1	0.92–1.08
Male (%)	71.4	81.8	0.481	Male vs. female	1.8	0.35–9.26
ALT (mean)	115.9 ± 105.6	126.2 ± 127.3	0.832	1-U increase	1	0.99–1.00
HBeAg (%)	66.1	81.8	0.307	Positive vs. negative	2.31	0.45–11.78
HBV-DNA						
Level (mean)	6.8 ± 1.6	7.1 ± 0.8	0.959	1-U increase	1.16	0.72–1.89
2-log-unit reduction at 1 yr (%)	98.2	63.6	<0.001	With vs. without	0.03	0.00–0.33
DNA <2.6 at 1 yr (%)	58.9	0.0	<0.001	With vs. without	NA[b]	
LVD refractory (%)	32.1	100.0	<0.001	With vs. without	NA	
Amino acid substitutions at baseline						
V80 (%)	3.6	18.2	0.063	With vs. without	6	0.74–48.17
I80 (%)	17.9	36.4	0.171	With vs. without	2.63	0.64–10.72
L173 (%)	3.6	18.2	0.063	With vs. without	6	0.75–48.18
M180 (%)	28.6	100.0	<0.001	With vs. without	NA	
V204 (%)	19.6	90.9	<0.001	With vs. without	10.91	4.72–354.28
I204 (%)	23.2	36.4	0.363	With vs. without	1.89	0.48–7.49
Amino acid substitutions during ETV therapy (3 yr)						
SCGA/ILFM184 (%)	1.8	72.7	<0.001	With vs. without	96.25	9.38–987.41
G202 (%)	1.8	63.6	<0.001	With vs. without	146.67	13.55–1,587.24

[a] Values are means ± standard deviations.
[b] NA, not applicable.

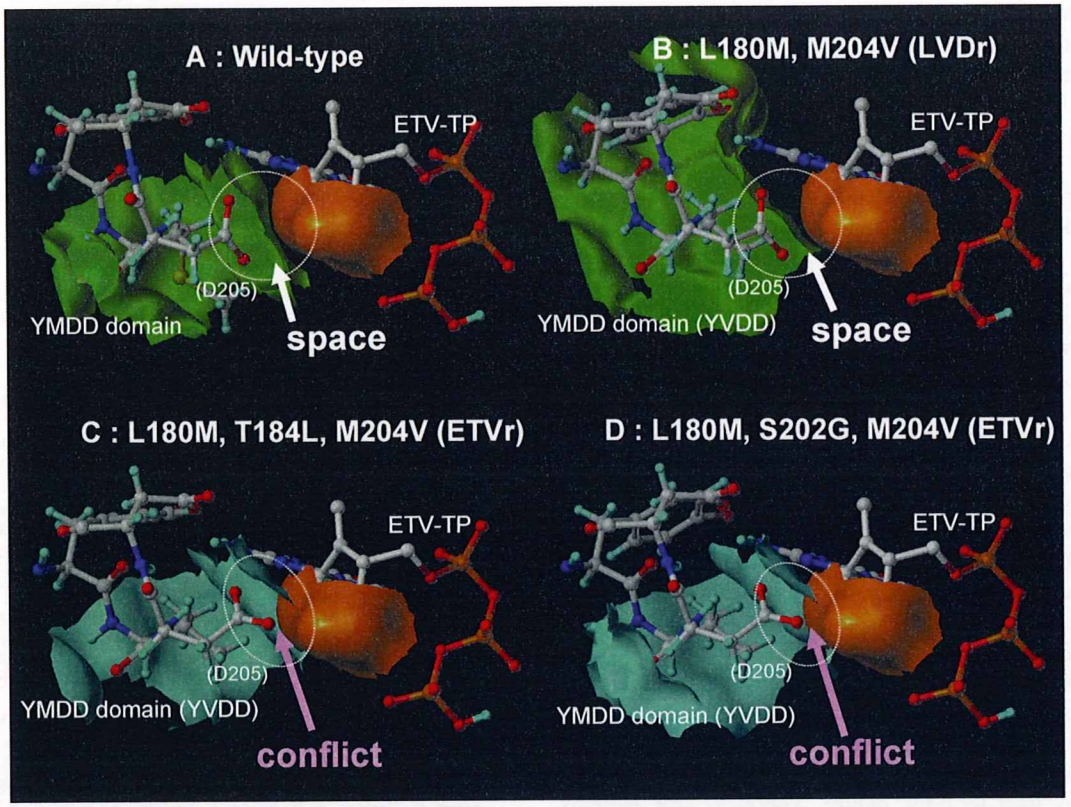


FIG. 3. 3D structures of the dNTP-binding domains of HBV RT of the wild type (A), an LVDr substitution (B), and ETVr substitutions (C and D). The molecular surfaces of the wild type and the LVDr mutant are drawn in green, those of LVDr plus ETVr mutants are drawn in blue, and that of ETV-TP is drawn in orange.

lower binding affinity for ETV-TP than the wild type. Molecular docking simulation in the present study showed that the L180M, M204V, S202G, and T184L substitutions can lessen the affinity of ETV-TP for HBV RT by heightening the potential energy between them, suggesting that S202G and T184L substitutions, in addition to M204V in the YMDD motif and L180M in domain C, could affect the initial polymerase binding of dNTP analog inhibitors.

DISCUSSION

Based on the combination of clinical observations and 3D docking simulation, this is the first report to suggest the mechanism by which ETVr substitutions (T184SCGA/ILFM and S202G, but not S202C), in addition to LVDr (L180M and M204V, but not M204I), can induce BT during ETV therapy.

TABLE 6. Minimal distances and binding potentials between ETV and the HBV RT domain in the wild type, one LVDr mutant, and 2 ETVr mutants

Strain	distance (Å)	Binding potential (GOLD score [Kcal/mol])	Reference (Fig. 3)
Wild type	1.3	−178.4	A
L180M, 204V	1.5	−141.3	B
L180M, T184L, 204V	2.2	−117.9	C
L180M, S202G, 204V	2.1	−99.8	D

First, an assessment of virological and biochemical events during a 3-year ETV treatment course showed that ETVr substitutions were absent among treatment-naïve patients but were detected in 44.8% of patients who were refractory to LVD during the preceding treatment period. Evidence of BT during ETV therapy was observed in 26.8% of LVD-refractory patients between weeks 60 and 144 of treatment. All 11 of the BT cases had both L180M and M204V/I substitutions at baseline (LVD refractory), as well as additional substitutions, such as T184 and/or S202G (not S202I/C), during the 3-year ETV treatment period.

Statistically significant risk factors for BT were the presence of LVDr (L180M and M204V) at baseline, detection of ETVr (S202G and T184SCGA/ILFM substitutions) during ETV treatment, and undetectable HBV DNA (<2.6) or more than a 2-$\log_{10}$-unit reduction in HBV DNA levels during the first year of ETV treatment. Detection of T184SCGA/ILFM and S202G was significantly associated with BT independent of age, gender, and LVDr (M204V and/or L180M) at baseline or nondetection or reduction in HBV DNA at the first year of treatment, indicating that these substitutions could be used as predictive markers for BT.

The mechanism by which combinations of ETVr (S202G and T184 SCGA/ILFM) and LVDr (L180M and M204V) can induce BT during ETV therapy is largely unknown. Note that T184L and S202G residues are located within domain B and domain C of the RT/polymerase, respectively, as well as

L180M and M204V. The modeling of HBV RT indicated that the combination changed the direction of the D205 residue (YMDD domain) and narrowed the dNTP-binding pocket in comparison with the wild type and LVDr substitutions (M204V and L180M) (Fig. 3). The results of docking simulation of HBV RT and ETV-TP showed that the ETVr (184L and S202G) plus LVDr (L180M and M204V) substitutions had significantly longer minimal distances for ETV-TP and steric conflict with the D205 residue (Fig. 3 and Table 6). These docking simulation results suggest that nucleotide analogs that have the exocyclic alkene moiety of ETV-TP replaced by a smaller atom may retain activity against ETV-resistant mutants. Differences in the mode of binding of nucleotide inhibitors to the dNTP-binding pocket of HBV polymerase, as predicted from the current modeling studies, may account for the complementary drug resistance profiles seen for different nucleotide analogs. Interestingly, a previous *in vitro* study showed that ETVr substitutions (S202I and T184G), in addition to LVDr (L180M and M204V), were associated with a >1,100-fold decrease in susceptibility to ET (20). Collectively, these data indicate that nucleoside-naïve patients treated with ETV were less likely to become resistant to ETV.

In an *in vitro* assay, the rtA181T/V clinical-isolate genome from patients refractory to LVD/ADV induced a decrease in susceptibility to LVD, ADV, and, to a lesser extent, TDF, but sensitivity to ETV remained (22). LVDr selected by LVD exposure may lead to ETV failure. Therefore, for patients refractory to LVD/ADV, a combination of emtricitabine/TDF (10) might be an effective option. Furthermore, since sequential antiviral therapy leads to the selection of multidrug-resistant HBV and fitness or maximal viral resistance (25), combination therapy using a nucleoside together with a nucleotide analog, such as emtricitabine/TDF (10), ADV/LVD, ADV/ETV, ADV/telbivudine, or TDF, would be a more appropriate treatment strategy for patients with the LVDr substitution.

Based on HBV DR v3, T184SCGA/ILMF and S202G substitutions were present at baseline in 4.8% of patients and were detected in 14.6%, 24.4%, and 44.8% during 48, 96, and 144 weeks, respectively, of ETV therapy (Fig. 2). The prevalence of ETVr in our cohort seems to be higher than that reported in previous studies, based on assessment of ETV treatment at weeks 48, 96, 144, 192, and 240 using direct sequencing, where ETVr emerged in 6%, 15%, 36%, 47%, and 51% of LVD-refractory patients, respectively (21). The differences might be attributable to the tools used to detect HBV DNA substitutions associated with drug resistance, which differed between the studies. HBV DR v.3 and v.2 performed better than direct sequencing, and monitoring of the nucleoside mutations by HBV DR v.3 and v.2 in patients before and during ETV therapy was good for selecting effective therapeutic strategies and new combination therapies.

In conclusion, the combination of clinical observations and 3D docking simulation in the present study indicated that the low binding affinity of ETV-TP for the dNTP-binding domains of HBV RT by the ETVr plus LVDr substitutions could induce BT and provides the mechanistic foundations for a mechanism of inhibition of ETV against HBV. This modeling would be useful for designing new antiviral drugs.

ACKNOWLEDGMENTS

This work was supported in part by a grant-in-aid from the Ministry of Health, Labor, and Welfare of Japan and a grant-in-aid from the Ministry of Education, Culture, Sports, and Science.

We thank Kenichi Fukai, Graduate School of Medicine, Chiba University, Chiba, Japan; Tatsuya Ide, Department of Internal Medicine, Kurume University School of Medicine, Kurume, Japan; Debbie Hana Yi, Department of Emergency Medicine, New York-Presbyterian Hospital Columbia/Cornell, New York, NY; and Robert G. Gish, California Pacific Medical Center, San Francisco, CA, for their help throughout this work.

We have no conflicts of interest to disclose, except for M. F. Yuen and C. L. Lai, who received research support from BMS.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Baldick, C. J., B. J. Eggers, J. Fang, S. M. Levine, K. A. Pokornowski, R. E. Rose, C. F. Yu, D. J. Tenney, and R. J. Colonno.** 2008. Hepatitis B virus quasispecies susceptibility to entecavir confirms the relationship between genotypic resistance and patient virologic response. J. Hepatol. **48:**895–902.
3. **Baldick, C. J., D. J. Tenney, C. E. Mazzucco, B. J. Eggers, R. E. Rose, K. A. Pokornowski, C. F. Yu, and R. J. Colonno.** 2008. Comprehensive evaluation of hepatitis B virus reverse transcriptase substitutions associated with entecavir resistance. Hepatology **47:**1473–1482.
4. **Brautigan, D. L., M. Brown, S. Grindrod, G. Chinigo, A. Kruszewski, S. M. Lukasik, J. H. Bushweller, M. Horal, S. Keller, S. Tamura, D. B. Heimark, J. Price, A. N. Larner, and J. Larner.** 2005. Allosteric activation of protein phosphatase 2C by D-chiro-inositol-galactosamine, a putative mediator mimetic of insulin action. Biochemistry **44:**11067–73.
5. **Chan, H. L., A. Y. Hui, M. L. Wong, A. M. Tse, L. C. Hung, V. W. Wong, and J. J. Sung.** 2004. Genotype C hepatitis B virus infection is associated with an increased risk of hepatocellular carcinoma. Gut **53:**1494–1498.
6. **Chang, T. T., R. G. Gish, S. J. Hadziyannis, J. Cianciara, M. Rizzetto, E. R. Schiff, G. Pastore, B. R. Bacon, T. Poynard, S. Joshi, K. S. Klesczewski, A. Thiry, R. E. Rose, R. J. Colonno, R. G. Hindes, and the BEHoLD Study Group.** 2005. A dose-ranging study of the efficacy and tolerability of entecavir in lamivudine-refractory chronic hepatitis B patients. Gastroenterology **129:**1198–1209.
7. **Degertekin, B., M. Hussain, J. Tan, K. Oberhelman, and A. S. Lok.** 2009. Sensitivity and accuracy of an updated line probe assay (HBV DR v. 3) in detecting mutations associated with hepatitis B antiviral resistance. J. Hepatol. **50:**42–48.
8. **Huang, H., R. Chopra, G. L. Verdine, and S. C. Harrison.** 1998. Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: implications for drug resistance. Science **282:**1669–1675.
9. **Jain, A. N.** 2003. Surflex: fully automatic flexible molecular docking using a molecular similarity-based search engine. J. Med. Chem. **46:**499–511.
10. **Keeffe, E. B., D. T. Dieterich, S. H. Han, I. M. Jacobson, P. Martin, E. R. Schiff, and H. Tobias.** 2008. A treatment algorithm for the management of chronic hepatitis B virus infection in the United States: 2008 update. Clin. Gastroenterol. Hepatol. **6:**1315–1341.
11. **Lai, C. L., D. Shouval, A. S. Lok, T. T. Chang, H. Cheinquer, Z. Goodman, D. DeHertogh, R. Wilber, R. C. Zink, A. Cross, R. Colonno, L. Fernandes, and the BEHoLD AI463027 Study Group.** 2006. Entecavir versus lamivudine for patients with HBeAg-negative chronic hepatitis B. N. Engl. J. Med. **354:**1011–1020.
12. **Lee, W. M.** 1997. Hepatitis B virus infection. N. Engl. J. Med. **337:**1733–1745.
13. **Marcellin, P., T. Asselah, and N. Boyer.** 2005. Treatment of chronic hepatitis B. Rev. Prat. **55:**624–632.
14. **Miyakawa, Y., and M. Mizokami.** 2003. Classifying hepatitis B virus genotypes. Intervirology **46:**329–338.
15. **Ono, S. K., N. Kato, Y. Shiratori, J. Kato, T. Goto, R. F. Schinazi, F. J. Carrilho, and M. Omata.** 2001. The polymerase L528M mutation cooperates with nucleotide binding-site mutations, increasing hepatitis B virus replication and drug resistance. J. Clin. Invest. **107:**449–455.
16. **Orito, E., M. Mizokami, H. Sakugawa, K. Michitaka, K. Ishikawa, T. Ichida, T. Okanoue, H. Yotsuyanagi, and S. Iino.** 2001. A case-control study for clinical and molecular biological differences between hepatitis B viruses of genotypes B and C. Japan HBV Genotype Research Group. Hepatology **33:**218–223.
17. **Petrey, D., X. Xiang, C. L. Tang, L. Xie, M. Gimpelev, T. Mitors, C. S. Soto, S. Goldsmith-Fischman, A. Kernytsky, A. Schlessinger, I. Y. Y. Koh, E. Alexov, and B. Honig.** 2003. Using multiple structure alignments, fast model building, and energetic analysis in fold recognition and homology modeling. Proteins **53:**430–435.
18. **Sherman, M., C. Yurdaydin, J. Sollano, M. Silva, Y. F. Liaw, J. Cianciara, A. Boron-Kaczmarska, P. Martin, Z. Goodman, R. Colonno, A. Cross, G. Denisky, B. Kreter, R. Hindes, and the AI463026 BEHoLD Study Group.** 2006.

Entecavir for treatment of lamivudine-refractory, HBeAg-positive chronic hepatitis B. Gastroenterology **130:**2039–2049.

19. **Szczesny, P., G. Wieczorek, and P. Zielenkiewicz.** 2005. MOFOID—not only the protein modeling server. Acta Biochim. Pol. **52:**267–269.
20. **Tenney, D. J., R. E. Rose, C. J. Baldick, S. M. Levine, K. A. Pokornowski, A. W. Walsh, J. Fang, C. F. Yu, S. Zhang, C. E. Mazzucco, B. Eggers, M. Hsu, M. J. Plym, P. Poundstone, J. Yang, and R. J. Colonno.** 2007. Two-year assessment of entecavir resistance in lamivudine-refractory hepatitis B virus patients reveals different clinical outcomes depending on the resistance substitutions present. Antimicrob. Agents Chemother. **51:**902–911.
21. **Tenney, D. J., R. E. Rose, C. J. Baldick, K. A. Pokornowski, B. J. Eggers, J. Fang, M. J. Wichroski, D. Xu, J. Yang, R. B. Wilber, and R. J. Colonno.** 2009. Long-term monitoring shows hepatitis B virus resistance to entecavir in nucleoside-naive patients is rare through 5 years of therapy. Hepatology **49:**1503–1514.
22. **Villet, S., C. Pichoud, G. Billioud, L. Barraud, S. Durantel, C. Trepo, and F. Zoulim.** 2008. Impact of hepatitis B virus rtA181V/T mutants on hepatitis B treatment failure. J. Hepatol. **48:**747–755.
23. **Xiang, Z., and B. Honig.** 2001. Extending the accuracy limits of prediction for side chain conformations. J. Mol. Biol. **311:**421–430.
24. **Xiang, Z., C. Soto, and B. Honig.** 2002. Evaluating conformational free energies: the colony energy and its application to the problem of loop prediction. Proc. Natl. Acad. Sci. U. S. A. **99:**7432–7437.
25. **Yim, H. J., M. Hussain, Y. Liu, S. N. Wong, S. K. Fung, and A. S. Lok.** 2006. Evolution of multi-drug resistant hepatitis B virus during sequential therapy. Hepatology **44:**703–712.
26. **Yuen, M. F., W. K. Seto, D. H. Chow, K. Tsui, D. K. Wong, V. W. Ngai, B. C. Wong, J. Fung, J. C. Yuen, and C. L. Lai.** 2007. Long-term lamivudine therapy reduces the risk of long-term complications of chronic hepatitis B infection even in patients without advanced disease. Antivir. Ther. **12:**1295–1303.

JOURNAL OF VIROLOGY, Oct. 2009, p. 10538–10547 Vol. 83, No. 20
0022-538X/09/$08.00+0 doi:10.1128/JVI.00462-09

A Genetic Variant of Hepatitis B Virus Divergent from Known Human and Ape Genotypes Isolated from a Japanese Patient and Provisionally Assigned to New Genotype J▽†

Kanako Tatematsu,[1] Yasuhito Tanaka,[1]* Fuat Kurbanov,[1] Fuminaka Sugauchi,[2] Shuhei Mano,[3] Tatsuji Maeshiro,[4] Tomokuni Nakayoshi,[5] Moriaki Wakuta,[6] Yuzo Miyakawa,[7] and Masashi Mizokami[1,8]

Department of Clinical Molecular Informative Medicine[1] and Department of Gastroenterology and Metabolism,[2] Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan; Nagoya City University Graduate School of Natural Sciences, Nagoya, Japan[3]; Control and Prevention of Infectious Diseases, Department of Medicine and Therapeutics, Faculty of Medicine, University of the Ryukyus, Okinawa, Japan[4]; Heart Life Hospital, Okinawa, Japan[5]; Wakusan Clinic. Okinawa, Japan[6]; Miyakawa Memorial Research Foundation, Tokyo, Japan[7]; and Research Center for Hepatitis and Immunology, International Medical Center of Japan Kohnodai Hospital, Chiba, Japan[8]

Received 5 March 2009/Accepted 24 July 2009

Hepatitis B virus (HBV) of a novel genotype (J) was recovered from an 88-year-old Japanese patient with hepatocellular carcinoma who had a history of residing in Borneo during the World War II. It was divergent from eight human (A to H) and four ape (chimpanzee, gorilla, gibbon, and orangutan) HBV genotypes, as well as from a recently proposed ninth human genotype I, by 9.9 to 16.5% of the entire genomic sequence and did not have evidence of recombination with any of the nine human genotypes and four nonhuman genotypes. Based on a comparison of the entire nucleotide sequence against 1,440 HBV isolates reported, HBV/J was nearest to the gibbon and orangutan genotypes (mean divergences of 10.9 and 10.7%, respectively). Based on a comparison of four open reading frames, HBV/J was closer to gibbon/orangutan genotypes than to human genotypes in the P and large S genes and closest to Australian aboriginal strains (HBV/C4) and orangutan-derived strains in the S gene, whereas it was closer to human than ape genotypes in the C gene. HBV/J shared a deletion of 33 nucleotides at the start of preS1 region with C4 and gibbon genotypes, had an S-gene sequence similar to that of C4, and expressed the *ayw* subtype. Efficient infection, replication, and antigen expression by HBV/J were experimentally established in two chimeric mice with the liver repopulated for human hepatocytes. The HBV DNA sequence recovered from infected mice was identical to that in the inoculum. Since HBV/J is positioned phylogenetically in between human and ape genotypes, it may help to trace the origin of HBV and merits further epidemiological surveys.

Worldwide, an estimated 400 million people are infected with hepatitis B virus (HBV) persistently, of whom three quarters live in the Southeast and Far East Asia, and one million die of decompensated cirrhosis and/or hepatocellular carcinoma (HCC) annually (8, 15). HBV is the smallest animal DNA virus and has a genome made of approximately 3,200 nucleotides (nt) that contains four open reading frames for P, C, S, and X genes; they code for DNA polymerase/reverse-transcriptase, core protein, surface protein, and X protein, respectively (49). The S gene is divided into preS1 and preS2 regions and the small S gene, and the C gene splits into PreC and C.

Eight genotypes of HBV have been recognized by a sequence divergence of >8% in the entire genome and named by capital alphabet letters (A to H) in the order of discovery (3, 26, 29, 42). HBV genotypes are further classified into subgenotypes, such as B1/Bj and B2-5/Ba (44), as well as C1/Cs, C2/Ce, and C3-5 (36). A systematic nomenclature is proposed for designating HBV subgenotypes using Arabic numbers, such as A1, A2, and A3 (25). HBV genotypes have distinct geographical distribution (16, 23). Genotype A is prevalent in Africa, Europe and India, genotypes B and C are common in Asia, and genotype E is common in sub-Saharan Africa. Genotypes F and H are restricted to Central and South American continents, whereas genotype D is distributed all over the world. HBV genotypes have clinical application, and they influence severity and progression of liver disease and the response to antiviral therapies. Previous reports indicate that HCC is more frequent in the patients infected with genotype C than B (7, 47), and interferon is more effective in those infected with genotype B than C in Asia and more effective in those infected with genotype A than D in Europe (18, 34, 51).

Recently, a ninth genotype (I) was tentatively proposed for HBV strains detected in Laos (31). These strains are phylogenetically similar to aberrant Vietnamese strains that display complex recombination over the genome (10). In the present study, an HBV isolate was recovered from a Japanese patient with HCC, who was involved in military actions in Borneo during the World War II. The isolated strain was compared against eight human (A to H) and four ape (chimpanzee, gorilla, gibbon, and orangutan) genotypes and was provisionally designated genotype J. The new genotype was assigned based on a sequence diver-

* Corresponding author. Mailing address: Department of Clinical Molecular Informative Medicine, Nagoya, City University Graduate School of Medical Sciences, Kawasumi, Mizuho, Nagoya 467-8601, Japan. Phone: (81) 52-853-8292. Fax: (81) 52-842-0021. E-mail: ytanaka@med.nagoya-cu.ac.jp.

† Supplemental material for this article may be found at http://jvi.asm.org/.

▽ Published ahead of print on 29 July 2009.

TABLE 1. Nucleotide divergence in the full-genome sequence estimated from pairwise comparison between the Ryukyu 34 strain of a provisional genotype J and 1,440 HBV strains from the database entered by September 2008

Genotype	No. of strains	Divergence (%)		
		Range	Mean	SD
A	202	12.1–15.9	13.0	0.4
B	309	11.1–13.6	11.9	0.5
C	396	11.2–13.1	11.9	0.5
D	264	12.6–15.0	13.4	0.2
E	90	12.3–13.4	12.7	0.3
F	56	15.2–16.5	15.6	0.2
G	23	12.8–14.6	13.7	0.3
H	21	15.4–16.3	15.7	0.3
I	16	11.4–12.0	11.7	0.2
Chimpanzee	14	11.6–12.7	12.1	0.3
Gorilla	1	12.2		
Gibbon	34	9.9–11.7	10.9	0.5
Orangutan	12	10.4–11.2	10.7	0.4
Woolly monkey	2	27.2–27.4	27.3	0.1

gence of 10.7 to 15.7% from other genotypes, a unique phylogenetic position between human and ape genotypes, and the absence of strong evidence of recombination.

MATERIALS AND METHODS

Patient. A Japanese man, 88 years old, developed HCC in 2006. He had a history of residing in Borneo during the World War II. No HBV infections were recorded in his family members. In October 1996, he was diagnosed with chronic hepatitis B. Hepatitis B surface antigen (HBsAg) was detected in serum, and the aspartate aminotransaminase and alanine aminotransferase levels were elevated to 83 and 73 U/liter, respectively (normal levels, <30 U/liter for both). Thereafter, the transaminase levels were normalized, and he had been monitored as an asymptomatic HBV carrier. In August 2000, the level of a tumor marker (des-γ-carboxy prothrombin) was elevated to 52 mAU/ml (normal, <40 mAU/ml), while another tumor marker (alpha-fetoprotein) remained within normal range (<10 ng/ml) as alanine aminotransferases. In October 2006, a tumor (4.3 by 4.1 cm) was detected in the liver by ultrasonography, and he received treatment with transarterial embolization. Des-γ-carboxy prothrombin was elevated to 419 mAU/ml, while the aminotransferase levels remained within normal limits. Hepatitis B e antigen (HBeAg) was negative, and the corresponding antibody (anti-HBe) was detected in his serum. The subtype of HBsAg in this serum was *ayw*.

HBV DNA was extracted from his serum specimen obtained in 2006, and the full-length genome sequence was determined for phylogenetic and biological analyses. An informed consent had been obtained from the patient, and the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee.

Markers of HBV infection. HBeAg and anti-HBe were determined by enzyme-linked immunosorbent assay (ELISA) with commercial kits (HBeAg EIA; Institute of Immunology, Tokyo, Japan), and subtypes of HBsAg by ELISA with commercial kits (HBsAg Subtype EIA; Institute of Immunology). Hepatitis B core-related antigen (HBcrAg) was determined by chemiluminescence enzyme immunoassay (13). The method allows more sensitive detection of core protein and, as was shown in previous studies, HBcrAg levels reflect HBV DNA loads and well correlate with intrahepatic covalently closed circular DNA (cccDNA) levels. The measurement of serum HBcrAg is a useful noninvasive tool for monitoring intrahepatic HBV viral status (52). HBV DNA was quantified by the S gene-targeted real-time detection PCR with a sensitivity of 100 copies/ml (equivalent to 20 IU/ml) (1). However, due to small volumes of sera available from the challenged mice, HBV DNA was extracted from 10-fold-diluted specimens, resulting in reduced assay sensitivity in the present study (1,000 copies/ml [200 IU/ml]).

Determination of the complete nucleotide sequence of HBV/J isolate. HBV DNA was extracted by using the QIAamp DNA blood kit (Qiagen, GmbH, Hilden, Germany) from 100 μl of serum that had been stored at −80°C. The complete genome sequence of an HBV/J isolate recovered from the patient was determined by the strategy previously reported (43). In brief, two sets of primers were designed to amplify overlapping fragments (A and B) covering the entire HBV genome (stat not shown). Nested PCR was carried out for 35 cycles (95°C, 30 s; 57°C, 30 s; and 72°C, 2 min) using TaKaRa LA *Taq* polymerase (Takara Biochemicals, Kyoto, Japan). Amplified fragments were inserted into the pGEM-T Easy vector (Promega, Madison, WI), and cloned in DH5a cells (Toyobo, Osaka, Japan). Obtained HBV DNA clones were confirmed to have the sequence identical to the major-clone consensus sequence determined directly on PCR products by Prism BigDye (Applied Biosystems, Foster City, CA) in the ABI 3100 automated sequencer.

Phylogenetic analysis. Full-length sequences of HBV isolates were aligned with use of the CLUSTAL W software program (48) (available at www.ebi.ac.uk), and the alignment was confirmed by visual inspection. Genetic distances were estimated by the six-parameter method, and phylogenetic trees were constructed with the neighbor-joining method (35). To confirm the reliability of phylogenetic trees, bootstrap resampling and reconstruction were carried out 1,000 times using the program

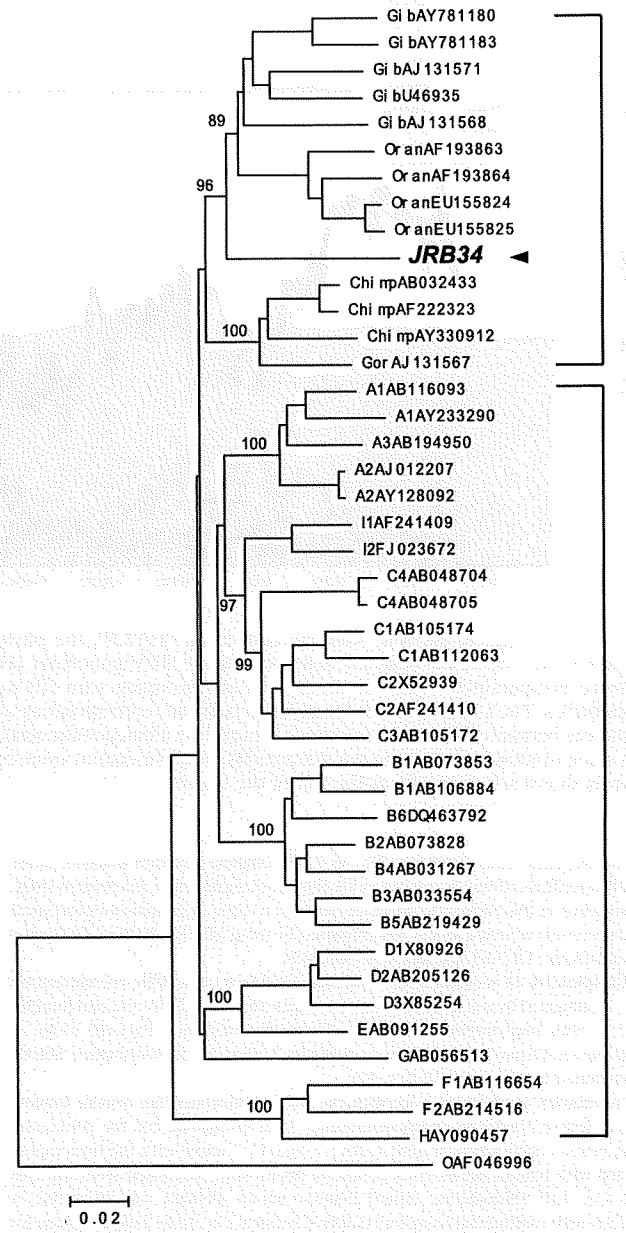


FIG. 1. Phylogenetic tree constructed on the entire genome sequences of 44 HBV isolates representing four ape and eight human genotypes. A woolly monkey HBV isolate serves as an outgroup. The HBV/J isolate (JRB34) is indicated by an arrowhead, and the genetic distance is indicated by a bar below.

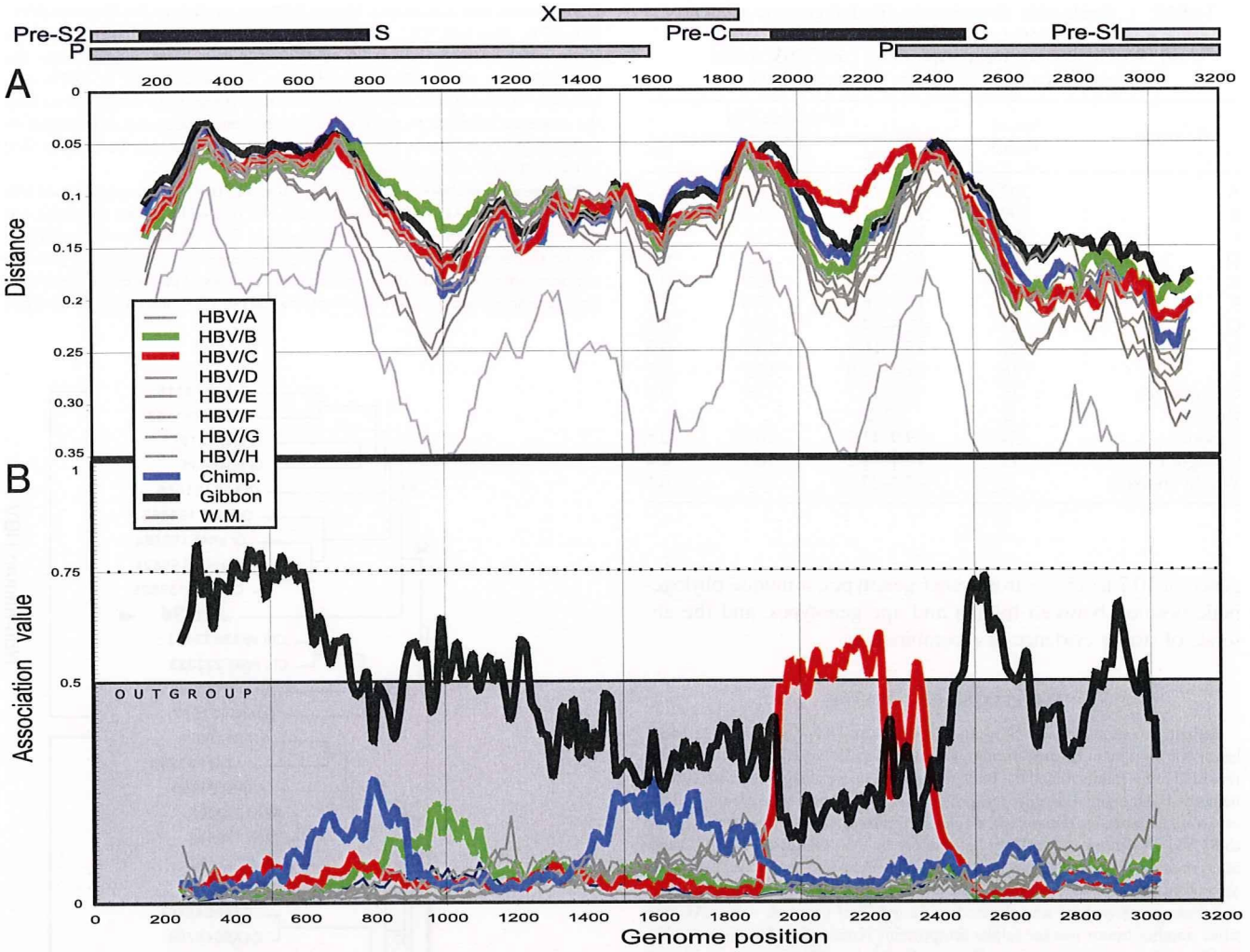


FIG. 2. Complete genome scanning carried by PHYLIP, the phylogeny inference package implemented in the Simmonic software, for the JRB34 strain versus 228 selected nonrecombinant HBV genotypes (HBV/Ba and HBV/I not included) reference strains grouped by genotype. Kimura two-parameter distance model (A) and grouping scan (B) were determined with a 300-nt size window sliding by an increment of 15 nucleotides. The *x* axis indicates the genome position (corresponding to the midpoint of the scanning fragment), and the *y* axis indicates the mean distances between JRB34 and reference groups (A). Phylogenetic association (*y* axis) was evaluated throughout entire HBV genome (*x* axis) with the same window and step size parameters (B). The association value below 0.5 was considered to represent an outgroup. The open reading frame map is shown schematically at the top of the figure.

of the Hepatitis Virus Database (39). All 1,440 complete genomes available in the DDBJ/GenBank served as references for the initial alignment in the present study. Divergence in the nucleotide sequence between a strain of provisional genotype J and previously reported strains was estimated by using MEGALIGN v.6.00 (Lazergene package; DNASTAR, Inc., Madison, WI).

Examination of recombination evidence. Evidence of possible recombination was investigated by using the software packages Simmonic 2005 v1.6 and SimPlot v3.5.1, both implementing PHYLIP (Phylogeny Inference Package v3.68; J. Felsenstein, Department of Genome Sciences, University of Washington, Seattle [distributed by the authors]) (19, 40).

Inoculation of chimeric mice with the liver repopulated for human hepatocytes. Severe combined immunodeficiency mice transgenic for the urokinase-type plasminogen activator gene (uPA$^{+/+}$/SCID$^{+/+}$ mice) with the liver repopulated with human hepatocytes (chimeric mice) were purchased from Phoenix Bio Co., Ltd. (Hiroshima, Japan). Human serum albumin was measured by ELISA with commercial assay kits (Eiken Chemical Co., Ltd., Tokyo, Japan) for estimating the extent of repopulation. The research complied with all relevant federal guidelines and institutional policies.

Immunofluorescence. Freshly prepared liver tissues were snap-frozen in isopentane precooled in liquid nitrogen. Frozen specimens were cut at 5 to 6 μm by cryostat, mounted on glass slides, air dried, and fixed in 100% acetone at room temperature for 10 min. Sections were blocked with antibody diluent (Dako, Tokyo, Japan) and stained for hepatitis B core antigen (HBcAg). They were incubated with rabbit anti-HBc (Dako) at room temperature for 1 h, washed in phosphate-buffered saline, and then incubated with goat anti-rabbit immunoglobulin G conjugated with Cy3 (Chemicon International, Inc., Temecula, CA) or goat anti-human albumin antibody labeled with fluorescein isothiocyanate (Bethyl Laboratories, Inc., Montgomery, TX). Sections were washed with phosphate-buffered saline and observed in a fluorescence microscope (Eclipse E800M; Nikon, Tokyo, Japan).

Nucleotide sequence accession numbers. The nucleotide sequence data reported in the present study will appear in the DDBJ/EMBL/GenBank databases under accession no. AB486012.

RESULTS

Composition of the HBV genome of genotype J. HBV DNA was extracted from serum of a patient with HCC. It was named JRB34 ("J" for Japanese; "R" after the southernmost island [Ryukyu] where the patient has spent most of his life now

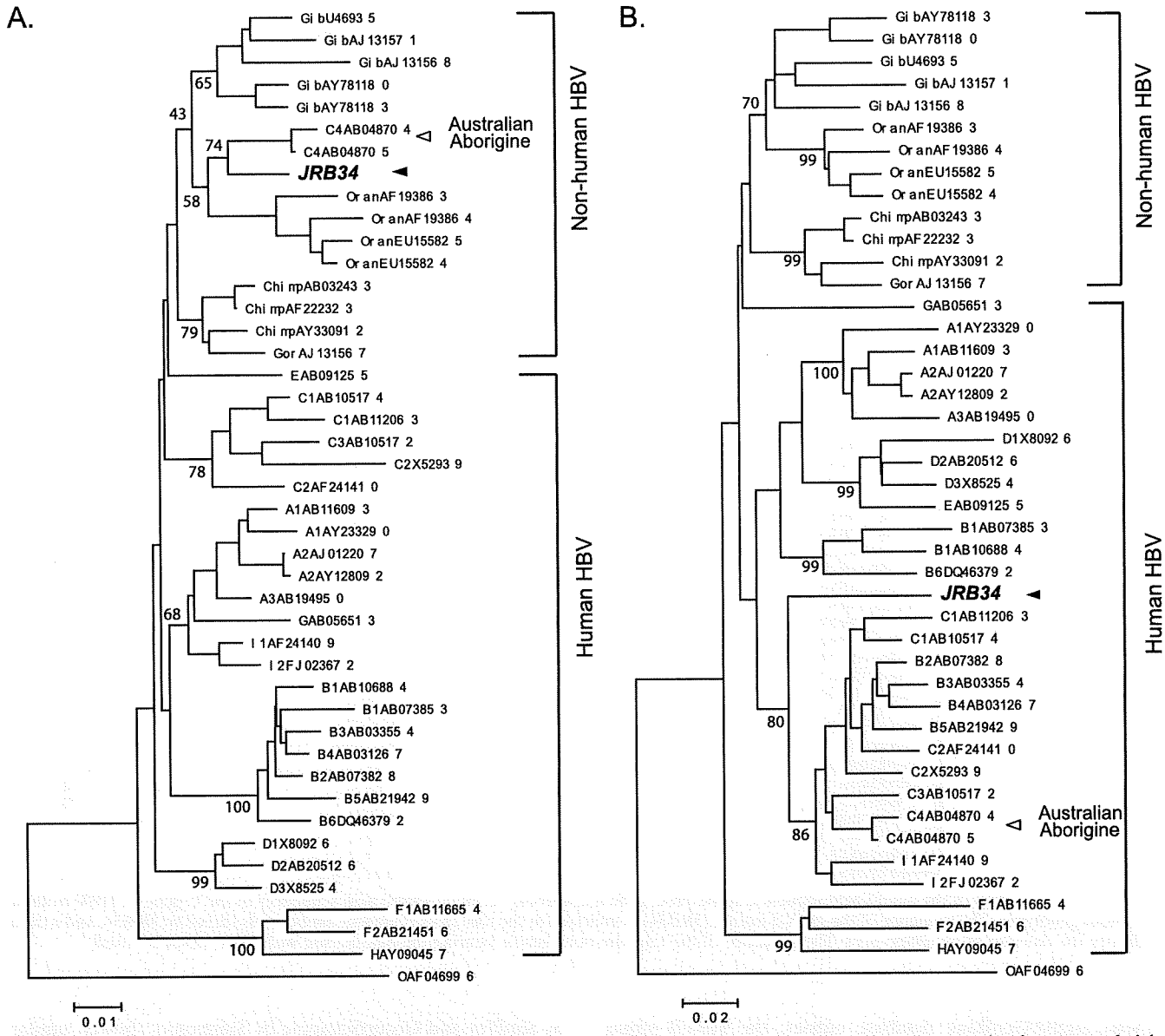


FIG. 3. Phylogenetic tree constructed on the preS/S gene (A) and C gene (B) sequences of 44 HBV isolates representing four ape and eight human genotypes. A woolly monkey HBV isolate serves as an outgroup. The HBV/J isolate (JRB34) is indicated by an arrowhead, and an HBVC4 isolate from Australian aborigine is indicated by an open triangle. The genetic distance is indicated by a bar below.

exceeding 90 years; and "B" for Borneo where he is suspected to have contracted the HBV infection). The entire nucleotide sequence was determined for the JRB34 isolate of genotype J (HBV/J). It had a genomic length of 3,182 nt, which consisted of envelope gene containing preS1 region (nt 2848 to 3171, coding for 108 amino acids [aa]), preS2 region (nt 3172 to 154 [55 aa]), and the small S gene (nt 155 to 835 [226 aa]), X gene (nt 1374 to 1838 [154 aa]), preC region (nt 1814 to 1897 [27 aa]), C gene (nt 1901 to 2452 [183 aa]), and P gene (nt 2307 to 1623 [832 aa]).

Sequence divergence of the JRB34 strain from other genotypes. The complete genome sequence of the JRB34 strain obtained in the present study was compared against those of 1,440 HBV genomes registered in the Viral Hepatitis Database (39). Estimated nucleotide sequence divergence of the JRB34 strain from four ape and nine human genotypes is summarized in the Table 1. The mean divergence by genotypes ranged from 10.7 and 10.9% (from orangutan and gibbon, respectively) to 15.6 and 15.7% (from genotypes F and H, respectively). Surprisingly, the minimum divergence of 9.9% was observed in comparison with a nonhuman HBV isolate from *Hilobates agilis* gibbon confiscated in Taiwan in 1993 (AY330917) (41). Since the sequence divergence from any documented genotypes, including recently proposed genotype I, exceeded 8%, the JRB34 strain was tentatively classified into a novel genotype J of HBV.

Phylogenetic analysis of the entire genomic sequence. In the phylogenetic tree constructed on 1,440 complete genome