延する重症肝炎，それ以外の5例(10.9%)も劇症肝炎例である．④E型肝炎の発症月に偏りがなく，年間を通して発生している．⑤症例の多くは，北日本を中心に分布している．我が国のE型肝炎の感染地域，流行地域は，B型，C型肝炎とは逆の東高西低といってよい．とくに関東以北のNon-ABC型急性肝炎例では，Okamotoらが報告した特徴に該当する場合には積極的にE型肝炎感染を疑うべきであろう．

第二は，流行の時期である．北海道でのE型急性肝炎症例の多くは2000年以後の症例が多く，今回の国立病院の調査結果でも10例中6例が2000年以後に発生している．A型肝炎同様，E型肝炎も経口感染で伝播することから，2000年以後のこの2～3年の間に急速に流行している可能性は否定できない．

第三は，今回の解析対象者の中でHEV-IgG抗体陽性者が約15%存在する点である．今回の検討では342例中64例(18.7%)がHEVIgG抗体陽性であったが，うち10例(2.9%)はE型急性肝炎例でHEVIgM抗体も陽性であることから，残りの54例(15.8%)がHEVIgG抗体のみ陽性であった．HEVIgG抗体のみ陽性例の解釈・理解を深めるために，E型急性肝炎治癒後も4年間経過を観察し，HEV抗体価の推移を観察した症例を図2に提示する．HEV-RNAの陰性化を確認後の経過では，HEV特異IgM抗体は感染後急速に抗体力価が低下して1年ほどで陰性化したが，IgG抗体の減衰はゆるやかで，感染後4年目の時点でも高力価を示していた．この症例の抗体価の推移から，HEVIgG抗体のみ陽性は，既往の感染，E型肝炎感染のメモリーを表していると考えられる．我々の解析対象例中，40代から60代の世代でHEVIgG抗体陽性率は20%を超えており，その世代の対象者は過去にHEVに曝露され抗体を獲得したと考えられる．我が国の一般人口におけるHEVIgG抗体陽性率に関してTanakaらも，地域によって抗体陽性率に偏るがあるものの1.9%から14.1%と報告している[4]．すなわち，我が国においてもかつてE型肝炎は常在し，流行していた可能性が十分考えられる．

イノシシバーベキュー摂取後のE型肝炎の集団感染

2003年3月，イノシシ肉を焼肉バーベキューとして食した2人が，ほぼ40日の潜伏期の後，長崎県内の病院に入院し，E型急性肝炎と診断された．イノシシ肉が保存されていなかったことから直接的な証拠はないものの，同時にイノシシ肉を食した計12人の調査結果から，イノシシバーベキュー摂取後のE型肝炎の集団感染であることが判明した[5]．以下にその詳細を報告する．

発端となった2例はともに69歳男性で，入院時HEV-RNA陽性，HEVIgM抗体，HEVIgG抗体ともに陽性でE型急性肝炎と診断した．2例とも一峰性のALT値の変動を示したのち自然に治癒したが，血液中のウイルス遺伝子解析結果では塩基配列は99.4%一致し，genotype Ⅲ型であった．同時にイノシシ肉を食した12人中発端となった2人以外に3人が急性肝炎で他の病院に受診し，いずれもHEV-IgMが陽性．12人中8人(66.7%)がIgM抗体陽性，11人(91.7%)がIgG抗体高力価陽性であった．発症した5例は通常型急性肝炎で経過するも，感染が成立したと考えられる6例はまったく症状がなく，不顕性感染で経過した．バーベキューではあったが，十分な加熱処理が行われなかったことが感染の原因と考えられる．イノシシ，シカなどの野生動物を食用に用いる場合には，十分な加熱処理が必要であることを警告した事例である．

おわりに

厚生労働省は緊急に解決すべき課題として，「本邦に於けるE型肝炎の診断・予防・疫学に関する研究(主任研究者：三代俊治 東芝病院研究

部長)」を2003年から発足させた．現在，ウイルス学的なアプローチと臨床疫学的なアプローチの両側面から多くの班員の協力のもとに我が国のE型肝炎の実態を解明すべく調査と研究が進行しつつある．今後の研究の進展に期待したい．

文　献

1) 厚生省非A非B型肝炎研究班：平成4年度報告書，97頁，1992.
2) 厚生労働科学研究費補助金，肝炎等克服緊急対策研究事業(肝炎分野)肝炎ウイルス等の標準的治療困難例に対する治療法の確立に関する研究，本邦の急性肝炎の疫学調査および欧米型B型急性肝炎(genotype A)とE型肝炎の発生状況に関する研究報告書，22頁，2003.
3) Okamoto H *et al*：Features of hepatitis E virus infection in Japan. Intern Med **42**：1065, 2003.
4) Tanaka E *et al*：Seroepidemiological study of hepatitis E virus infection in Japan using a newly developed antibody assay. J Gastroenterol **36**：317, 2001.
5) Tamada Y, Yano K, Yatsuhashi H *et al*：Consumption of wild boar linked to cases of hepatitis E. J Hepatol **40**：869, 2004.

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＜短　報＞

B型慢性肝炎に対するラミブジン治療のウイルス学的効果予測式の作成

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緒言：B型慢性肝炎の治療薬としてラミブジンが登場し，hepatitis B virus（HBV）に対する優れた抗ウイルス効果を示すことから臨床の場で広く使われてきている．しかし使用期間が長期化するにつれて，HBVゲノムのポリメラーゼ領域のBおよびCドメインに変異を有するラミブジン耐性株の出現が問題となってきている[1,2]．耐性株はラミブジン投与前が高ウイルス量，HBe抗原陽性，またalanine aminotransferase（ALT）高値などの症例に早期に出現し易く[2,3]，さらに投与6カ月目のHBV DNA量が10^3 copies/mlより多い症例では，有意にYMDD変異株の出現頻度が高まることが報告されている[3]．我々はラミブジン投与前に効果予測を行う目的にてウイルス量低下に関与する因子の検討を行い，投与6カ月目のウイルス学的効果を予測する判別式の作成を試みたので報告する．

方法：対象は2000年11月から2002年6月までにラミブジン治療を受けたB型慢性肝炎患者78例中，投与開始後6カ月以上観察し，臨床データおよび保存血清が利用可能であった53例（男性40例，女性13例，年齢33～80歳，中央値49歳，CH 38例，LC 15例，HBe抗原陽性26例，陰性27例）とした．投与直前の患者背景，血液生化学検査値，HBV DNA量［transcription-mediated amplification（TMA）法］，pre-C変異（nt 1896；G to A），core promoter（CP）変異（nt 1762；A to T, 1764；G to A）を測定し，ラミブジンの効果との関連を検討した．pre-CおよびCP変異の検出は血清からDNAを抽出後，PCRを行いダイレクトシークエンスにて塩基配列を決定した[4]．HBVジェノタイプはPCR-RFLP法[5]にて判定した．統計にはχ^2検定，Mann-Whitney U test，変数増加法によるロジスティック回帰分析を用いた．

結果：投与開始6カ月の時点で，53例中40例（75.5%）にTMA法によるHBV DNA陰性化が認められた．陰性化群と持続陽性群で年齢，性別，病型，既往歴，ALT値，血小板数，総ビリルビン値に有意差はみられなかった．しかし，HBe抗原陽性率（陰性化群 vs. 持続陽性群；35.0 vs. 92.0%，P=0.001），HBV DNA量（TMA法）（以下同様に；5.8±1.3 vs. 8.0±1.3 LGE/ml，P<0.001），pre-C変異陽性率（45.0 vs. 0%，P=0.008）の3項目は両群間で有意差がみられた．HBV DNA陰性化率は，投与開始前HBV DNA量が7.0 LGE/ml以上では42.9%，未満では97.0%であり，後者で有意に高かった（P<0.001）．HBVジェノタイプ，CP変異陽性率は両群間で差はなかった．平均21カ月の観察期間中，肝炎再燃を持続陽性群13例中3例（23.1%）に認め，陰性化群からの肝炎再燃はみられなかった（P<0.05）．多変量解析ではHBV DNA量（≧7 LGE/ml），HBe抗原陽性，ALT値（≧120 IU/l）が，治療6カ月後のDNA陰性化に有意に関与していた（表）．

ロジスティック回帰分析による6カ月後のHBV DNA陰性化予測式を作成した．

$$y=\frac{1}{1+e^{-4.838+3.869\times(HBV\ DNA)+2.691\times(HBeAg)-3.136\times(ALT)}}$$

HBV DNA：TMA法にて7.0 LGE/ml以上は1，未満は0，HBeAg：陽性は1，陰性は0，ALT：ALT 120 IU/l以上は1，未満は0を代入し，この値が0.5以上を6カ月後に陰性化すると予測した．

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表　ラミブジン治療開始6カ月後のHBV DNA陰性化に関与する因子

説明変数		95%信頼区間	オッズ比	P
HBV DNA				
<7 LGE/m*l*	(n=32)		1	
≧7 LGE/m*l*	(n=21)	0.002～0.244	0.021	0.0002
HBe抗原				
陰性	(n=27)		1	
陽性	(n=26)	0.005～0.895	0.068	0.0410
ALT				
<120 IU/*l*	(n=43)		1	
≧120 IU/*l*	(n=10)	1.648～321.6	23.020	0.0197

この予測式を用い，2例追加した55例で検討したところ，感度95.1%，特異度78.6%，正診率90.9%であった．また投与前HBV DNA量が7 LGE/m*l*以上の高ウイルス群(n=23)に限って検討したところ，感度80.0%，特異度84.6%，正診率82.6%と良好な結果であった．

考察：今回我々は，従来言われているラミブジンの効果と関連があるHBV量，HBe抗原，血清ALT値から6カ月後のウイルス量低下を予測する判別式を作成することができた．ラミブジンは速やかに血清中HBVを低下させ，生化学データを改善するが，投与中ウイルス量が十分に低下しない例はウイルスの複製が盛んであり，従って耐性株も出現し易い[3]．今回の結果では，HBeAg陽性でウイルス量がTMA法で7 LGE/m*l*以上の症例ではウイルス学的効果に乏しく，その後の投与期間において持続陽性群の23%に肝炎再燃を認めた．このことから投与6カ月目のウイルス陰性化を予測することは，ブレイクスルーを起こさないための必要条件であると考えられる．

投与前HBV量と治療効果との関係をみると，TMA法で7 LGE/m*l*未満では6カ月後のウイルス陰性化率は97%と高率であるので，予測式を用いる有用性は低いと判断される．一方7 LGE/m*l*以上の高ウイルス量群においては，耐性株出現と投与中止が困難という問題が予想されるため，ラミブジン導入に際しては今回作成した予測式は有用であると考えられる．特に予測式の特異度が84.6%と良好な値を示しているので，陰性化が期待できない症例の予測に役立つと思われる．臨床的には予測式を併用し，病期など総合的に判断した上でラミブジン治療を開始すべきと思われる．

予測式については，今後症例を増やし更に検討する必要があり，また，投与中止基準である6カ月間のALT正常化を伴うウイルス持続陰性化についても，同様の検討が必要であると考えられる．

索引用語：B型慢性肝炎，ラミブジン，効果予測

文献：1) Allen MI, Deslauriers M, Andrews CW, et al： Hepatology 27：1670－1677, 1998　2) Lai CL, Chien RN, Leung NW, et al： N Engl J Med 339：61－68, 1998　3) Yuen MF, Sablon E, Hui HJ, et al： Hepatology 23：785－791, 2001　4) Rokuhara A, Tanaka E, Yagi S, et al： J Med Virol 62：471－478, 2000　5) Mizokami M, Nakano T, Orito E, et al： FEBS Lett 450：66－71, 1999

Practice of Interferon Therapy
—Chronic hepatitis C (Therapy with consensus interferon)—

JMAJ 47(2): 69–72, 2004

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Abstract: The consensus interferon, rIFN-αcon1, has been proved to be effective for the treatment of chronic hepatitis C (CH-C) with genotype 1b and high viral load (≧100 kc/m*l*) through randomized controlled studies performed in Japan. For patients with genotype 1b and high viral load, the complete response rate (16.7%) of rIFN-αcon1 (18MU) is significantly higher than that of IFN-nα (6MU), though it is 0% in those with a viral load of more than 700 kc/m*l*. rIFN-αcon1 is also effective for the treatment of CH-C patients with both low viral load and a history of prior IFN treatment. It will be concluded that rIFN-αcon1 is effective for CH-C patients with a viral load of less than 700 kc/m*l*.

Key words: Chronic hepatitis C; High viral load group; Consensus interferon

Introduction

The therapeutic strategy for chronic hepatitis C has changed dramatically since consensus interferon (rIFN-αcon1, Advaferon) and the ribavirin (Rebetol) + IFNα-2b (Intron A) combination therapy were approved in December 2001 and the limitations to the administration period of existing IFN-α preparations were abolished in February 2002.

This paper describes the indications and future issues for rIFN-αcon1 based on clinical study results.

Outline of rIFN-αcon1 (consensus interferon)

This preparation has 166 amino acid residues and a molecular weight of 19,500 Daltons. The number of amino acid residues is almost the same as that of existing IFN-α preparations. Thirteen subtypes of IFN-α with different activities were known in 1982. A new amino acid sequence was designed according to the hypothesis that a useful IFN preparation could be created by choosing a frequent amino acid at each site of amino acid sequences (consensus-sequence theory; Fig. 1).[1] rIFN-αcon1 was produced with synthetic DNA based on the amino

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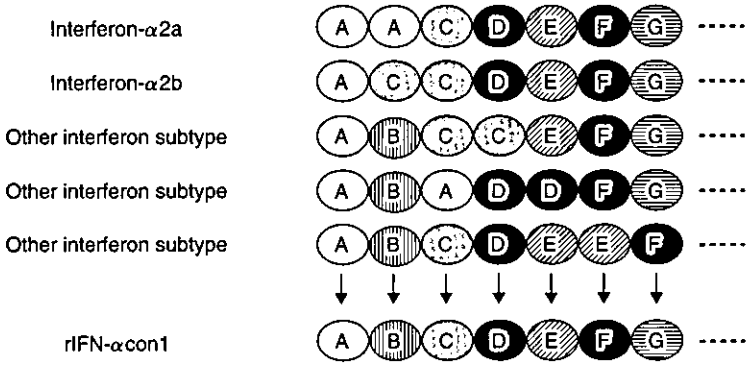

Fig. 1 Design of amino acid sequence for rIFN-αcon1

acid sequence in a gene-recombinant *Escherichia coli* expression strain. Since this preparation had a high affinity for Type 1 IFN receptors[2-4] and was superior to a control IFN preparation in antiviral effect,[5-7] cell growth-inhibiting effect,[8] and immuno-stimulating effect,[5,8] clinical studies were started in 1991 in USA and in 1993 in Japan.

Unlike existing IFN-α preparations, it is supplied as a subcutaneous injection at a small volume (0.4 m*l* for 12 MIU and 0.6 m*l* for 18 MIU) without human serum-derived albumin.

Clinical Results of rIFN-αcon1

Although rIFN-αcon1 has been clinically applied, no major results have been published yet. Therefore, the results of a randomized controlled study using the IFN-αn1 preparation as a control are described here.[9,10] rIFN-αcon1 was subcutaneously administered at 18 or 12 MIU/day for 2 weeks consecutively, followed by thrice weekly for 22 weeks to determine the rate of patients with virologic complete response (CR) (defined as negative for amplicor determination) and normal ALT at the time after 24 weeks from the end of treatment.

1. Group with high viral load (≥100 kcopies/m*l* or ≥1 Meq/m*l*)

rIFN-αcon1 produced a CR rate of 26.3%

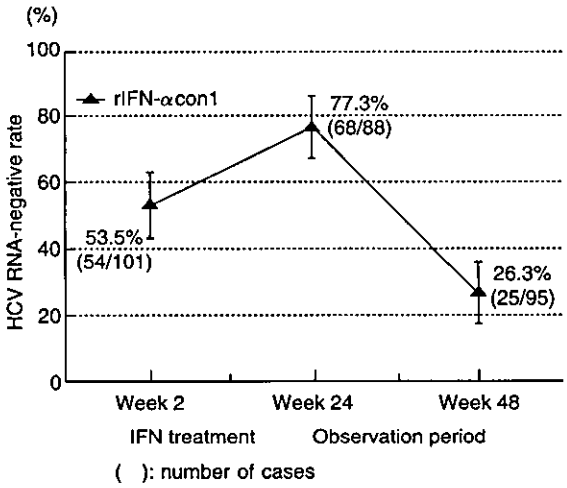

Fig. 2 Change in HCV RNA-negative rate in patients with high viral load

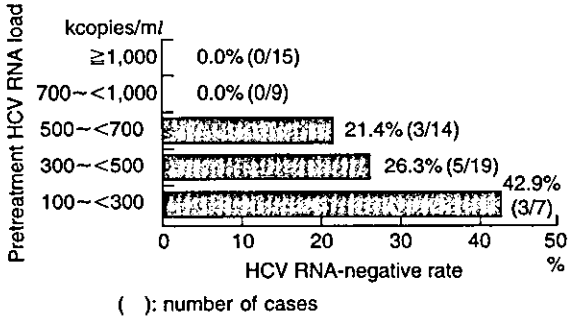

Fig. 3 HCV RNA-negative rate by viral loads in patients with genotype 1b and high viral load

Table 1 HCV RNA-Negative Rate by Genotypes in Patients with Low Viral Load

	Genotype			Total
	1b	2a	2b	
HCV RNA-negative rate (%) (No. of cases)	71.4 (10/14)	75.0 (12/16)	—	73.3 (22/30)

Table 2 HCV RNA-Negative Rate by Viral Loads and Genotypes in Patients Treated with IFN for the Second Time

Pretreatment viral load (kcopies/m*l*)	Genotype			Total
	1b 12.5% (2/16)	2a 100% (9/9)	2b 30.0% (3/10)	40.0% (14/35)
≥100	6.7% (1/15)	100% (3/3)	37.5% (3/8)	26.9% (7/26)
<100	100% (1/1)	100% (6/6)	0.0% (0/2)	77.8% (7/9)

( ): number of cases

(25/95) (Fig. 2) and a normal ALT rate of 43.8% (35/80) in the 18 MIU treated group. It produced a CR rate of 16.7% (11/60) and normal ALT rate of 35.1% (20/57) in the patients with hepatitis of genotype 1b, who are considered particularly intractable among those with high blood viral load. The CR rate was significantly higher than that of the control group ($p<0.05$). The CR rate was also higher than that of existing IFN-α preparations (0 to 8.6%). Figure 3 shows the CR rate by pretreatment viral load in patients with hepatitis of genotype 1b and high viral load. Although the CR rate tended to fall as the viral load was increased, a high CR rate of 27.5% (11/40) was obtained for the group with a viral load from 100 to less than 700 kcopies/m*l*. In contrast, rIFN-αcon1 produced a CR rate of 0% in patients with a very high viral load of 700 kcopies/m*l* or higher.

2. Group with low viral load (<100 kcopies/m*l* or <1 Meq/m*l*)

rIFN-αcon1 at 12 MIU produced a CR rate of 73.3% (22/30), with no difference among genotypes (Table 1). It was more effective than existing IFN-α preparations (49.1 to 66.0%) in the patients with low viral load as well.

3. IFN re-treatment group

rIFN-αcon1 was administered at 18 MIU in patients in whom previous IFN therapy made HCV RNA negative or ALT return to normal, but did not achieve CR. The results showed it achieved CR in 40.0% (14/35) and made ALT persistently normal in 51.5% (17/33) (Table 2). This indicates that the preparation is clinically significant for those re-treated with IFN as much as for those treated for the first time. In addition, rIFN-αcon1 produced a CR rate of 0% in patients with hepatitis of 1b genotype and a high viral load of 300 kcopies/m*l* or higher.

4. Safety

All the 227 patients treated with rIFN-αcon1 experienced at least one adverse reaction: fever developed in 98.2%, general malaise in 45.4%, anorexia in 39.6%, headaches in 39.2%, arthralgia in 32.6%, insomnia in 27.8%, alopecia in 27.8%, and gastric discomfort in 20.3%. Although all these adverse reactions

were already known, it should be noted that they tended to develop at higher incidences than with previous IFN-α preparations. Depression, a serious adverse reaction to IFN, was observed in 5% or higher. Sho-saiko-to is contraindicated in patients treated with rIFN-αcon1. Further, it is recommended that the combination of rIFN-αcon1 with theophylline, antipyrine, or warfarin be performed with care because the IFN preparation inhibits the activities of enzymes responsible for drug metabolism in the liver.

Indications and Future Issues of rIFN-αcon1

Considering the above clinical results, this preparation is indicated for patients with 1b genotype and a high viral load of up to 700 KIU/m*l*, in addition, it is expected to show higher efficacy than existing IFN-α preparations in the groups with 2a genotype and high viral load, or with low viral load. Although the ribavirin + IFN-α2b combination therapy is also indicated for patients with a high viral load, it has a drawback in that ribavirin causes hemolytic anemia. Therefore, rIFN-αcon1 is recommended as the first line treatment for the patients with blood Hb around 12 g/d*l* or lower. Since the clinical study of this preparation was performed in a relatively small number (less than 300) of patients, it goes without saying that verifying the efficacy and safety of rIFN-αcon1 is required.

To further improve the therapeutic results of rIFN-αcon1 in patients in the intractable 1b/high viral load group, it would be valuable to create a polyethylene glycol (PEG) preparation of rIFN-αcon1 or combine rIFN-αcon1 with ribavirin.

REFERENCES

1) Blatt, L.M., Davis, J.M., Klein, S.B. *et al.*: The biologic activity and molecular characterization of a novel synthetic interferon-alpha species, consensus interferon. *J Interferon Cytokine Res* 1996; 16: 489–499.
2) Pfeffer, L.M.: Biologic activities of natural and synthetic type 1 interferons. *Semin Oncol* 1997; 24(S9): 63–69.
3) Klein, S.B., Blatt, L.M. and Taylor, N.W.: Cell surface binding characteristics correlate with consensus type I interferon enhanced activity. *J Interferon Cytokine Res* 1996; 16: 1–6.
4) Walter, M.R.: Three-dimensional models of interferon-α subtypes IFN-con1, IFN-α_8, and IFN-α_1 derived from the crystal structure of IFN-α_{2b}. *Semin Oncol* 1997; 24(S9): 52–62.
5) Onzes, O.N., Reiter, Z., Klein, S. *et al.*: A comparison of interferon-con1 with natural recombinant interferon-α: Antiviral, antiproliferative, and natural killer-inducing activities. *J Interferon Res* 1992; 12: 55–59.
6) Van der Meide, P.H., Harteveld, N., Stebbing, N. *et al.*: The effects of recombinant and natural human interferons in vaccinia virus infected rhesus monkeys. *Antiviral Res* 1985; S1: 199–207.
7) Altrock, B.W., Fagin, K.D., Hockman, H.R. *et al.*: Antiviral and antitumor effects of a human interferon analog, IFN-αCon1, assessed in hamsters. *J Interferon Res* 1986; 6: 405–415.
8) Tanaka, S., Yasuda, S., Miyata, K. *et al.*: Effects of interferon-αcon1 (YM643) to inhibit tumor cell proliferation and stimulate the immunity. *Pharmacology and Clinic* 2000; 10: 411–419. (in Japanese)
9) Suzuki, H. and Kumada, H.: New drug for chronic hepatitis C — Consensus interferon. *Liver, Gallbladder, and Pancreas* 2001; 43: 281–288. (in Japanese)
10) Suzuki, H. and Tango, T.: Consensus Interferon Research Group. A multicenter, randomized, controlled clinical trial of interferon alphacon-1 in comparison with lymphoblastoid interferon-alpha in patients with high-titer chronic hepatitis C virus infection. *Hepatology Res* 2002; 22: 1–12.

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Characteristics of Core Promoter and Precore Stop Codon Mutants of Hepatitis B Virus in Vietnam

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In Asia, genotypes B and C are the most common genotypes of hepatitis B virus (HBV); and genotype C causes more severe liver disease. Core promoter/precore (CP/PC) mutants, known to be linked to these genotypes, could have an impact on the progression and severity of liver disease. Sera of 115 patients, including 39 acute and 76 chronic Vietnamese HBV infected patients, were tested for their liver profile, HBeAg, HBV genotypes, and HBV DNA level. Fragments of 282 nucleotides covering CP/PC were amplified, sequenced, and analysed. In the acute group, CP/PC mutants accounted for 38.4 and 25.6%, respectively. Genotype B was found to be predominant (74.3%, $P < 0.05$) and linked to the PC mutant (A1896) ($P < 0.05$). In the chronic group, CP/PC mutants accounted for 61.7 and 32.8%. CP mutants, especially the T1762/A1764 double mutant, were found to correlate with genotype C (81%, $P < 0.001$), liver cirrhosis, and hepatocellular carcinoma ($P < 0.05$). Therefore, genotype C in Vietnam, which carried high rate of C-1858 (70%), could play an important role in causing severe chronic liver disease. ***J. Med. Virol. 74:228–236, 2004.*** © 2004 Wiley-Liss, Inc.

KEY WORDS: HBV in Vietnam; HBV variant; corepromoter and precore mutant of HBV; HBV genotype

INTRODUCTION

Hepatitis B virus (HBV) infection is a global public health problem [Lee, 1997]. The course of HBV infection and HBV-related liver injury depend on several host-viral factors [Lok, 2000]. Due to the lack of proof-reading capacity in the DNA polymerase, HBV carries high mutation rate, which is not limited to any open reading frame. Mutations in the core promoter (CP) region of HBV can be found in many hepatitis B e antigen (HBeAg)-negative patients [Chan et al., 2000; Lok et al., 2000]. The well-known mutation is the double nucleotide substitution: A to T at nucleotide 1762 and G to A at nucleotide 1764 (A1762T/G1764A). The CP mutants have been linked to the severity of liver diseases, especially hepatocellular carcinoma (HCC) [Kramvis and Kew, 1999]. Alternatively, the mutation in the precore (PC) region, substitution of G to A at nucleotide 1896 (A1896), creates a premature stop codon at codon 28 in the *PC* gene. This mutation prevents translation of the PC protein and completely abolishes the production of HBeAg. The role of A1896 to the HBV infection course is still controversial; although it has been thought to aggravate liver disease severity, especially fulminant hepatitis (FH) [Lok et al., 1994; Hunt et al., 2000]. In addition, different HBV genotypes have been associated with different mutant rates in the CP/PC regions of HBeAg-negative chronic hepatitis B [Funk et al., 2002]. Genotypes B and C are the most common genotypes in Asia, and the role of genotype C in the etiology of more severe liver disease has been demonstrated [Kao et al., 2002]. Recently, it has been found that genotype C, especially the C-1858 variant in Southeast Asia, is associated with a higher rate of CP mutants [Chan et al., 1999; Lindh et al., 1999].

A recent study in Vietnam showed that HBV was the most important causative agent correlated with liver disease [Tran et al., 2003]. To clarify the importance of HBV variants and HBV genotypes on the course of HBV infection, the correlation between CP/PC mutants, HBV genotypes and liver disease was analysed in Vietnamese patients.

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PATIENTS AND METHODS

Patients

A cross-sectional study was performed in 115 consecutive Vietnamese HBV infected patients within 1 year, between January 2000 and December 2000. There were 87 men and 28 women, aged from 16 to 83, with the mean age 45.62 ± 15.8 years. These patients were recruited from in-patients Gastroenterology wards at Cho Ray Hospital (Ho Chi Minh city, Vietnam) and Bach Mai Hospital (Hanoi, Vietnam). The diagnoses of HBV-related liver diseases were established based on clinical data, laboratory tests, and imaging studies (ultrasonography, computerised tomography (CT-scan), and/or Magnetic Resonance Imaging (MRI)). Among 115 patients, 39 were diagnosed as acute hepatitis B based on either the novo appearance of HBsAg or the presence of Immunoglobulin M antibody to hepatitis B core antigen (IgM anti-HBc). Patients with a prolonged prothrombin time over than 50% of control and/or hepatic encephalopathy during their acute hepatitis (AH) were diagnosed as FH. Seventy-six patients were diagnosed as chronic HBV infection; and the persistence of HBsAg of these patients were followed in more than 1 year. The chronic group included asymptomatic carriers (ASCs) with normal or mild elevated alanine transferase (ALT) (<2 times of upper normal limit); chronic hepatitis (CH) with mild symptoms and abnormal ALT; liver cirrhosis (LC), and HCC. Cirrhosis and HCC were defined on liver function test, alpha-fetoprotein level, imaging studies, and histology. None of the patients had co-infection with hepatitis C virus (HCV) and/or hepatitis D virus (HDV) and their serum samples were stored at −70°C until used. Informed consent was obtained from all patients, and the study was approved by the local ethical committee.

Serologic Markers

All sera were screened for HBsAg, HBeAg, anti-HCV antibody, and anti-HDV antibody by enzyme linked immunosorbent assay (ELISA), using commercially available kits from Abbott (Abbott Laboratories, North Chicago, IL). Diagnosis of acute hepatitis was reconfirmed by IgM anti-HBc assay in all cases.

Extraction of DNA

Viral DNA was extracted from 100 μl of serum using the DNA/RNA extraction Kit (SepaGene RV-R, Sanko Junyaku Co., Ltd., Tokyo, Japan). The resulting pellet was eluted in 50 μl of RNase-free water and kept in −20°C until use.

HBV Genotyping by PCR

Genotyping of HBV was identified by PCR using type-specific primers designed from pre-S1 through S genes of HBV [Naito et al., 2001]. Six genotypes (A to F) of HBV could be identified by specific bands of second PCR. To avoid false-positive results, instructions to prevent cross contaminations were strictly followed, and the results were considered valid only when they were consistently obtained in duplicate.

Amplification of the CP/PC Regions

Partial gene covering 282 nucleotides (nt) (from nt 1689 to 1970) of CP/PC region were amplified by nested PCR. Primer pair eP11: 5′-GCATGGAGACCACCGTGAAC-3′ (sense) and BG1R: 5′-ATAGGGGCATTTGGTGGTCT-3′ (antisense) was used for the first round PCR; and primer pairs PC1: 5′-CATAAGAGGACTCTTGGACT-3′ (sense), PC2: 5′-AAAGAATTCAGAAGGCAAAAAAGA-3′ (antisense) for the second round PCR. The PCR reaction was performed in 40 cycles (94°C 20 sec, 55°C 20 sec, and 72°C for 30 sec) followed by extension at 72°C for 7 min. PCR products were separated by 2% agarose gel electrophoresis and purified using the QIAquick gel extraction kit (Qiagen, Inc., Chatsworth, CA).

Nucleotide Sequencing and Phylogenetic Analysis

Purified PCR products were subjected to direct sequencing using the ABI PRISM™ Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA). The inner primer pair was used as sequencing primers. Sequences of amplified DNA were determined using automated DNA sequencer ABI 377 (Perkin Elmer, Norwalk, CT). Nucleotide sequences were multiple-aligned, analysed using Genetyx for Windows ver. 6.0 software (GENETYX, Tokyo, Japan), and corrected manually by visual inspection. Nucleotide consensus sequences of CP/PC regions of HBV genotypes B and C were taken from GenBank for multi-aligment and mutant analysis. Tree construction was analysed by neighbour-joining method with bootstrap resampling (1,000 times), using MEGA version 2.1 [Kumar et al., 2001].

Quantitation Assay of Viral Load

Quantitation of HBV DNA was performed by real-time PCR method [Chen et al., 2001]. The detection limit of this assay was 3.73×10^2 genome equivalents per ml. Sequences of primers and probes were HBc1: 5′-AGTGTGGATTCGCACTCCT-3′ (sense, nt 2269–2287), HBc1R: 5′-GAGTTCTTCTTCTAGGGGACCTG-3′ (antisense, nt 2387–2365) and HBcP1: 5′-CCAAATGCCCCTATCTTATCAACACTTCC-3′ (TaqMan probe, nt 2303–2331).

Statistical Analysis

Proportions of each factor were compared between the groups using Fisher exact 2-tail test, and the group means were compared using the Student's *t*-test. Differences were considered to be significant for $P < 0.05$. Mean of HBV DNA levels were compared after logarithmic transformation of the HBV DNA values from the real-time PCR assay.

RESULTS

Patients With Acute and Chronic HBV-Infected Diseases

Among 39 acute HBV-infected patients including acute hepatitis and fulminant hepatitis, there were 29 (74.3%) with genotype B and 10 (25.7%) with genotype C. Their mean age was 35.2 ± 12.8 years and 36 of them were men. In 76 chronic HBV-infected patients, there was an equal distribution of genotype B (39; 51%) and genotype C (37; 48.7%). The mean age of this group was 50.9 ± 14.4 years. The characteristics of the patients in each group and each diagnosis category were described in Table I. The acute group had a younger age ($P < 0.001$), a higher ALT ($P < 0.001$), and a higher HbeAg +ve rate ($P < 0.05$) than that of the chronic group. HBV DNA level among acute HBV infected patients was higher than that of chronic infection, however, there was no statistically significant difference ($P = 0.26$). Of note, the frequency of genotype B was found to be higher than that of genotype C in the acute group, in comparison with the chronic group (74.3% vs. 51.3%, $P < 0.05$).

CP/PC Mutant in Acute HBV-Infected Patients

In the CP region, the occurrence rate of the CP mutant was 15/39 (38.4%), in which the T1762/A1764 and deletion mutants accounted for 30.7 and 7.6%, respectively (Table II). Two out of three cases of fulminant hepatitis had a deletion in this region, spanning from 20 to 21 nucleotides (Fig. 1). The total rate of CP mutants in genotype B (9; 31%) was lower than that of genotype C (6; 60%) but the difference was not statistically significant ($P = 0.14$). In the PC region, 70% of genotype C isolates carried the C at nucleotide 1858 (C-1858) ($P < 0.001$), and there was no A1896 mutant among these isolates (Fig. 1B and Table II). However, genotype B isolates carried only T at nucleotide 1858 (T-1858), and the A1896 mutant was determined in 34.4% cases ($P < 0.05$) (Fig. 1A and Table II).

CP/PC Mutant in Chronic HBV-Infected Patients

In the CP region, the occurrence rate of the CP mutant was 51/76 (67.1%). Three kinds of mutants were detected in genotype B, i.e., T1762/A1764 (33.3%), T1762A alone (10.2%) and deletion mutant (7.6%) (Table III). The frequency of the T1762/A1764 double mutant was found to be higher in genotype C (81%) than in genotype B isolates (33.3%) ($P < 0.001$). In the PC region, the A1896 mutant was seen in 25/76 (32.8%). As reported in the acute group, C-1858 also possessed a strong link to genotype C (70.2%) ($P < 0.001$). The A1896 mutant, therefore, was less detectable in genotype C (5.4%) than in genotype B (58.9%) ($P < 0.001$). Furthermore, when only the T-1858 isolates were taken into account, the A1896 mutant rate was also lower in genotype C than in genotype B (2/11 (22.2%) and 23/39 (58.9%), respectively). In addition, there were correlations between cirrhosis and HCC with a high occurrence rate of T1762/A1764 in genotype C ($P < 0.01$); and A1896 in genotype B ($P < 0.05$) (Table III).

CP/PC Mutant, Virological Manifestations, and Liver Injury

In the acute group, the mean age, HBV DNA, and ALT level were not significantly different between the wild type and the CP/PC mutant type (Table IV). Conversely, in the chronic group, the CP mutant was detected more frequently in older age cases and associated with a lower HBV DNA level than that of the wild type. However, there was no statistically significant difference ($P = 0.6$ and 0.6, respectively). The same insignificant different finding was observed with the A1896 mutant, although it was detected in cases with a higher mean age, higher HBV DNA, and ALT level ($P = 0.7$; $P = 0.4$; and $P = 0.6$,

TABLE I. Characteristic of Patients of Acute and Chronic HBV Infection

Diagnosis	n	Sex (M/F)	Age (year)[a]	ALT (UI/L)	HBeAg (+ve/−ve)	HBV DNA (log_{10} copies/ml)	Genotype	
							B	C
Acute infection	39	36/3	35.2 (12.8)*	1,089 (892)**	9/30***	5.48 (1.33)****	29 (74.3)†	10 (25.7)
AH	36	26/10	35.2 (12.9)	1,137 (908)	8/28	5.50 (1.36)	28 (77.7)	8 (22.3)
FH	3	2/1	34.6 (15.1)	516 (373)	1/2	5.31 (1.38)	1 (33.3)	2 (66.7)
Chronic infection	76	59/17	50.9 (14.4)*	85 (120)**	5/71***	4.98 (1.21)****	39 (51.3)†	37 (48.7)
ASC	10	8/2	36.5 (18.1)	40 (5)	2/8	6.30 (0.74)	6 (60)	4 (40)
CH	4	3/1	47.2 (8.3)	69 (16)	0/4	4.45 (0.54)	0	4 (100)
LC	39	29/10	52.9 (12.8)	108 (163)	1/38	4.98 (1.36)	21 (53.8)	18 (46.2)
HCC	23	19/4	54.3 (12.7)	61 (28)	2/21	4.79 (0.93)	12 (52.1)	11 (47.9)

[a]Age, ALT, HBV DNA were denoted in mean with the standard deviation in parenthesis; sex, HBeAg were denoted in number of cases; and genotype was denoted in number of cases with percentage in parenthesis.
*$P < 0.001$.
**$P < 0.001$.
***$P < 0.05$.
****$P = 0.26$.
†$P < 0.05$.

TABLE II. Core Promoter and Precore Mutant in Acute HBV Infected Patients

	Core promoter (CP) region[a]			Precore region	
	T1762/A1764	T1762 alone	Deletion in CP	C-1858	A1896
Genotype B (n = 29)	8 (27.5)*	0	1 (3.4)	0***	10 (34.4)**
AH (n = 28)	7 (25.0)	0	1 (3.5)	0	9 (32.1)
FH (n = 1)	1 (100)	0	0	0	1 (100)
Genotype C (n = 10)	4 (40.0)*	0	2 (20.0)	7 (70.0)***	0**
AH (n = 8)	4 (50.0)	0	0	6 (75.0)	0
FH (n = 2)	0	0	2 (100)	1 (100)	0

[a]Number of cases with percentage in parenthesis.
*$P = 0.69$.
**$P < 0.05$.
***$P < 0.0001$.

respectively). HBeAg was still detected in patients with CP/PC mutants, however, the rate of HBeAg loss was found more frequent in CP mutant infected patients than those with A1896 mutant.

Phylogenetic Analyses

As shown in Figure 2, all of 115 analysed CP/PC sequences (282 nucleotides) were clustered in major branches of genotype B (68 isolates) and C (47 isolates). All genotype C isolates in this study was belonged to sub-branches that differed from Japanese isolates of genotype C (Accession D50520 and D50517). These genotype C isolates were closely related to branches including isolates from Vietnam and Thailand strains from database (AF223957 and AF068756, respectively). Genotype B isolates, however, were shown branching off from genotype C branch rather than from a more proximal node, with low bootstrap value (28%). The C-1858 strains which were only detected in genotype C isolates, assembled closely but did not form a unique phylogenetic entity.

DISCUSSION

It is known that the CP/PC plays a central role in HBV replication. CP directs the transcription of both pre-genomic RNA and precore mRNA [Kramvis and Kew, 1999]. PC and core genes are essential for the pre-genome encapsidation signal and for the core protein assembly [Tong et al., 1992]. The CP mutants have been found to correlate with the HBV genotypes, viral replication, and liver damage in East Asian HBV carriers [Lindh et al., 1999]. The PC stop codon mutant, A1896, has been considered an important factor for fulminant hepatitis and progressive liver disease [Lok et al., 1994; Hunt et al., 2000]. On the other hand, Vietnam has a high rate of endemic HBV infection, with an HBsAg carrier rate between 9–14% in urban areas [Tran et al., 1993; Nakata et al., 1994] and 12–20% in rural areas [Hipgrave et al., 2003]. More than 3.5 million Vietnamese are currently at risk of a premature death due to HBV infection [Ngoan Le et al., 2002; Hipgrave et al., 2003]. Therefore, virologic characterisation of this virus and the CP/PC mutant may be helpful for the understanding of HBV pathogenesis in this country.

In this study, genotype B was found to be the predominant genotype in the acute group (74.3%). As reported previously, genotypes B and C of HBV were equally distributed in Vietnam [Tran et al., 2003] and a similar result was also confirmed in chronic infected patients in the present study (51.3 and 48.7%, respectively). Interestingly, it was known that genotype B in Japan was linked to the acute form, specifically to fulminant but not acute hepatitis [Imamura et al., 2003]. Moreover, genotype B in Hong Kong patients was strongly associated with chronic hepatitis B exacerbations [Yuen et al., 2003a]. This finding suggested a correlation between genotype B with the acute forms of HBV infection. Recent studies have shown that genotype B might be more immunogenic, and patients infected with this genotype have earlier HBeAg seroconversion, in comparison to patients with genotype C [Chu et al., 2002; Yuen et al., 2003b]. Hence, an in-depth genomic sequence analysis of HBV in acute cases could be required to address this matter.

Among the investigated sequences of 39 acute and 76 chronic HBV-infected patients, there were different effects of genotypes on the CP/PC mutants. In the acute group, genotype B was found to correlate with the A1896 mutant. In the chronic group, genotype B was associated with the A1896 mutant, whereas genotype C was correlated with CP mutants. Interestingly, the T1762/A1764 double mutant in genotype C was found to be associated with cirrhosis and HCC. However, due to the small number of asymptomatic carriage and chronic hepatitis in this study, this result needs further confirmation. Nevertheless, similar findings were also reported by other Asian studies, suggesting that the high prevalence of the CP mutant in genotype C isolates could be one of the important factors causing a detrimental effect on the evolution of HBV infection [Takahashi et al., 1995; Lindh et al., 1999; Fang et al., 2002; Yotsuyanagi et al., 2002].

It has also been known that genotype C in Southeast Asian countries has a high prevalence of the C-1858 variant, which is base-paired to nucleotide 1896 and prevents the occurrence of the A1896 mutant [Lok et al.,

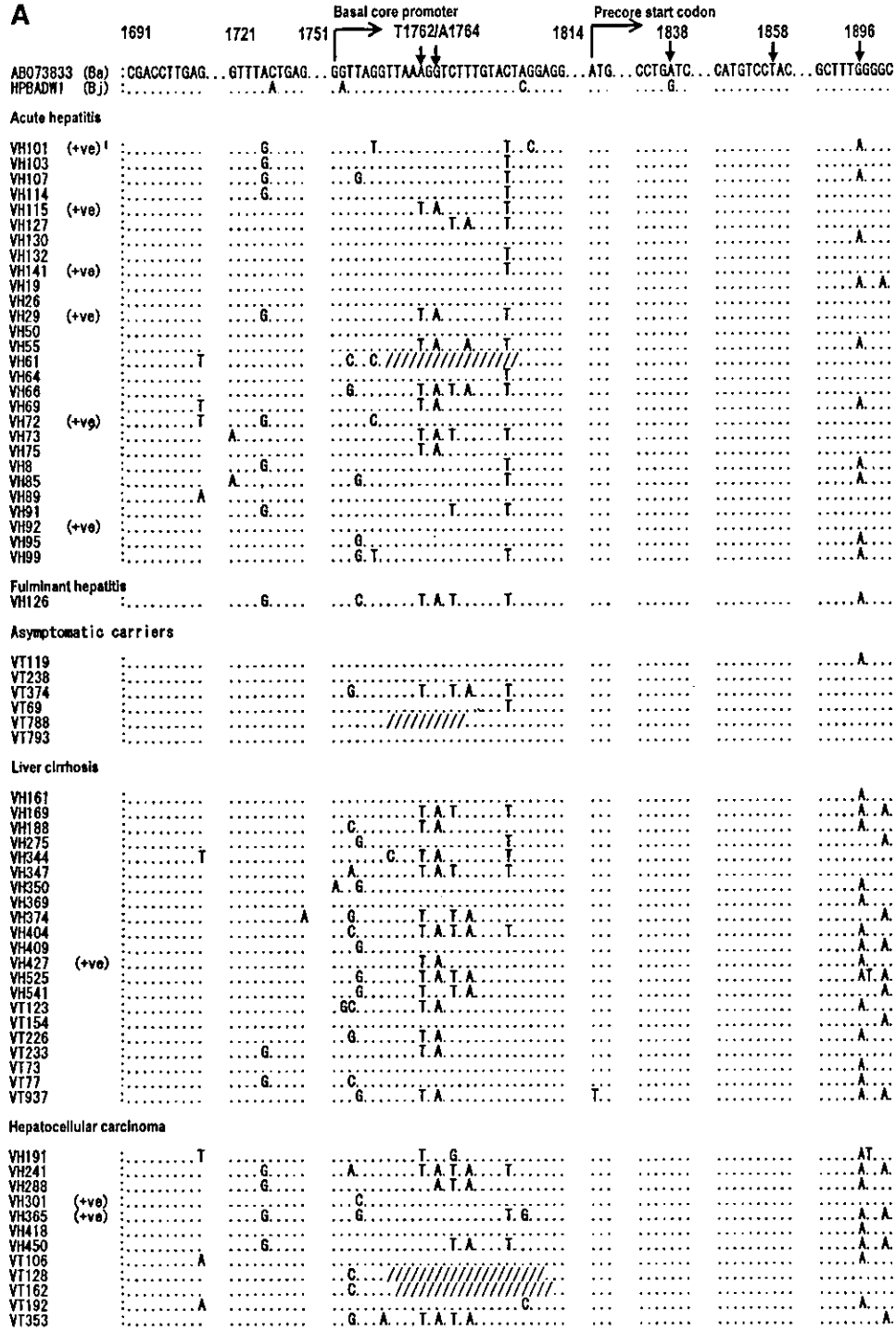

Fig. 1. Alignment of the CP/PC region (nt 1689–1970) from 68 Vietnamese genotype B (A) and 47 genotype C isolates (B). Dots represented nucleotides identical to the consensus sequences while deletions were represented as forward slashes. Isolates from each genotype were grouped based on clinical diagnosis. The top line(s) in each figure were wild type consensus sequences using nucleotide numbered by Okamoto et al. [1988]. The consensus of Ba and Bj subgroup of genotype B reported by Sugauchi et al. [2002] were shown (Accession No. AB073833 and D50521, respectively). The consensus of genotype C was the one reported in Japan (Accession No. D50517). Specific changes from G to A in nucleotide 1721 and 1727 were found in genotype C detected in Vietnam.

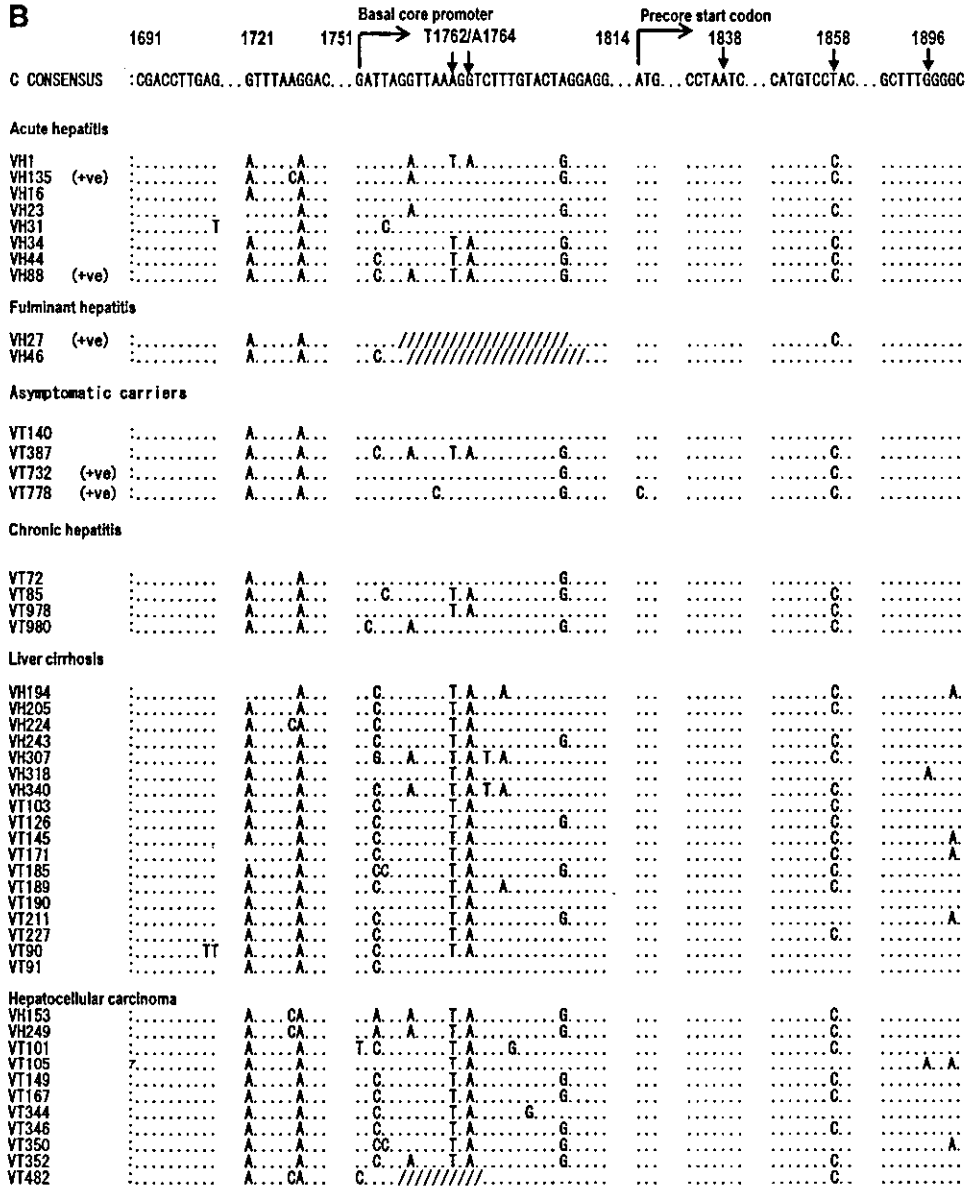

Fig. 1. (*Continued*)

1994]. C-1858 was also thought to correlate with the CP mutants [Chan et al., 1999]. In the present study, about 70% of genotype C isolates in Vietnamese patients possessed this variant. The result was remarkably different from a recent study in Japan [Kobayashi et al., 2004], in which no C-1858 variant was found in either genotype B or C. One of the possibly explanations is that genotypes B and C in Southeast Asia belong to subgroups that have a distinctive phylogenetic entity [Sugauchi et al., 2002; Huy et al., 2004]. In fact, the consensus nucleotide sequence at nt 1838 (genotype B, Ba specific) [Sugauchi et al., 2003] or nt 1721 and nt 1727 (genotype C) supports the notion that HBV genotypes B and C detected in Vietnam belong to subgroups of genotype B and C. In addition, phylogenetic tree analysis showed that C-1858 isolates have been grouped to each other, but cannot form a unique subgroup of C-1858 in genotype C (Fig. 2) as mentioned previously [Alestig et al., 2001]. In the present study, the analysed region is rather short (282 nucleotides) and partly covered the region of genotype B and C recombination in Southeast Asia (genotype Ba), which is spanned from nt 1740 to 2443 [Sugauchi et al., 2003]. In fact, all genotype B in this study were confirmed as subgroup Ba (data not shown). The resulting sub-branches reflect the recombination of genotype B and C in this region;

TABLE III. Core Promoter and Precore Mutant in Chronic HBV Infected Patients

	Core promoter (CP) region[a]			Precore region	
	T1762/A1764	T1762 alone	Deletion in CP	C-1858	A1896
Genotype B (n = 39)	13 (33.3)*	4 (10.2)	3 (7.6)	0***	23 (58.9)**
ASC (n = 6)	0	1 (16.6)	1 (16.6)	0	1 (16.6)
LC (n = 21)	11 (52.3)	2 (9.5)	0	0	14 (66.6)†
HCC (n = 12)	2 (16.6)	1 (8.3)	2 (16.6)	0	8 (66.6)†
Genotype C (n = 37)	30 (81.0)*	0	1 (27.0)	26 (70.2)***	2 (5.4)**
ASC (n = 4)	1 (25.0)	0	0	3 (75.0)	0
CH (n = 4)	2 (50.0)	0	0	3 (75.0)	0
LC (n = 18)	17 (94.4)****	0	0	12 (66.6)	1 (5.5)
HCC (n = 11)	10 (90.9)****	0	1 (9.0)	8 (72.7)	1 (9.0)

[a]Number of cases with percentage in parenthesis.
*$P < 0.001$.
**$P < 0.001$.
***$P < 0.0001$.
****$P \lesssim 0.01$.
†$P < 0.05$.

therefore it was associated with the low bootstrap values to differentiate the two genotypes; as well as the C-1858 variant.

Seven cases of deletion mutants, which spanned the TA-rich regions of the CP region, were found. These deletions have been known to result in a frame-shift and/or truncation of the X protein at the C terminal end [Kidd-Ljunggren et al., 1997]. Although two out of three cases of fulminant hepatitis had a deletion in the CP region, this mutant was also found in other diagnoses, and might have no significant role [Kramvis and Kew, 1999]. G1899A, a mutant that changes glycine at codon 29 to aspartic acid, has been linked to G1896A [Yuan et al., 1995]. However, in the present study, G1899A occurred independently with G1896A in 5/13 cases (38.4%) of genotype B and 5/6 cases (83.3%) of genotype C, respectively. Therefore, its role is not clearly identified in Vietnamese isolates.

In this study, the correlation between the HBV DNA level and CP/PC mutant was unclear in both the acute and chronic groups, although the HBV DNA level was found to be insignificantly lower in CP mutant isolates; and higher in the PC mutant isolates. In addition, the HBV DNA level was not significantly different between the acute and chronic states; and was in a lower range than in the previous studies, in which the level was usually around 10^8 copies/ml in patients with HBeAg +ve [Lindh et al., 1999]. One explanation relates to the time of collecting serum samples from these patients in the acute group. In the present study, up to 10 cases of acute group had A1896. These low viral titer samples might be approaching the period of seroconversion [Parekh et al., 2003] at the time of investigation.

In conclusion, mutants in the HBV CP/PC regions prevailed in chronic and acute hepatitis B patients in Vietnam. In chronic infection, CP mutants, especially

TABLE IV. Characteristic of CP/PC Mutant in Both Acute and Chronic Forms

	Core promoter		Precore	
	WT	MUT	WT	MUT
Acute forms				
Age[a]	34.7 (14.4)	36 (10.1)	33.6 (13.7)	39.0 (9.8)
HBeAg (+ve/−ve)	5/19**	4/11**	8/21	1/9
HBV DNA	5.27 (1.44)	5.78 (1.17)	5.34 (1.29)	5.81 (1.46)
ALT	1,171 (966)	959 (772)	1,120 (975)	1,011 (667)
Chronic Forms				
Age	46.8 (18.0)*	52.9 (11.9)*	50.5 (15.4)	51.7 (12.2)
HBeAg (+ve/−ve)	4/21***	1/50***	3/48	2/23
HBV DNA	5.50 (1.22)****	4.77 (1.15)****	4.69 (1.08)†	5.42 (1.29)†
ALT	91 (177)	81 (81)	69 (60)‡	116 (190)‡

[a]Age, ALT, HBV DNA were denoted in mean with the standard deviation in parenthesis; HBeAg was denoted in number of cases.
*$P = 0.465$.
**$P = 0.734$.
***$P < 0.05$.
****$P = 0.605$.
†$P = 0.490$.
‡$P = 0.606$.

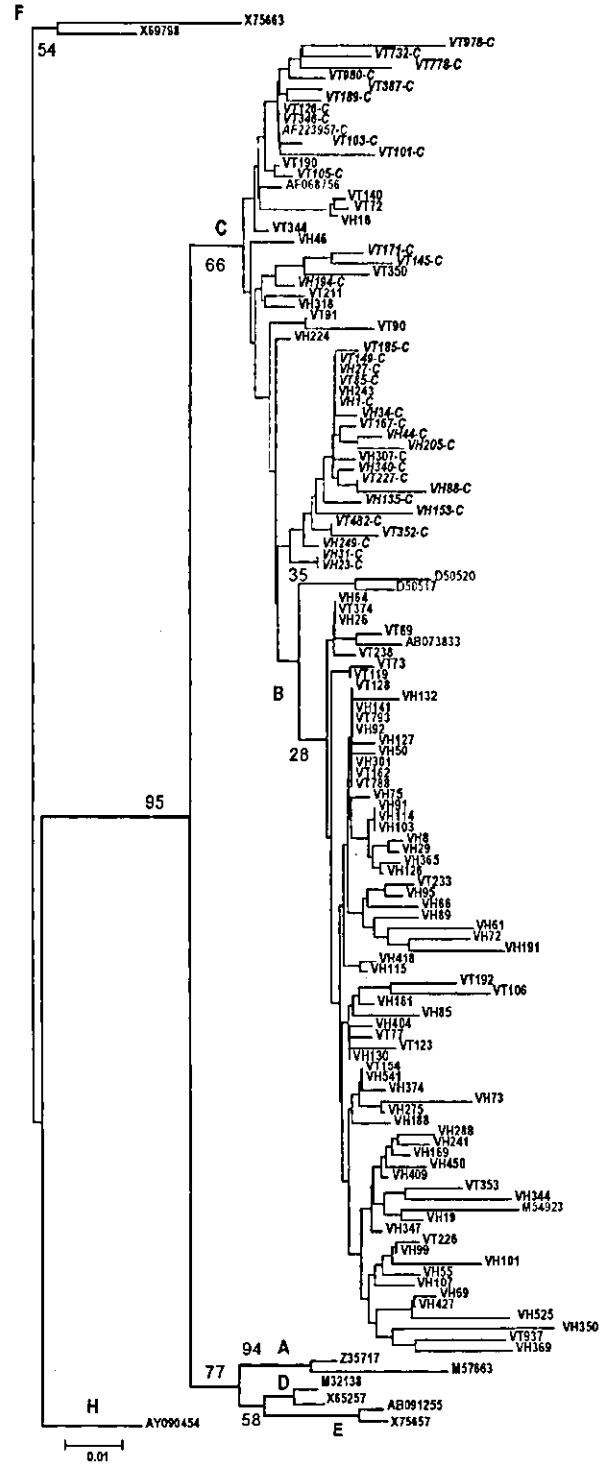

Fig. 2. Phylogenetic tree. This tree was constructed by the neighbour-joining method based on the partial nucleotide sequence of the CP/PC gene (282 nt, from nt 1691–1972) of 115 HBV Vietnamese isolates (VH and VT) and 15 reported isolates from genotype A–H in database. Bootstrap values were indicated in major branches and subbranches of genotype B and C. Genotype G was excluded from this analysis due to their common 36-nucleotide insertion in the core gene. Sequences of genotype C written in italic-C were those with C-1858.

the T1762/A1764 double mutant, were linked to genotype C of HBV, which had a high rate of C-1858 variants and could be associated with the more severe diseases. In acute infection, the influence of HBV genotypes on CP mutants was not clear, although genotype B, possessing a higher rate of the A1896 mutant, was linked to acute hepatitis manifestation in Vietnam.

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REFERENCES

Alestig E, Hannoun C, Horal P, Lindh M. 2001. Phylogenetic origin of hepatitis B virus strains with precore C-1858 variant. J Clin Microbiol 39:3200–3203.

Chan HL, Hussain M, Lok AS. 1999. Different hepatitis B virus genotypes are associated with different mutations in the core promoter and precore regions during hepatitis B e antigen seroconversion. Hepatology 29:976–984.

Chan HL, Leung NW, Hussain M, Wong ML, Lok AS. 2000. Hepatitis B e antigen-negative chronic hepatitis B in Hong Kong. Hepatology 31:763–768.

Chen RW, Piiparinen H, Seppanen M, Koskela P, Sarna S, Lappalainen M. 2001. Real-time PCR for detection and quantitation of hepatitis B virus DNA. J Med Virol 65:250–256.

Chu CJ, Hussain M, Lok AS. 2002. Hepatitis B virus genotype B is associated with earlier HBeAg seroconversion compared with hepatitis B virus genotype C. Gastroenterology 122:1756–1762.

Fang ZL, Yang J, Ge X, Zhuang H, Gong J, Li R, Ling R, Harrison TJ. 2002. Core promoter mutations (A(1762)T and G(1764)A) and viral genotype in chronic hepatitis B and hepatocellular carcinoma in Guangxi, China. J Med Virol 68:33–40.

Funk ML, Rosenberg DM, Lok AS. 2002. World-wide epidemiology of HBeAg-negative chronic hepatitis B and associated precore and core promoter variants. J Viral Hepat 9:52–61.

Hipgrave DB, Van NT, Huong VM, Long HT, Dat DT, Trung TN, Jolley D, Maynard JE, Biggs B-A. 2003. Hepatitis B infection in rural Vietnam and the implications for a national program of infant immunization. Am J Trop Med Hyg 69:288–294.

Hunt C, McGill J, Allen M, Condreay L. 2000. Clinical relevance of hepatitis B viral mutations. Hepatology 31:1037–1044.

Huy TT-T, Ushijima H, Quang VX, Win KM, Luengrojanakul P, Kikuchi K, Sata T, Abe K. 2004. Genotype C of hepatitis B virus can be classified into at least two subgroups. J Gen Virol 85:283–292.

Imamura T, Yokosuka O, Kurihara T, Kanda T, Fukai K, Imazeki F, Saisho H. 2003. Distribution of hepatitis B viral genotypes and mutations in the core promoter and precore regions in acute forms of liver disease in patients from Chiba, Japan. Gut 52:1630–1637.

Kao JH, Chen PJ, Lai MY, Chen DS. 2002. Genotypes and clinical phenotypes of hepatitis B virus in patients with chronic hepatitis B virus infection. J Clin Microbiol 40:1207–1209.

Kidd-Ljunggren K, Oberg M, Kidd AH. 1997. Hepatitis B virus X gene 1751 to 1764 mutations: Implications for HBeAg status and disease. J Gen Virol 78:1469–1478.

Kobayashi M, Arase Y, Ikeda K, Tsubota A, Suzuki Y, Saitoh S, Suzuki F, Akuta N, Hosaka T, Someya T, Matsuda M, Sato J, Miyakawa Y, Kumada H. 2004. Wild-type precore and core promoter sequences in patients with acute self-limited or chronic hepatitis B. Scand J Gastroenterol 39:53–59.

Kramvis A, Kew MC. 1999. The core promoter of hepatitis B virus. J Viral Hepat 6:415–427.

Kumar S, Tamura K, Jakobsen IB, Nei M. 2001. MEGA2: Molecular evolutionary genetics analysis software. Bioinformatics 17:1244–1245.

Lee WM. 1997. Hepatitis B virus infection. N Engl J Med 337:1733–1745.

Lindh M, Hannoun C, Dhillon AP, Norkrans G, Horal P. 1999. Core promoter mutations and genotypes in relation to viral replication

and liver damage in East Asian hepatitis B virus carriers. J Infect Dis 179:775–782.

Lok AS. 2000. Hepatitis B infection: Pathogenesis and management. J Hepatol 32:89–97.

Lok AS, Akarca U, Greene S. 1994. Mutations in the pre-core region of hepatitis B virus serve to enhance the stability of the secondary structure of the pre-genome encapsidation signal. Proc Natl Acad Sci USA 91:4077–4081.

Lok AS, Hussain M, Cursano C, Margotti M, Gramenzi A, Grazi GL, Jovine E, Benardi M, Andreone P. 2000. Evolution of hepatitis B virus polymerase gene mutations in hepatitis B e antigen-negative patients receiving lamivudine therapy. Hepatology 32:1145–1153.

Naito H, Hayashi S, Abe K. 2001. Rapid and specific genotyping system for hepatitis B virus corresponding to six major genotypes by PCR using type-specific primers. J Clin Microbiol 39:362–364.

Nakata S, Song P, Duc DD, Nguyen XQ, Murata K, Tsuda F, Okamoto H. 1994. Hepatitis C and B virus infections in populations at low or high risk in Ho Chi Minh and Hanoi, Vietnam. J Gastroenterol Hepatol 9:416–419.

Ngoanle T, Mizoue T, Yoshimura T. 2002. Estimates of cancer mortality in Hanoi and Ho Chi Minh City, Viet Nam in the 1990s. J Epidemiol 12:179–187.

Okamoto H, Tsuda F, Sakugawa H, Sastrosoewignjo RI, Imai M, Miyakawa Y, Mayumi M. 1988. Typing hepatitis B virus by homology in nucleotide sequence: Comparison of surface antigen subtypes. J Gen Virol 69:2575–2583.

Parekh S, Zoulim F, Ahn SH, Tsai A, Li J, Kawai S, Khan N, Trepo C, Wands J, Tong S. 2003. Genome replication, virion secretion, and e antigen expression of naturally occurring hepatitis B virus core promoter mutants. J Virol 77:6601–6612.

Sugauchi F, Orito E, Ichida T, Kato H, Sakugawa H, Kakumu S, Ishida T, Chutaputti A, Lai CL, Ueda R, Miyakawa Y, Mizokami M. 2002. Hepatitis B virus of genotype B with or without recombination with genotype C over the precore region plus the core gene. J Virol 76:5985–5992.

Sugauchi F, Orito E, Ichida T, Kato H, Sakugawa H, Kakumu S, Ishida T, Chutaputti A, Lai CL, Gish RG, Ueda R, Miyakawa Y, Mizokami M. 2003. Epidemiologic and virologic characteristics of hepatitis B virus genotype B having the recombination with genotype C. Gastroenterology 124:925–932.

Takahashi K, Aoyama K, Ohno N, Iwata K, Akahane Y, Baba K, Yoshizawa H, Mishiro S. 1995. The precore/core promoter mutant (T1762A1764) of hepatitis B virus: Clinical significance and an easy method for detection. J Gen Virol 76:3159–3164.

Tong SP, Li JS, Vitvitski L, Trepo C. 1992. Replication capacities of natural and artificial precore stop codon mutants of hepatitis B virus: Relevance of pregenome encapsidation signal. Virology 191:237–245.

Tran VB, Buu M, Nguyenthi M, Morris GE. 1993. Hepatitis B in Ho Chi Minh City, Viet Nam. Trans R Soc Trop Med Hyg 87:262.

Tran HT, Ushijima H, Quang VX, Phuong N, Li TC, Hayashi S, Xuan Lien T, Sata T, Abe K. 2003. Prevalence of hepatitis virus types B through E and genotypic distribution of HBV and HCV in Ho Chi Minh City, Vietnam. Hepatol Res 26:275–280.

Yotsuyanagi H, Hino K, Tomita E, Toyoda J, Yasuda K, Iino S. 2002. Precore and core promoter mutations, hepatitis B virus DNA levels and progressive liver injury in chronic hepatitis B. J Hepatol 37:355–363.

Yuan TT, Faruqi A, Shih JW, Shih C. 1995. The mechanism of natural occurrence of two closely linked HBV precore predominant mutations. Virology 211:144–156.

Yuen MF, Sablon E, Wong DK, Yuan HJ, Wong BC, Chan AO, Lai CL. 2003a. Role of hepatitis B virus genotypes in chronic hepatitis B exacerbation. Clin Infect Dis 37:593–597.

Yuen MF, Sablon E, Yuan HJ, Wong DK, Hui CK, Wong BC, Chan AO, Lai CL. 2003b. Significance of hepatitis B genotype in acute exacerbation, HBeAg seroconversion, cirrhosis-related complications, and hepatocellular carcinoma. Hepatology 37:562–567.

＜速　報＞

C型慢性肝炎における遺伝子発現－cDNAマイクロアレイを用いて－

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はじめに：C型慢性肝炎では，リンパ球を主体とする免疫反応の関与が想定されている．我々は，C型慢性肝炎患者の末梢血リンパ球の網羅的な遺伝子発現プロファイルの検討を行った．

方法：各施設の倫理委員会の承認下に，文書で同意を得たC型慢性肝炎10例を検討した．男女比7：3，平均年齢50±9歳(35～61歳)で，ALT平均139±97 IU/l(33～309 IU/l)，HCV RNA平均237±258 KIU/ml (0.8～850＜KIU/ml)，HCV血清型はgroup 1，2それぞれ5例だった．肝生検8例の組織所見は，F 0～1，2，3がそれぞれ2，4，2例，A 1，2，3がそれぞれ3，3，2例だった．cDNAマイクロアレイはClontech社製のAtlas™ Human 1.2 Arraysで，1枚に1,176個の遺伝子cDNAがプロットされている汎用チップである．患者末梢血リンパ球と対照のmRNAをそれぞれCy 3，Cy 5で蛍光標識して反応させ，両者の蛍光強度比(発現比)を求めた．対照の2倍以上を活性化された遺伝子，0.5倍以下を抑制された遺伝子と見做した．なお，対照mRNAは健常者30例の末梢血を混合したリンパ球から抽出した．

結果と考察(表1)：活性化された遺伝子のうち共通性が最も高いのはmigration inhibitory factor-related protein 14(MRP-14)で10例中6例に認めた．次いで，interleukin-8(IL-8)precursorと，puromycin-sensitive amino-peptidase(PSA)とを10例中5例に認めた．MRP-14は乾癬や関節炎などの慢性炎症に関係し[1]，IL-8はC型慢性肝炎患者血中で高値を示し[2]，PSAは細胞増殖への関与が知られている[3]．抑制された遺伝子で最も共通性が高いのはplatelete basic protein(PBP)precursorで10例中7例に認めた．次いで，monoamine oxidase(MAO-A)が10例中5例に認められた．PBPは血小板α顆粒から放出後に活性化されてneutrophil activating peptide 2(NAP 2)となり[4]，MAO-Aはセロトニンなどの代謝に関わる[5]．以上の結果は，C型慢性肝炎の肝組織を用いたHondaらの報告とは共通点がなかった[6]．彼らは肝生検組織を検体とし健常人肝組織を対照とした点，自製のcDNAマイクロアレイを用いた点で我々と異なっていた．次に，症例2－5，10は遺伝子発現プロファイルが類似しているが，これらの症例のHCV血清型やRNA量，ALT値，肝組織など臨床的背景には共通点がなかった．今後さらに，遺伝子発現プロファイルと臨床経過などとの関連を検討する必要がある．

索引用語：C型慢性肝炎，cDNAマイクロアレイ，遺伝子発現

文献：1) Odink K, et al：Nature 330：80－82, 1987　2) Polyak SJ, et al：J Virol 75：6209－11, 2001　3) Constam DB, et al：J Biol Chem 270：26931－39, 1995　4) Ehlert JE, et al：Blood 96：2965－72, 2000　5) Li M, et al：Protein Express Purif 24：152－62, 2002　6) Honda M, et al：Gastroenterology 120：955－66, 2001

表1　対照に比して遺伝子発現の異なる遺伝子と発現比

(1) 発現比が2倍以上(網かけ)の遺伝子										
	症例1	2	3	4	5	6	7	8	9	10
MRP-14	2.89	2.42	2.31	2.94	4.50	1.81	0.87	0.76	0.32	3.02
IL-8 prec.	1.13	17.54	3.81	5.84	4.90	1.37	0.95	0.48	1.23	4.60
PSA	0.96	2.30	5.10	2.04	1.18	2.26	1.94	0.83	1.19	2.26
(2) 発現比が0.5倍以下(下線)の遺伝子										
	症例1	2	3	4	5	6	7	8	9	10
PBP prec.	1.50	0.46	0.40	0.40	0.44	0.43	0.83	0.69	0.31	0.42
MAO-A	1.16	0.46	0.11	0.24	0.69	0.40	1.87	0.67	1.24	0.39

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Changes in virus loads and precore mutations in chronic hepatitis B patients treated with 4 weeks of daily interferon alfa-2a therapy

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Abstract

Interferon (IFN) alfa-2a was administered to 23 patients with chronic hepatitis B daily for 4 weeks and the relation between the efficacy of the treatment and changes in total hepatitis B virus (HBV) DNA and precore mutant levels was investigated. At 6 and 12 months after the completion of IFN therapy, 39.1% (9/23) and 36.8% (7/19) of patients, respectively, showed alanine transaminase (ALT) normalization; 31.3% (5/16) and 50.0% (7/14), respectively, became negative for HBe-antigen (HBeAg); and 42.1% (8/19) and 41.2% (7/17), respectively, became undetectable for HBV DNA. All 18 of the patients who were positive for HBeAg at baseline nevertheless had the precore mutation. The level of precore mutant as a proportion of the total HBV DNA level was constant at baseline, and 3 and 6 months after the completion of therapy. Thus, the investigation showed that in chronic hepatitis B, the precore mutation occurs at a constant proportion beginning in the HBeAg-positive phase, and IFN therapy inhibits the growth of the wild-type and precore mutant viruses equally.

Keywords: Chronic hepatitis B; rIFNα-2a; HBV DNA; Precore mutant

1. Introduction

Drugs that have been used to treat chronic hepatitis B include interferon (IFN), propagermanium, and steroids. Recently, lamivudine has been introduced and its use in combination with IFN has attracted interest. IFN therapy was first reported by Greenberg et al. [1] in 1976, whose work shows that IFN inhibits viral growth. In the US and Europe, IFN monotherapy generally consists of long-term administration of 5–10MU per day three times per week for 4–6 months [2,3]. In Japan, in 1986, the National Health Insurance coverage established 4 weeks as the standard treatment period. Consequently, in the present study, rIFN alfa-2a was administered daily for 4 weeks at a dose of 9MU per day for the first 3 days and 18MU per day thereafter.

The efficacy of IFN therapy is estimated by seroconversion from HBe-antigen (HBeAg) to HBe-antibody (HBeAb), undetectable response for hepatitis B virus (HBV) DNA, and normalization of alanine transaminase (ALT). Factors reported to be associated with response to IFN therapy are the baseline levels of HBV DNA and ALT [2,3]. Moreover, the clinical significance of infection with the precore mutant virus, which does not produce HBeAg, has recently drawn attention. We therefore, quantitatively analyzed precore mutant levels and examined the changes in these levels with IFN monotherapy.

2. Materials and methods

The subjects were 23 patients with chronic hepatitis B, 16 males and 7 females, with a mean age of 36.3 ± 9.8 years. Eighteen of the patients were positive for HBeAg and five were negative. Although all 23 patients were positive for HBV DNA in the polymerase chain reaction (PCR) assay, two patients were below detection limits by the bDNA probe assay. The precore mutant level was not less than 10^7 copies/ml in 12 patients and less than 10^7 copies/ml in 11

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Table 1
Baseline characteristics of patients

Patients number	Sex	Age	Grading[a]	Staging[a]	Interval from prior IFN (Month)	ALT (IU/l)	HBeAg (index)	HBeAb (%)	HBVDNA bDNA-p (Meq/ml)	HBVDNA PCR (copy/ml)	Precore mutant (copy/ml)
1	Female	35	–	–	38	287	0.8	96.4	96	8×10^7	8×10^7
2	Male	46	A1	F1	–	67	0.8	95.1	<0.7	5×10^3	<100
3	Female	52	A2	F1	10	310	179.8	0	1900	3×10^8	3×10^8
4	Female	29	–	–	24	304	3.6	57.8	3.4	3×10^6	3×10^6
5	Male	34	A1	F0	–	74	53.5	0	44	3×10^7	8×10^6
6	Female	22	A1	F1	–	259	407.7	0	78	3×10^7	3×10^7
7	Female	34	A1	F1	7	116	385.4	0	190	7×10^7	4×10^7
8	Male	43	A2	F2	14	206	113.3	0	1800	4×10^8	7×10^8
9	Male	56	A3	F2	–	148	7.2	58.0	1.6	9×10^5	9×10^5
10	Male	33	A1	F0	–	142	271.9	0	350	1×10^8	1×10^5
11	Male	33	–	–	41	87	292.2	0	150	3×10^7	3×10^7
12	Male	40	A2	F0	–	995	451.8	0	2100	4×10^8	4×10^8
13	Male	28	A1	F1	–	42	0.8	82.1	710	8×10^8	8×10^8
14	Male	44	A2	F2	49	57	1.8	17.2	2.8	5×10^6	8×10^5
15	Female	30	A2	F1	–	211	183.4	0	3800<	8×10^8	8×10^8
16	Female	27	A2	F2	–	87	9.2	23.3	<0.7	9×10^4	5×10^3
17	Male	36	–	–	10	448	231.8	0	25	1×10^7	1×10^6
18	Male	33	A3	F3	7	44	5.2	54.7	1	3×10^6	1×10^6
19	Male	27	A1	F2	5	184	53.9	0	400	3×10^7	3×10^7
20	Male	58	A3	F3	13	367	0.8	87.6	74	3×10^7	3×10^7
21	Male	26	A1	F3	8	115	172.8	0	1000	2×10^8	1×10^8
22	Male	28	A2	F0	10	338	174.7	0	490	1×10^7	7×10^6
23	Male	42	–	–	96	191	2.2	78.6	2.1	1×10^5	5×10^3

[a] Five cases were not measured.

patients, 1 of whom had a precore mutant level of less than 10^2 copies/ml. Fourteen patients had previously received IFN therapy for intervals from 5 to 96 months, during which they had been administered 477MU daily for 4 weeks. Baseline ALT was 34 to 66 IU/l in three patients and not less than 67 IU/l in 20 patients. Eighteen patients underwent liver biopsy, of whom four had a fibrosis score of F0, six a score of F1, five a score of F2, and three a score of F3 (Table 1).

rIFN alfa-2a was initially administered at a dose of 9MU per day for three consecutive days and 18MU per day for the subsequent 25 days (total dose, 477MU). Excluded were patients who had received an antiviral agent or immunomodulator within 3 months before the study; those who had received an injectable agent containing glycyrrhizin/cysteine/glycine or shosaiko-to (Chinese herbal medicine) within 1 month before the study; and those with a white blood cell (WBC) count of less than 3000/mm^3 or a platelet count of less than 100,000/mm^3.

The virological tests performed were the total amount of HBV DNA, using a bDNA probe assay (Quantiplex, Chiron) and competitive polymerase chain reaction assay (nested-PCR, Otsuka Assay), and the HBV precore mutant levels, using a quantitative mutation-site specific polymerase chain reaction assay (PCR-MSSA, Otsuka Assay). Using PCR-MSSA assay, precore point mutation (G–A, 83rd base of precore region) was examined using a mutation-trapped oligonucleotide primer, which yields a polymerase chain reaction amplification product only with precore mutantsand within the detection limits of 10^2 to 10^9 copies/ml [4]. Each measurement was performed immediately before treatment initiation, at treatment completion, and 6 months after treatment completion. HBeAg and HBeAb levels were measured immediately before treatment initiation, at treatment completion, and 3, 6, and 12 months after treatment completion. They were measured by radioimmunoassay (RIA), and a cutoff index higher than 2.1 for HBeAg was judged to be positive, and an inhibition percent higher than 50 for HBeAb was judged to be positive. Liver histology findings were assessed according to the Knodell histologic activity index [5] and the Desment scoring system [6].

The efficacy of the treatment was evaluated at its completion and at 6 and 12 months after completion according to ALT normalization and loss of HBeAg and HBV DNA.

The statistical analysis was performed using Fisher's exact test and the Wilcoxon 2-sample test.

3. Results

3.1. Efficacy

The rate of patients with normalized ALT levels was 4.3% (1/23) at treatment completion, 39.1% (9/23) at 6 months after completion, and 36.8% (7/19) at 12 months after completion. Although all measurement rates changed during the follow-up, there were many cases with normalized ALT levels after the treatment completion. Of the patients who were positive for HBeAg at baseline, the rate of patients who