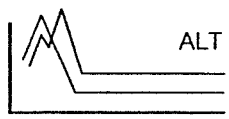
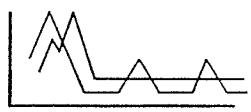
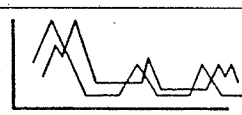
症例 11. ラミブジンを７年間投与したＢ型慢性肝炎症例（YMDD 変異ウイルス未出現）

●表１　ラミブジン長期投与例（７年以上）の反応性からみた治療成績

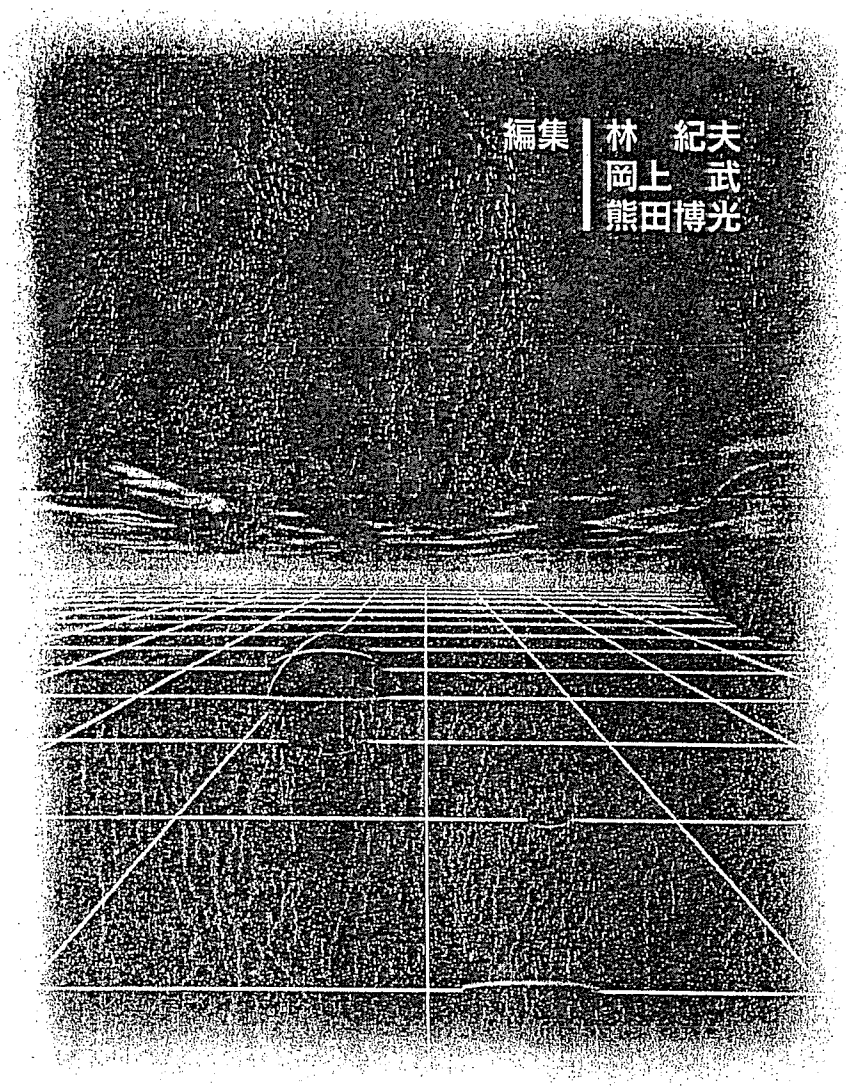
n = 14

治療反応性	ラミブジン開始時 HBe 抗原（＋）	ラミブジン開始時 HBe 抗原（−）	計
type 1 完全著効型 	2	5	7 (50.0%)
type 2 ウイルス再燃型 	1	1	2 (14.3%)
type 3 肝炎再燃型 	4	1	5 (35.7%)

果は明らかである。本例のように組織学的にも進行の認められるHBe 抗体陽性例には肝炎の鎮静化および組織学的改善を期待し，ラミブジン投与を試みるべきと考える。

C型慢性肝炎治療の新たなストラテジー

－インターフェロン治療の今後－



編集　林　紀夫
岡上　武
熊田博光

先端医学社

8

C型慢性肝炎の肝組織診断と治療

山田剛太郎

はじめに

慢性肝炎の長期予後の予測は，治療方針を決定するうえできわめて重要である．とくに，C型慢性肝炎患者は急速に高齢化を迎えており，肝発癌のリスクを考慮して治療戦略を決定する必要がある．ここ数年C型慢性肝炎の治療としてコンセンサスインターフェロン（コンセンサスIFN）やインターフェロン（IFN）/リバビリン（Rib）併用治療が新しく登場し，一方では従来型のIFN治療についても再投与や投与期間の枠が撤廃され，症例に応じた治療法の選択が可能となってきた．治療効果の改善とともに，症例によっては予想以上の副作用が認められることもあり，治療法の選択が非常に重要となっている．

したがって，C型慢性肝炎の治療戦略を決定するにあたっては，IFNなどの治療効果の予測に必要なC型肝炎ウイルス（hepatitis C virus：HCV）側因子に加えて，肝発癌リスクを予測する因子として年齢や肝線維化ステージ診断の重要性が増している．

そこでここでは，C型慢性肝炎の治療を進める際の肝組織診断の意義について述べる．

1. 肝組織診断によるC型慢性肝炎の経過観察

新犬山分類[1]を用いた線維化ステージ診断により，C型慢性肝炎における肝硬変への進展や肝細胞癌発症の自然経過が明らかにされてきた．それとともに，線維化ステージ診断による肝硬変への進展経過や肝発癌リスクなどの長期予後の予測が可能となっている．したがって，C型慢性肝炎の治療方針を決定する際には線維化ステージの判定がますます重要となっている．

1）肝線維化ステージ診断

慢性肝炎の肝組織診断は線維化のstagingと炎症のgradingによる．新犬山分類[1]では線維化ステージは以下の5段階に分類している．線維化なし：F0（fibrosis 0），門脈域の線維性拡大：F1，線維性架橋形成：F2，小葉のひずみを伴う線維性架橋形成：F3，偽小葉形成（肝硬変）：F4に分類する．Desmetら[2]の国際分類ではportal-portal septa（P-P結合）をF2に，portocentral septa（P-C結合）をF3の目安にしているが，線維化ステージ診断の基本的な考えは同じである．

2）血液検査による線維化ステージの予測

臨床の場では侵襲を伴う検査はできるだけ避け

表❶ 各種血液検査の肝線維化ステージの判別における有用性について
(山田剛太郎, 2002[3]より引用)

線維化ステージ	血小板数 ($\times10^4/\mu l$)	ICG クリアランス	アルブミン (g/dl)	PT (%)	ヒアルロン酸 (ng/ml)	AST/ALT 比
F0 (n=11)	21.8±3.9	0.165±0.027	4.6±0.3	95.9±17.2	51.3±54.0	0.947±0.410
F1 (n=200)	18.7±5.2	0.164±0.038	4.3±0.4	88.5±18.2	85.4±129.7	0.793±0.278
F2 (n=118)	15.8±4.9	0.153±0.032	4.2±0.3	79.1±19.6	101.1±71.9	0.814±0.231
F3 (n=70)	12.7±4.3	0.132±0.027	4.0±0.3	78.1±19.2	201.5±283.3	0.912±0.322
F4 (n=46)	9.7±3.6	0.110±0.037	3.8±0.5	61.3±14.6	255.6±141.4	1.016±0.369
ステージとの相関係数	r=0.552	r=0.466	r=0.414	r=0.407	r=0.349	r=0.208

肝線維化ステージ診断と各種血液検査との相関を検討したが，血小板数が最もよい相関を認めた．

たいとの願いから，末梢血液検査や肝生化学検査による肝線維化ステージの判別が検討されてきた．著者ら[3]もC型慢性肝炎455例で，血小板数，ICGクリアランス，血清アルブミン，プロトロンビン時間(PT)，ヒアルロン酸，AST/ALT比と線維化ステージとの相関性について検討した．血小板数の減少，ICGクリアランスの低下，血清アルブミンの減少，PTの延長はそれぞれ線維化ステージとの間に相関性がみられたが，ヒアルロン酸の増加やAST/ALT比の低下は相関係数が低かった（表❶）[3]．

さらに各検査法がいずれのステージの判別に有用かをt検定で検討したが，血小板，ICGクリアランス，血清アルブミン，PTは慢性肝炎，肝硬変のいずれのステージ間でも判別に有用であった．しかしながら，ヒアルロン酸やAST/ALT比はF4ステージ（肝硬変）と慢性肝炎のF1，F2ステージ間の判別には有用であるが，慢性肝炎間でのステージ判別には適さなかった[3]．

3）線維化ステージの進展と病態

B型慢性肝炎では慢性肝炎から肝硬変への進展とともにB型肝炎ウイルス(HBV)量の減少が指摘されてきたが，C型肝炎から肝硬変ではHCV-RNA量の減少は認めないとする報告が多く，著者らの線維化ステージ別の検討でも減少傾向は認められない(表❷)．また，HCVの遺伝子型によって長期予後に差が認められるとの報告[4]もあるが必ずしも一致していない．著者らの検討でもステージの進展とともにセロタイプ2型の頻度が多少低くなる傾向を認めたが(表❷)，有意な差は得られていない．

C型慢性肝炎では線維化の進展とともに炎症所見が増強する例が多く，サイトカインも線維化の進展とともにTh1/Th2比が高まるとの報告が多い．IL-10などのTh2サイトカイン優位の状態では細胞性免疫応答が抑えられ，IFNγやIL-2などの産生が増加し，Th1サイトカインが優位の状態では炎症が増強するものと推測されている．著者ら[5]の肝組織中の検討ではケモカインである

表❷ C型慢性肝炎における肝線維化ステージ別のHCV-RNA量と遺伝子型

線維化ステージ	HCV-RNA量*	遺伝子型（%）		
		1	2	ND
F0	5.9±0.6	37.5	50.0	12.5
F1	6.5±0.8	52.6	46.1	1.3
F2	6.4±0.8	61.9	35.4	2.7
F3	6.6±0.7	56.8	38.6	4.5
F4	6.5±0.7	63.6	31.8	4.5

肝線維化ステージが進展しても平均HCV-RNA量に大差は認められない．線維化ステージの進展とともに遺伝子型2型の減少傾向がみられる．
＊分岐DNAプローブ法による（log Eq/ml）

表❸ C型慢性肝炎における肝線維化ステージ別肝硬変への進展率（平均年率）

線維化ステージ	平均年間進展率（%）
F1	2.2（0.9～3.4）
F2	3.2（1.7～4.9）
F3	8.6（5.8～11.4）

肝線維化ステージの進展とともに肝硬変への年間進展率が明らかに高まる．

IL-8の発現が線維化の進展とともに有意に増加しており，C型慢性肝炎の病態を考えるうえで興味深い．

4）肝硬変への進展予測

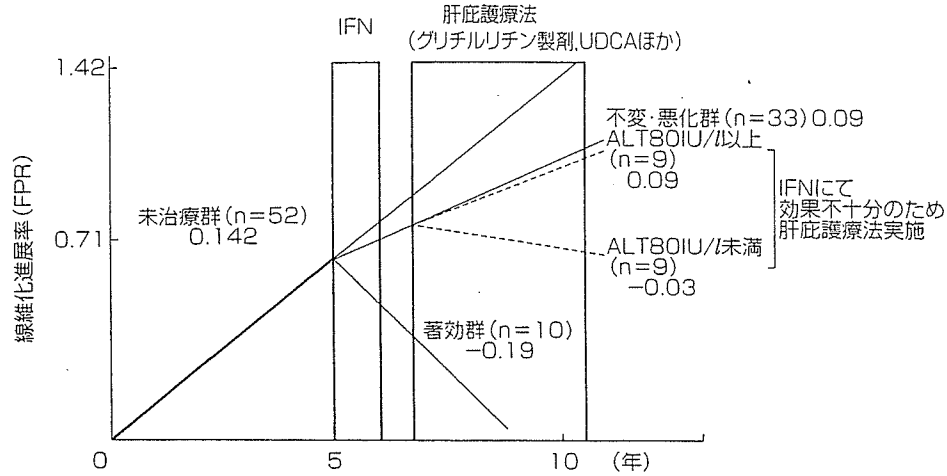
C型慢性肝炎から肝硬変への進展経過を，IFN治療を受けていないC型慢性肝炎で，くり返して肝生検を実施した57例で検討した[6]．初回の線維化ステージ診断別に肝硬変への進展率を計算すると，F1ステージでは平均11年で10%が，F2ステージでは平均9年で19%が，F3ステージでは平均7年で71%がそれぞれF4ステージへと進展した[6]．Yanoらの報告[7]では平均8.8年でF1の29.6%，F2の42.9%，F3の100%がそれぞれF4へ進展している．さらに，Kobayashiら[4]の報告では平均9.6年でF1の20%，F2の16%，F3の56%がF4へと進展している．したがって，ステージ別の肝硬変への進展率を年率に換算すると表❸に示すようにF1で0.9～3.4%，F2で1.7～4.9%，F3で5.8～11.4%である．

Poynardら[8]は2回の肝生検を基に，C型慢性肝炎における肝線維化の進展速度（fibrosis progression rate：FPR）を計算している．すなわち肝線維化ステージをF0-F4の5段階に分類し，2回の肝生検の線維化ステージの差を肝生検の間隔（年数）で除した値を年間あたりの平均進展率としている．自然経過例における平均進展率は0.133 fibrosis unitと報告している．すなわち，C型慢性肝炎では10年間に線維化ステージが平均1.3高まることになる．平均進展率を高める因子としては感染時の年齢（40歳以上），アルコール消費量（50 g/日以上），男女の性差をあげている．

著者らも，3年以上の間隔で2回肝生検を実施した自然経過例（未治療群）52例でFPRを算定したが0.142 fibrosis unitとほぼ同様の進展率である（図❶）．

5）肝細胞癌の発症リスク

C型慢性肝炎の肝生検診断では肝線維化ステージの進展とともに年間肝発癌率は高くなる．Yoshidaら[9]の多数例での検討（表❹）[9]をはじめとし



図❶ くり返し肝生検の観察によるインターフェロン治療と肝庇護療法による肝線維化進展率の抑制

C 型慢性肝炎の未治療群での肝線維化進展速度 0.142 に対して，IFN 治療を実施した症例では著効群では−0.19 とむしろ線維化は軽快し，不変・悪化群でも 0.09 と線維化進展速度が遅くなっている．不変・悪化群で肝庇護療法を受けた症例で ALT 80 IU/*l* を長期間持続した症例では−0.03 と線維化の進展は認められない．

Part 1

表❹ C 型慢性肝炎における肝線維化ステージ別肝細胞癌発症率（年率）(Yoshida H *et al*, 1999[9]より引用)

線維化ステージ	未治療群	IFN 治療群
F 0・F 1	0.45 (3/160)	0.08 (2/710)
F 2	1.99 (11/164)	0.54 (16/896)
F 3	5.34 (13/59)	1.95 (38/564)
F 4	7.88 (32/107)	4.16 (33/230)
計	3.17 (59/490)	1.10 (89/2400)

肝線維化ステージの進展とともに肝発癌リスクが高まる．IFN 治療群全体では肝発癌は明らかに減少するが，無効群ではなお発癌を認めるため，F 3，F 4 でのリスクはなお高い．

て多くの報告で肝硬変である F 4 ステージで年率 7〜8%，慢性肝炎の F 3 ステージで年率 3〜5%の発癌が認められ，著者[10]の検討でも同様である．C 型慢性肝炎の F 3，F 4 ステージはまさに肝細胞癌の高危険群としてその予防をめざした治療方針を急いでたてる必要がある．

2. IFN 治療における肝組織診断の有用性

1）IFN の治療効果因子

C 型慢性肝炎における IFN 治療効果は，HCV-RNA の持続的陰性化（SVR）率で評価する．そのような治療効果を左右する因子としてはウイルス側因子と宿主側因子がある．一番重要な因子は HCV-RNA 量と遺伝子型である．しかし，遺伝子型 2 型かつ低ウイルス量の症例でも難治例があり，逆に 1 型で 20 Meq/m*l* 以上の高ウイルス量でも，SVR となる症例がある．固体側因子としては，線維化ステージの進展した F 3，F 4 症例や高齢者では SVR 率が低下するとの報告が多い．

最近 6 年間に従来型の IFN 製剤による単独治療を実施した 183 例を遺伝子型と HCV-RNA 量によって 4 群に分けた後，肝線維化ステージを F 0-2 の非進展群と，F 3-4 の進展群に分けて比較した（表❺）．この検討結果より遺伝子型 2 型かつ高ウイルス群では肝線維化の進展の有無が IFN

表❺ C型慢性肝炎における遺伝子型，HCV-RNA量，肝線維化ステージとインターフェロンの治療効果
IFN単独治療症例を遺伝子型，HCV-RNA量別に4群に分けて，線維化ステージによる治療効果を比較した．遺伝子型2型で1 Meq/mℓ以上の高ウイルス量群では肝線維化ステージの進展が治療効果に大きく影響する．

遺伝子型 / HCV-RNA量 / 肝線維化ステージ	1型		2型	
	1 Meq/mℓ以上	1 Meq/mℓ未満	1 Meq/mℓ以上	1 Meq/mℓ未満
F 0-2	4/29 (13.8%)	15/23 (65.2%)	18/36 (50%)	38/46 (82.6%)
F 3-4	1/15 (6.7%)	3/5 (60%)	2/13 (15.4%)	13/16 (81.3%)
p値	0.49	0.83	0.03	0.9

の治療効果に大きな影響を与えることが判明した．したがって，遺伝子型2型かつ高ウイルス群でしかも線維化の進展したF3，F4症例における治療法の選択にあたってはIFN/Rib併用治療や6ヵ月以上の長期IFN単独治療を考えたい．

2.) IFN治療の長期予後

a) 肝線維化の改善

IFN治療によってSVRになった症例では炎症所見のみでなく，長期的には肝線維化の改善も明らかになっている．IFN治療群で3年間隔以上で2回肝生検を実施した症例をSVR群とそれ以外の難治群にに分けてFPRを比較した（図❶）．著効例では−0.19と線維化は逆に減少して明らかに改善する．すなわち，線維化ステージは5年間に1ステージずつの速度で軽快改善する．したがって，肝硬変の肝線維化は不可逆性と長年考えられてきたが，現在では初期の肝硬変でも，IFNによってHCVが完全に排除され，肝炎が根治すれば線維化は徐々に吸収改善することが判明している．さらに，IFN治療が無効であった例でも0.09と自然経過例の0.142に比較して，線維化進展は遅くなる．さらに，IFNにて効果不十分な多数の症例に長期にわたってグリチルリチン製剤やウルソデオキシコール酸（UDCA）による肝庇護療法を実施した[11]．血清ALTが持続的に80 IU/ℓ（年平均）以下で3年以上の間隔で2回肝組織像を検索できた症例では，FPRは−0.03と肝線維化の進展も抑制されていた．

b) 肝発癌の抑制

C型慢性肝炎のIFN治療の長期予後で，HCV-RNAの陰性化したSVR例ではもちろんのこと，HCV-RNA陽性ながら血清ALTの正常化した生化学的著効例でも肝細胞癌の発症は有意に少ない[9][10]．しかしながら，無効例では自然経過例に比較して発症頻度は減少するものの，線維化の進展したF3，F4ステージではなお発癌の機会は高い．著者らも，IFNの無効例を6年間の長期にわたってグリチルリチン製剤やUDCAによる肝庇護療法を実施し，ALTの改善に努めるとともに厳重な画像などのフォローアップを実施してきた．F3，F4ステージで種々の治療にかかわらず血清ALTの改善が認められない症例では，発癌率はなお高く，超高危険群と考えて画像などの厳重な追跡が必要である[11]．

3. 超高危険群に対する治療

IFN単独治療に加えて，IFN/Ribの併用治療が可能になったが，なお，遺伝子型1bかつ高ウイルス量でのSVRは20%程度である．したがって，かつてIFN単独治療受けたが無効であったF2，F3症例から，すでにF3，F4ステージへ進

展し，現在も血清 ALT の高値が持続して超高危険群ともよぶべき症例がなお多数存在する．しかも，このような症例は高齢化を迎え，血小板数も減少している症例が多く，IFN/Rib の併用治療が不可能な例も少なくない．

IFN の少量長期投与が血清 ALT の改善に有用なことが報告され，保険診療での IFN 治療の期間や再投与の制限が取り除かれた現状では，HCV の完全排除の難しい患者はもちろん，肝細胞癌のハイリスクステージである F 3, F 4 ステージにあるような高齢者に対しても，患者にやさしい少量長期投与法が期待できる．著者らも IFNα 250～300 万単位の週 2～3 回の少量投与をこのような症例に実施し，多くの症例で血清 ALT の改善を認めている．今後，肝発癌の抑制効果が期待できるものと考えられるが，長期予後を厳重に見守りたい．

おわりに

C 型慢性肝炎における治療の選択肢が増え，治療効果も改善しているが，一方では患者の高齢化が進み，副作用の少ない患者にやさしい治療法の選択が重要である．C 型慢性肝炎の治療方針をたてるうえでの肝組織診断の意義について紹介したが，今後は慢性肝炎の線維化ステージを正しく診断し，肝細胞癌発症に備えた長期予後を認識したうえで，個々の患者に応じたより細かな治療方針をたてる必要がある．

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C型慢性肝炎治療の新たなストラテジー
―インターフェロン治療の今後―

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編集者●林　紀夫
岡上　武
熊田　博光

発行者●鯨岡　哲

発行所　株式会社 先端医学社
〒103-0004　東京都中央区東日本橋1-9-7
G1東日本橋ビル
電　話　(03) 5820-2100(代)
FAX　(03) 5820-2501
振　替　00190-0-703930
http://www.sentan.com
E-mail:book@sentan.com
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Single nucleotide polymorphism of the MxA gene promoter influences the response to interferon monotherapy in patients with hepatitis C viral infection

F. Suzuki,[1] Y. Arase,[1] Y. Suzuki,[1] A. Tsubota,[1] N. Akuta,[1] T. Hosaka,[1] T. Someya,[1] M. Kobayashi,[1] S. Saitoh,[1] K. Ikeda,[1] M. Kobayashi,[2] M. Matsuda,[2] K. Takagi,[2] J. Satoh[2] and H. Kumada[1] [1]*Department of Gastroenterology, Toranomon Hospital, Tokyo; and* [2]*Research Institute for Hepatology, Toranomon Branch Hospital, Kawasaki, Japan*

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SUMMARY. The biological activity of interferon (IFN) is mediated by the induction of intracellular antiviral proteins, such as 2′–5′ oligoadenylate synthetase, dsRNA-activated protein kinase and MxA protein. Among these, MxA protein is assumed to be the most specific surrogate parameter for IFN action. This study was performed to elucidate whether a single nucleotide polymorphism (SNP) (G/T at nt-88) in the promoter region of the MxA gene influences the response to IFN therapy in patients with chronic hepatitis C virus (HCV) infection. Polymorphisms of the MxA gene in 235 HCV patients were determined by polymerase chain reaction-restriction fragment length polymorphism. The frequency of SNP was compared between sustained-responders ($n = 78$) and nonresponders ($n = 157$), as determined by biochemical and virological responses to IFN. Multivariate analysis showed that among all patients, HCV genotype, HCV RNA level and the SNP of the *MxA* gene were independent and significant determinants of the outcome of IFN therapy [odds ratio 3.8 (95% confidence interval 2.0–7.0), $P < 0.0001$; 0.27 (0.15–0.50), $P < 0.0001$; 1.8 (1.0–3.4), $P = 0.0464$, respectively]. Furthermore, among patients with a low viral load (≤2.0 Meq/mL), MxA-T-positive patients were more likely to show a sustained response compared with MxA-T-negative patients [2.87 (1.3–6.3); 62% *vs* 36%; $P = 0.0075$]. Our findings suggested that the SNP of the MxA gene is one of the important host factors that independently influences the response to IFN in patients with chronic HCV infection, especially those with a low viral load.

Keywords: chronic hepatitis C, interferon therapy, MxA promoter gene, single nucleotide polymorphism.

INTRODUCTION

Interferon (IFN) has been used for treatment of patients with chronic hepatitis C virus (HCV) infection. However, <30% of patients treated with IFN monotherapy show a sustained response [1, 2]. Specifically, viral factors, such as genotype 1 and high HCV RNA levels are associated with a higher incidence of resistance to IFN therapy [3]. Furthermore, several host factors, such as old age, advanced fibrosis and long duration of disease can also adversely influence the response to IFN treatment.

The difficulty encountered in the detection of IFN-α in sera may be due to the fact that the biological half-life of this cytokine is short (a few hours) and it can be cleared rapidly from the circulation. Biological activity of IFN is mediated, in part, by the induction of intracellular antiviral proteins, such as 2′–5′ oligoadenylate synthetase (2′–5′ OAS), dsRNA-activated protein kinase (PKR) and MxA protein. The MxA protein influences IFN-induced antiviral activities of host cells against several viruses [4, 5]. Moreover, the MxA protein is assumed to be the most specific surrogate parameter for IFN action [6, 7]. Some reports have shown that increasing the MxA protein or mRNA levels were related to response to IFN therapy [8, 9]. However, the levels of the MxA protein or mRNA during IFN therapy differ between individuals. One cause of these differences may be associated with genomic factors.

Abbreviations: ALT, alanine aminotransferase; HCV, hepatitis C virus; IFN, interferon; IL, interleukin; ISRE, IFN-stimulated response elements; LMP, low molecular mass polypeptide; NR, nonresponders; OAS, oligoadenylate synthetase; PCR, polymerase chain reaction; PKR, dsRNA-activated protein kinase; SNP, single nucleotide polymorphism; SR, sustained responders; TGF-β, transforming growth factor-β; TNF-α, tumour necrosis factor-α.

Correspondence: Fumitaka Suzuki, MD, Department of Gastroenterology, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku, Tokyo 105-8470, Japan. E-mail: fumitakas@toranomon.gr.jp

Matsushita *et al.* [10] showed an association between polymorphism of the mannose-binding lectin gene and IFN responsiveness of Japanese patients with chronic hepatitis C. This was associated with the role mannose-binding lectin plays in opsonization of HCV particles through glycosylated coat proteins on the virion's surface. Moreover, the same group recently showed that a single nucleotide polymorphism (SNP) (G/T at nt -88) in the promoter region of the *MxA* gene was associated with the IFN response of HCV patients [11]. Although these findings indicated that MxA protein might be an important factor in the response to IFN therapy in patients with HCV infection, there has been only one study to date about this SNP and IFN responsiveness. Moreover, in that study, HCV RNA level, which is an important factor for IFN response, was not taken into consideration.

In the present study, we investigated a large population of patients with chronic HCV infection, and demonstrated that the previously described SNP in the promoter region of the *MxA* gene is associated with the response to IFN therapy. Moreover, we analysed the relationship between HCV viral load, genotype and this SNP.

PATIENTS AND METHODS

Patients

From November 1990 to August 2001, 235 Japanese adult patients with chronic hepatitis C infection were recruited for this study. All patients were positive for both anti-HCV antibody and serum HCV RNA by polymerase chain reaction (PCR). IFN treatment and clinical follow-up were performed at the Department of Gastroenterology of Toranomon Hospital. Before IFN therapy, patients had persistently elevated serum alanine aminotransferase (ALT) levels for at least 6 months and none of them had evidence of hepatitis B virus co-infection or other liver diseases (e.g. alcoholic-induced or autoimmune hepatitis). None of the patients had received prior antiviral treatment such as IFN and/or ribavirin. A pretreatment liver biopsy was carried out for histopathological examination, and histological staging was performed according to the classification proposed by Desmet *et al.* [12]. All patients gave written informed consent to participate in this study.

Patients were treated with IFN-α or IFN-β for periods from 8 to 26 weeks. Patients received 3–10 million units (MU) of IFN every day for the first 2–8 weeks, after which most patients received IFN three times a week for the following 16–20 weeks. The total period of IFN treatment was within 26 weeks, with the exception of 10 patients who received a total dose of <2000 MU IFN. All patients were followed for at least 6 months after completion of IFN therapy. Sustained responders (SR; $n = 78$) were defined as patients who showed normalization of the ALT level and who tested negative for HCV RNA by Amplicor HCV RNA™ (Roche Molecular Systems, Nutley, NJ, USA) for at least 6 months after completion of IFN therapy. All other patients, who did not show SR, were considered nonresponders (NR; $n = 157$).

Hepatitis C virus genotyping and HCV RNA quantity

HCV genotypes were determined by PCR and were expressed according to the nomenclature proposed by Simmonds *et al.* [13]. Serum HCV RNA was quantified using a branched DNA assay (bDNA probe assay, version 2.0; Chiron, Dai-ichi Kagaku, Tokyo, Japan).

Determination of MxA promoter single nucleotide polymorphism

The SNP was determined by PCR-restriction fragment length polymorphism as described previously by Hijikata *et al.* [11]. In brief, genomic DNA from peripheral blood mononuclear cells was isolated by a GFX™ Genomic Blood DNA Purification Kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). In the next step, 1 μg of genomic DNA was amplified in a reaction mixture containing 10x PCR buffer (Applied Biosystems Japan, Tokyo, Japan), 1.5 mmol/L $MgCl_2$, 160 μmol/L of each deoxynucleosidetriphosphate, 1 μmol/L of primer (No. MXAF01 and No. MXAR02), and one unit of AmpliTaq Gold DNA polymerase (Applied Biosystems Japan). The amplified products were subjected to restriction enzyme (*Hha*I) digestion for 4 h. Samples were electrophoresed in agarose gels, stained with ethidium bromide and observed under UV transillumination.

Statistical analysis

Differences between groups were examined for statistical significance using the Mann–Whitney *U*-test and chi-squared test where appropriate. All calculations were performed using StatView software (Version 4.5J; Abacus Concepts, CA, USA). Independent predictive factors associated with SR to IFN treatment were studied using multivariate multiple logistic regression. Potential predictive factors for SR to IFN treatment that were assessed included the following eight variables: age, sex, HCV genotype, HCV RNA level, histopathological stage (mild or not), baseline ALT, total dose of IFN and MxA genotype. All factors found to be at least marginally associated with SR to IFN therapy ($P < 0.15$) were entered into the multivariate multiple logistic regression. Multivariate multiple logistic regression was performed using the Windows SPSS software package (SPSS Inc., Chicago, IL, USA). The odds ratio (OR) and 95% confidence interval (CI) were calculated to assess the relative risk confidence. A two-tailed *P*-value <0.05 was considered statistically significant.

RESULTS

Clinical and virological features of patients with chronic hepatitis C infection

Comparison of the clinical and virological characteristics between the SR and NR are summarized in Table 1. Among the total of 235 patients, histopathological staging was performed on 229 patients before treatment; liver biopsies could not be performed on the remaining six patients. The univariate analysis of clinical and virological factors showed that HCV genotype other than 1b (2a or 2b) [OR 3.4 (95% CI 1.9–6.1), $P < 0.0001$] and lower serum HCV RNA levels (≤2 Meq/mL) [OR 0.3 (95% CI 0.17–0.53), $P < 0.0001$] were associated with a higher probability of SR. There were no significant differences in the sex ratio, age, histopathological stage, total dose of IFN and ALT level before treatment between the two groups.

Comparison of the MxA promoter allele frequencies between sustained-responders and nonresponders

Polymorphism of the MxA promoter gene and allele frequencies between SR and NR were compared among all patients (Table 2). Allele frequency of the MxA promoter gene in all chronic hepatitis C patients was the same as in healthy controls. For healthy controls, we examined another 56 normal subjects and merged their data with those of the original group [11]. MxA promoter gene G/T and T/T heterozygous (MxA-T-positive) tended to be more frequent than G/G homozygous, albeit statistically insignificant. The univariate analysis of the MxA polymorphism (MxA-T-positive *vs*-negative) showed borderline significance with a higher chance of sustained response among all patients ($P = 0.11$).

Table 1 Characteristics of patients with chronic hepatitis C viral infection treated with interferon

	Sustained responders ($n = 78$)	Nonresponders ($n = 157$)
Age (years)	50 (20–83)	51 (22–66)
Sex (male/female)	53/25	92/65
Baseline ALT (IU/L)	81 (16–683)	94 (27–452)
HCV RNA level (Meq/mL)*	0.7 (0.4–38)	3.5 (0.4–48)
HCV genotype*		
1b	38 (49%)	120 (76%)
2a	33 (42%)	29 (18%)
2b	7 (9%)	8 (5%)
Histological stage		
Mild	45 (62%)	91 (58%)
Moderate	23 (32%)	47 (30%)
Severe	2 (3%)	11 (7%)
Cirrhosis	3 (4%)	7 (4%)
Total dose of interferon (MU)	625 (267–1432)	626 (256–1972)

Data are median (range), or number of patients. All HCV RNA values below the lower limit of detection ($<0.5 \times 10^6$ Meq/mL) were set to 0.4. Histological examination was not performed in five and one patients of the sustained responders and nonresponders, respectively.
*$P < 0.0001$, between responders and nonresponders.

Multivariate analysis of predictive factors for interferon treatment among all patients

The significance of the response to IFN therapy was investigated by multivariate logistic regression analysis. Both HCV RNA level and genotype independently and significantly influenced the outcome of IFN therapy (Table 3). Moreover, the SNP of the *MxA* gene was also independently associated with the outcome of IFN therapy. We also analysed the relationship between SNP of the MxA and HCV viral load or genotype (Tables 4 and 5). Table 4 shows that the SNP of the MxA did not correlate with the viral road and that the distribution of this SNP was unbalanced in each viral load. Therefore, this SNP was an independent factor as determined by multivariate logistic regression analysis.

The single nucleotide polymorphism of the MxA gene and interferon response among patients with low viral loads

Figure 1 shows the relationship among IFN response, viral load and the SNP of the *MxA*. Differences in viral load (high or low viral load) influenced the response to IFN therapy. Therefore, we analysed the response with respect to high and low viral loads.

Among the patients with a high viral load (>2.0 Meq/mL, $n = 127$), univariate analysis of clinical and virological factors showed that HCV genotype other than 1b (2a or 2b) [OR 5.1 (95% CI 2.1–12.8), $P = 0.0002$] was associated with a high probability of SR. Among this group, the SNP of the *MxA* gene was not related with SR. On the other hand, among the patients with a lower viral load (≤2 Meq/mL, $n = 108$), the univariate analysis of clinical and virological factors showed that the SNP of the *MxA* gene (MxA-T-positive) [OR 2.87 (95% CI 1.3–6.3), $P = 0.0075$] and HCV genotype other than 1b (2a or 2b) [OR 2.83 (95% CI 1.2–6.5), $P = 0.0121$] were associated with a higher probability of SR. These results indicated that the SNP of the *MxA* gene specifically influenced the efficacy of IFN treatment in patients with a low viral load.

DISCUSSION

Genetic studies to date have examined three aspects of HCV infection; viral clearance, liver disease progression

Table 2 Distribution of the MxA promoter genotypes in patients with hepatitis C viral infection treated with interferon

MxA promoter polymorphism at nt -88	Healthy controls*	All patients	Response to interferon	
			Sustained responders	Nonresponders
n	98	235	78	157
Alleles	196	470	156	314
Allele frequency				
G	143 (73%)	332 (71%)	106 (68%)	226 (72%)
T	53 (27%)	138 (29%)	50 (32%)	88 (28%)
Zygosity				
G/G heterozygous	51 (52%)	114 (49%)	32 (41%)	82 (52%)
G/T heterozygous	41 (42%)	104 (44%)	42 (54%)	62 (39%)
T/T heterozygous	6 (6%)	17 (7%)	4 (5%)	13 (8%)

*We included additional 56 normal subjects and reported the group data (by Hijikata et al. [11]) of the entire 98 subjects.

Table 3 Factors associated with response to interferon monotherapy

Variable	Multivariate odds ratio	95% Confidence interval*	*P*-value
HCV RNA levels (≤2 *vs* >2 Meq/mL)	0.27	0.15–0.50	<0.0001
Genotype (1b *vs* 2a or 2b)	3.8	2.0–7.0	<0.0001
The SNP of the MxA gene (G/G *vs* G/T or T/T)	1.8	1.0–3.4	0.0464

*Values are the odds of having a sustained response to interferon.

Table 4 Relationship between the single nucleotide polymorphism of the MxA and hepatitis C virus (HCV) load

	HCV Viral Load	
	≤2.0 Meq/mL	>2 Meq/mL
MxA-T-positive	50	71
MxA-T-negative	58	56

Table 5 Relationship between the single nucleotide polymorphism of the MxA and hepatitis C virus (HCV) genotype

	HCV genotype	
	1	2
MxA-T-positive	80	41
MxA-T-negative	78	36

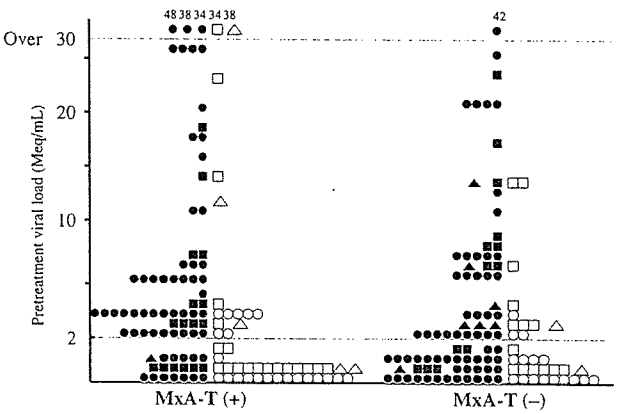

Fig. 1 SNP of the *MxA* gene and interferon response. HCV RNA levels in six patients were over 30 Meq/mL. Numbers above symbols represent the actual level of HCV RNA in these six patients. Open circles, sustained-responders with genotype 1b; open squares, sustained-responders with genotype 2a; open triangles, sustained-responders with genotype 2b; closed circles, nonresponders with genotype 1b; closed squares, nonresponders with genotype 2a; closed triangles, nonresponders with genotype 2b.

(cirrhosis), and host susceptibility to infection [14]. However, there is paucity of information about the host gene factors that could predict the outcome of IFN therapy.

We examined the relationship between polymorphism of the MxA and response to IFN therapy in 235 patients with chronic HCV infection. Our results showed that HCV RNA level and genotype were independent significant determinants of the outcome of IFN therapy. These findings are consistent with those reported previously [3]. Multivariate logistic regression analysis identified the SNP of the MxA as independent determinant of the outcome of IFN therapy although univariate analysis showed borderline significance among all patients. The reason for mismatched results of the

two types of regression analyses was probably the lack of relationship between the SNP of the MxA on one hand and viral load and/or genotype on the other. Moreover, the number of MxA-T-positive patients was less than MxA-T-negative patients among those with a lower viral load (≤2 Meq/mL). This bias was rectified by multivariate logistic regression analysis, which identified the SNP of the MxA as an independent factor. Our study also showed that all patients with genotype 1b and more than 5 Meq/mL in HCV RNA were nonresponders. Therefore, among patients with a higher viral load (>5 Meq/mL), the viral genotype (2a or 2b) was the most important factor associated with the outcome of IFN therapy. On the other hand, among patients with low viral load (≤2 Meq/mL), about half did not achieve a sustained determinant for the outcome of IFN therapy. Moreover, the SNP of the MxA gene was a more important factor than viral genotype in determining the outcome; a significantly higher proportion of patients positive for MxA-T showed a sustained response than those who were negative for MxA-T.

MxA is an IFN-inducible protein that contains consensus IFN-stimulated response elements (ISRE) within the promoter region [11, 15]. This polymorphic site (MxA promoter at nt -88) is involved in a genetic element with high homology to the ISRE consensus sequence and has been shown to play a role in regulation of a downstream reporter gene by a luciferase reporter assay [16, 17]. This suggests that the SNP of the MxA promoter might affect the expression of MxA and that carriers of MxA-T-positive might express the protein more efficiently when treated with IFN than those who are MxA-T-negative [11]. In previous clinical trials, the IFN-induced incremental levels of the MxA mRNA or protein were significantly higher in responders than in nonresponders to treatment [8, 9]. Hijikata *et al.* [11] and our findings suggested that MxA-T-positive patients who had a higher IFN-induced increment of expression of the MxA protein, might respond more efficiently than MxA-T-negative patients. Moreover, a recent report also showed that this SNP of MxA influenced the IFN response among patients with chronic hepatitis B infection [18].

Hijikata *et al.* [11] reported that the SNP of the *MxA* gene was an important factor in predicting outcomes of IFN therapy, independent of HCV genotype. However, in that study, HCV RNA levels were not considered in the IFN response. Our findings showed that HCV RNA levels and genotype were significant factors, especially in patients with high viral load. On the other hand, host factors like MxA may influence the IFN response in patients with low viral load. Recently, the SNP of low molecular mass polypeptide (LMP)7 has been identified as an important host factor that independently influences the response to IFN in patients with chronic hepatitis C [19]. LMP7 within the HLA class II region plays a pivotal role in the HLA class I-restricted antigen-presenting pathway [20, 21]. In this report, among patients with a low viral load (≤2.0 Meq/mL, bDNA probe assay), a higher ratio of LMP7-K-positive patients showed a sustained response compared with those negative for LMP7-K. Although the mechanism(s) of this SNP in the response to IFN therapy remain unknown, host factors like MxA or LMP7 may influence the IFN response among chronic HCV patients with a low viral load.

Other host factors that may determine the response to IFN therapy, such as cytokine gene expression, have been investigated. Polymorphisms in the genes of interleukin (IL)-10, tumour necrosis factor-α (TNF-α), TNF-β and transforming growth factor-β1 (TGF-β 1) in chronic HCV patients treated with IFN monotherapy or in combination with ribavirin have been examined [22– 24]. Yee *et al.* [22] reported that the SNPs of the IL-10 promoter gene were associated with SR to a combination of interferon alfa-2b and ribavirin. However, another report [23] showed that the SNP of the TGF-β 1 gene may be associated with resistance to combination therapy while that of the IL-10 promoter was not. Therefore, the relationship between these polymorphisms and IFN response were not consistent and the findings differed among the studies. Further studies of the involvement of these factors are required.

In summary, we investigated the association of the SNP in the promoter region of the *MxA* gene, HCV viral load and viral genotype with IFN response in patients with chronic HCV infection. The SNP of the *MxA* gene influenced the IFN response among patients with a low viral load. However, viral genotype and HCV RNA levels were important factors in IFN response among patients with a high viral load. Recently, therapy either with IFN combined with ribavirin or with pegylated IFN has been reported to result in higher rates of sustained virological and biochemical responses than IFN monotherapy, even in patients with a high viral load [25, 26]. For such therapy, host factors such as MxA and LMP7 may help identify patients more or less likely to respond.

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Epidemiological and sequence differences between two subtypes (Ae and Aa) of hepatitis B virus genotype A

Fuminaka Sugauchi,[1,2] Hiromitsu Kumada,[3] Subrat A. Acharya,[4] Santosh Man Shrestha,[5] Maria Teresita A. Gamutan,[6] Mobin Khan,[7] Robert G. Gish,[8] Yasuhito Tanaka,[1] Takanobu Kato,[1] Etsuro Orito,[2] Ryuzo Ueda,[2] Yuzo Miyakawa[9] and Masashi Mizokami[1]

Correspondence
Masashi Mizokami
mizokami@med.nagoya-cu.ac.jp

[1,2]Departments of Clinical Molecular Informative Medicine[1] and Internal Medicine and Molecular Science[2], Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

[3]Department of Gastroenterology, Toranomon Hospital, Tokyo, Japan

[4]Department of Gastroenterology, All India Institute of Medical Sciences, New Delhi, India

[5]Liver Foundation Nepal, Nepal

[6]SanJuan de Dios Hospital, The Philippines

[7]Department of Hepatology, Bangabandhu Sheikh Mujib Medical University, Dhaka, Bangladesh

[8]Hepatology and Gastroenterology, California Pacific Medical Center, San Francisco, USA

[9]Miyakawa Memorial Research Foundation, Tokyo, Japan

Complete nucleotide sequences of 19 hepatitis B virus (HBV) isolates of genotype A (HBV/A) were determined and analysed along with those of 20 previously reported HBV/A isolates. Of the 19 HBV/A isolates, six including three from Japan and three from the USA clustered with the 14 HBV/A isolates from Western countries. The remaining 13 isolates including four from The Philippines, two from India, three from Nepal and four from Bangladesh clustered with the six HBV/A isolates reported from The Philippines, South Africa and Malawi. Due to distinct epidemiological distributions, genotype A in the 20 HBV isolates was classified into subtype Ae (e for Europe), and that in the other 19 into subtype Aa (a for Asia and Africa) provisionally. The 19 HBV/Aa isolates had a sequence variation significantly greater than that of the 20 HBV/Ae isolates (2·5 ± 0·3 % vs 1·1 ± 0·6 %, $P<0·0001$); they differed by 5·0 ± 0·4 % (4·1–6·4 %). The double mutation (T1762/A1764) in the core promoter was significantly more frequent in HBV/Aa isolates than in HBV/Ae isolates (11/19 or 58 % vs 5/20 or 25 %, $P<0·01$). In the pregenome encapsidation (ε) signal, a point mutation from G to A or T at nt 1862 was detected in 16 of the 19 (84 %) HBV/Aa isolates but not in any of the 20 HBV/Ae isolates, which may affect virus replication and translation of hepatitis B e antigen. Subtypes Aa and Ae of genotype A deserve evaluation for any clinical differences between them, with a special reference to hepatocellular carcinoma prevalent in Africa.

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INTRODUCTION

Hepatitis B virus (HBV) has been classified into seven genotypes based on a sequence divergence over the entire genome exceeding 8 % (Norder *et al.*, 1994; Okamoto *et al.*, 1988; Stuyver *et al.*, 2000), and they are designated by upper-case letters from A to G. A possible eighth genotype is proposed with a tentative designation of H that is closely related to genotype F phylogenetically (Arauz-Ruiz *et al.*, 2002). The six major HBV genotypes (A–F) have distinct geographical distributions (Lindh *et al.*, 1997; Magnius & Norder, 1995). HBV genotypes A and D are predominant in Europe, North America and Africa, while genotypes B and C are prevalent in east and south Asia. On clinical fronts, there have been increasing lines of evidence to indicate influences of HBV genotypes on the outcome of liver diseases in hosts and the

The sequences reported in this article have been deposited in the DDBJ/EMBL/GenBank databases under accession numbers AB116076–AB116094.

response to antiviral therapies, especially between genotypes A and D prevalent in Western countries as well as B and C common in Asia (Chu *et al.*, 2002; Kao *et al.*, 2002; Kobayashi *et al.*, 2002; Mayerat *et al.*, 1999; Orito *et al.*, 2001; Sugauchi *et al.*, 2002a; Wai *et al.*, 2002). Information is limited for geographical distribution or clinical relevance of genotypes G and H which were discovered recently (Arauz-Ruiz *et al.*, 2002; Kato *et al.*, 2002a, b; Stuyver *et al.*, 2000).

Virological characteristics and clinical manifestations may differ, however, even amongst HBV isolates of the same genotype. We have reported two subtypes of genotype B, designated Ba (a for Asia) and Bj (j for Japan), of which Ba has the recombination with genotype C over the precore region plus core gene, while Bj does not (Sugauchi *et al.*, 2002b). Response to antiviral therapies and the prevalence of hepatitis B e antigen (HBeAg) differed amongst patients with chronic liver diseases who were infected with HBV/Ba and HBV/Bj (Akuta *et al.*, 2003; Sugauchi *et al.*, 2003b). Likewise, amongst isolates of HBV genotype A (HBV/A), two subtypes have been reported, one of which distributes widely in European countries and the USA, while the other prevails in sub-Saharan Africa (Bowyer *et al.*, 1997; Kramvis *et al.*, 2002; Sugauchi *et al.*, 2003a). The subtype of genotype A, designated A′ by Bowyer *et al.* (1997), seems to be virologically distinct from the original genotype A and associated with reduced serum levels of HBV DNA and a low prevalence of HBeAg in serum (Kramvis *et al.*, 1997, 1998). In addition, subtype A′ may have an association with hepatocellular carcinoma prevalent in Africa (Attia, 1998; Edman *et al.*, 1980; Olweny, 1984).

Complete nucleotide sequences were determined for 19 HBV/A isolates recovered from the USA and Asian countries. Including the sequences of 20 HBV/A isolates retrieved from the DNA databases, 20 isolates of the original genotype A and 19 isolates of subtype A′ were compared phylogenetically and for unique mutations in their nucleotide sequences. Due to distinct epidemiological distributions, together with marked virological differences, we would like to propose the classification of the original genotype A prevalent in European countries into subtype Ae (e for Europe) and A′ common in African and Asian countries into subtype Aa (a for Africa/Asia).

METHODS

Serum samples. Nineteen serum samples containing HBV/A were collected from native HBV carriers in various countries [Bangladesh, $n=4$; India, $n=2$; Japan, $n=3$; Nepal, $n=3$; The Philippines $n=4$; USA, $n=3$ (none of African ethnicity)]. Samples from India, Nepal and The Philippines were submitted by doctors who attended the Second Workshop on Hepatocellular Carcinoma in Asia held on 21 February 2002 in Tokyo by the Miyakawa Memorial Research Foundation. HBV genotypes were determined by ELISA with a commercial kit (HBV GENOTYPE EIA, Institute of Immunology Co., Ltd) involving monoclonal antibodies to type-specific epitopes in the preS2 region product (Kato *et al.*, 2002b; Usuda *et al.*, 1999, 2000) as well as by RFLP on the small-S gene sequence amplified by PCR with nested primers (Mizokami *et al.*, 1999). The entire nucleotide sequences of the HBV/A isolates in the 19 serum samples, which had been stored at −20 °C, were determined. The study protocol was approved by the Ethics Committees of the institutions, in accordance with the 1975 Declaration of Helsinki, and an informed consent was obtained from each HBV carrier.

Determination of the full-length sequence of HBV. Nucleic acids were extracted from serum (100 µl) using a DNA extractor kit (Genome Science Laboratory). HBV DNA fragments covering the entire genome sequence in 19 samples were amplified by the method reported previously (Sugauchi *et al.*, 2001). Amplified HBV DNA fragments were sequenced directly by the dideoxy method using a *Taq* Dye Deoxy Terminator cycle sequencing kit and a fluorescent 3100 DNA sequencer (Applied Biosystems).

Phylogenetic analysis. Complete genome sequences of 46 HBV isolates were aligned using the CLUSTAL W software program (Thompson *et al.*, 1994), and the alignment was confirmed by visual inspection. The genetic distances were calculated with the 6-parameter method (Saitou & Nei, 1987), and the phylogenetic tree was constructed by the neighbour-joining method using the ODEN program of the National Institutes of Genetics (Mishima, Japan) (Ina, 1994). To confirm the reliability of the phylogenetic tree, bootstrap resampling tests were performed 1000 times.

Statistical analyses. Frequencies between groups were compared by the chi-square test or by Fisher's exact test. Differences were considered significant for P values less than 0·05.

RESULTS

Phylogenetic relatedness and genetic diversity of the two subtypes of HBV/A

Complete nucleotide sequences of 19 HBV/A isolates were determined. Of the 19 HBV/A genomes, 18 had a genome length of 3221 bp and one (HBV-NEP40) possessed a deletion of 21 nt in the preS1 region, as did three HBV/A isolates retrieved from the DDBJ/EMBL/GenBank databases (accession nos AF297623, AF297625 and V00866). The insertion of 6 nt characteristic of genotype A was present in the core region in all 19 HBV/A isolates. Together with the 20 complete genome sequences of HBV/A isolates retrieved from the databases, the 19 determined in the present study were subjected to phylogenetic analysis along with seven HBV isolates representative of genotypes B, C, D, E, F, G and H (Fig. 1). Three recombinant HBV strains of genotypes A and D [AF297621 (Kramvis *et al.*, 2002); AF418674 and AF418682 (unpublished)], as well as a single recombinant strain of genotypes A and A′ (Bowyer *et al.*, 1997), were excluded from the phylogenetic analysis.

Of the 19 HBV/A isolates for which full-length sequences were determined in the present study, 13 including four from The Philippines, two from India, three from Nepal and four from Bangladesh were classified into subtype Aa and clustered with the HBV isolates from The Philippines, South Africa and Malawi retrieved from the databases; they differed from one another in 2·5 ± 0·3 % (range 1·1–4·6 %) of the entire genome sequence by pairwise comparison. The remaining six HBV/A isolates including three from the USA and three from Japan were classified into subtype Ae, and clustered with the 14 HBV isolates from Western countries

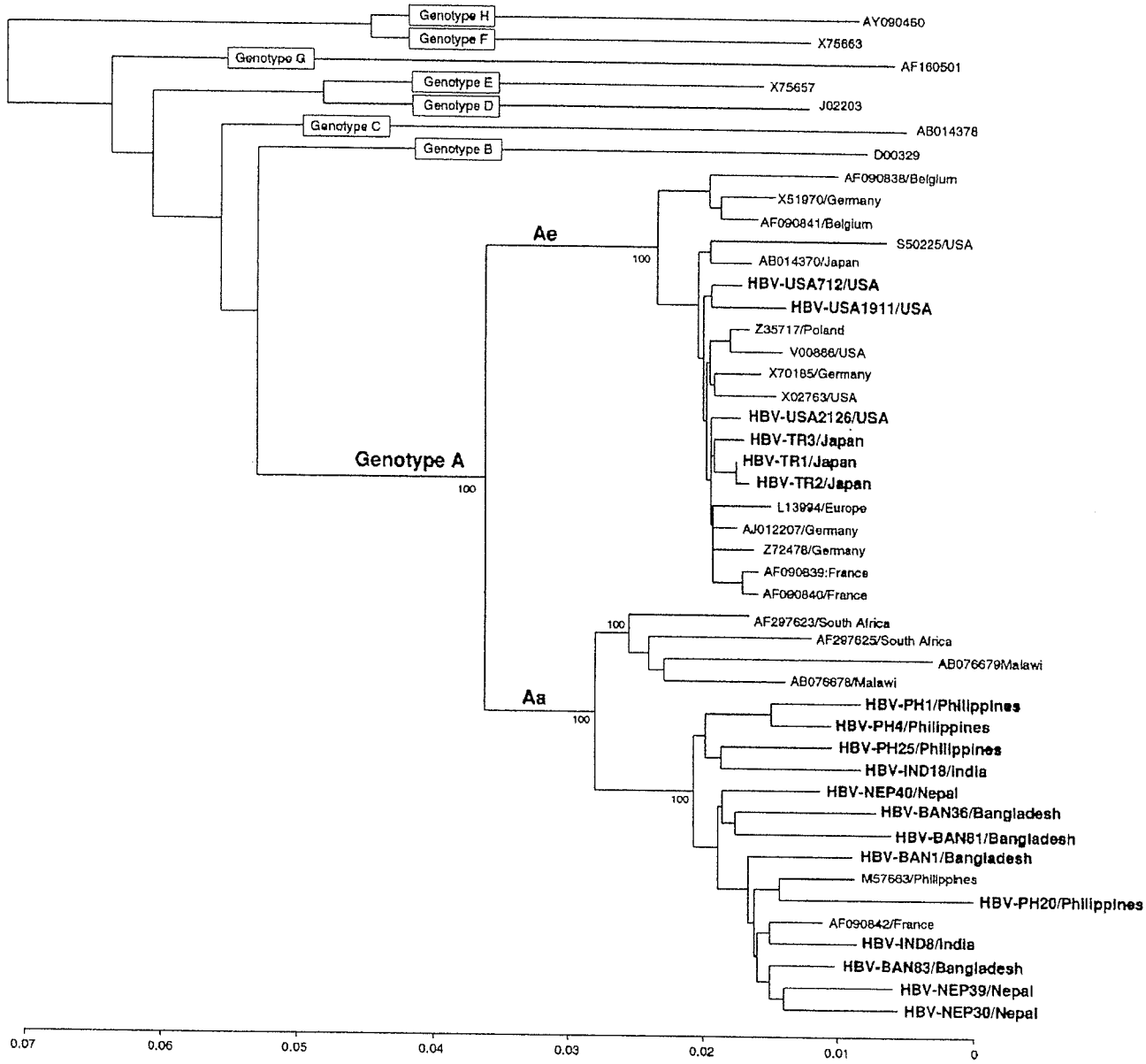

Fig. 1. Phylogenetic tree constructed using the entire nucleotide sequences of 46 HBV isolates. The 39 HBV/A isolates were compared with seven HBV isolates representing genotypes B–H. HBV/A isolates clustered on two branches, Ae (the original European genotype A) and Aa (the new African/Asian genotype A corresponding to A′ proposed by Bowyer *et al.*, 1997). The 19 HBV/A isolates, the sequences of which were determined in this study, are shown in boldface; accession numbers are given for sequences of the other 27 HBV isolates. The country of origin is indicated after a solidus for each HBV/A isolate. Genetic distance is indicated below the tree. Bootstrap values are shown at the nodes of the main branches.

and Japan whose sequences were retrieved from the databases. These 20 HBV/Ae isolates had a sequence variation of $1{\cdot}1 \pm 0{\cdot}6$ %, which was significantly smaller than the $2{\cdot}5 \pm 0{\cdot}3$ % in the 19 HBV/Aa isolates ($P < 0{\cdot}0001$) (Table 1). The inter-group sequence divergence between the 20 and 19 isolates of genotypes Ae and Aa, respectively, was $5{\cdot}0 \pm 0{\cdot}4$ % (4·1–6·4 %) by pairwise comparison.

Phylogenetic analyses were performed on 19 HBV/Aa isolates and six HBV/Ae isolates, the sequences of which were determined in the present study and retrieved from DNA databases, within four reading frames, i.e. the preS1/preS2 region, the S gene, the X gene and the precore/core region (Fig. 2). A clear separation of subtype Ae from Aa is seen in the tree topology for the preS1/preS2 region, the X gene and

Table 1. Mean number of differences in nucleotide sequences of the entire genome and its reading frames within 20 HBV/Ae and 19 HBV/Aa isolates as well as between them

The mean ± SD values are shown with the ranges in parentheses.

Reading frames	Differences within Ae or Aa isolates (%)		Differences between Ae and Aa isolates (%)
	Ae	Aa	
Entire genome	1·1 ± 0·6 (0·1–3·6)	2·5 ± 0·3 (1·1–4·6)	5·0 ± 0·4 (4·1–6·4)
preS1/preS2	1·4 ± 0·2 (0–4·6)	3·3 ± 0·4 (0·8–6·9)	6·6 ± 0·9 (5·0–8·6)
S gene	0·5 ± 0·4 (0–2·1)	1·4 ± 0·6 (0·3–2·9)	1·9 ± 0·5 (0·9–3·7)
X gene	0·9 ± 0·2 (0–1·9)	2·4 ± 0·4 (0·4–4·1)	4·1 ± 0·7 (2·2–5·6)
Precore/core	1·3 ± 0·2 (0–5·4)	3·0 ± 0·3 (0·6–6·4)	4·6 ± 0·6 (2·8–7·9)
Polymerase gene	1·0 ± 0·1 (0·1–2·4)	2·5 ± 0·2 (1·2–4·3)	5·0 ± 0·4 (4·1–6·0)

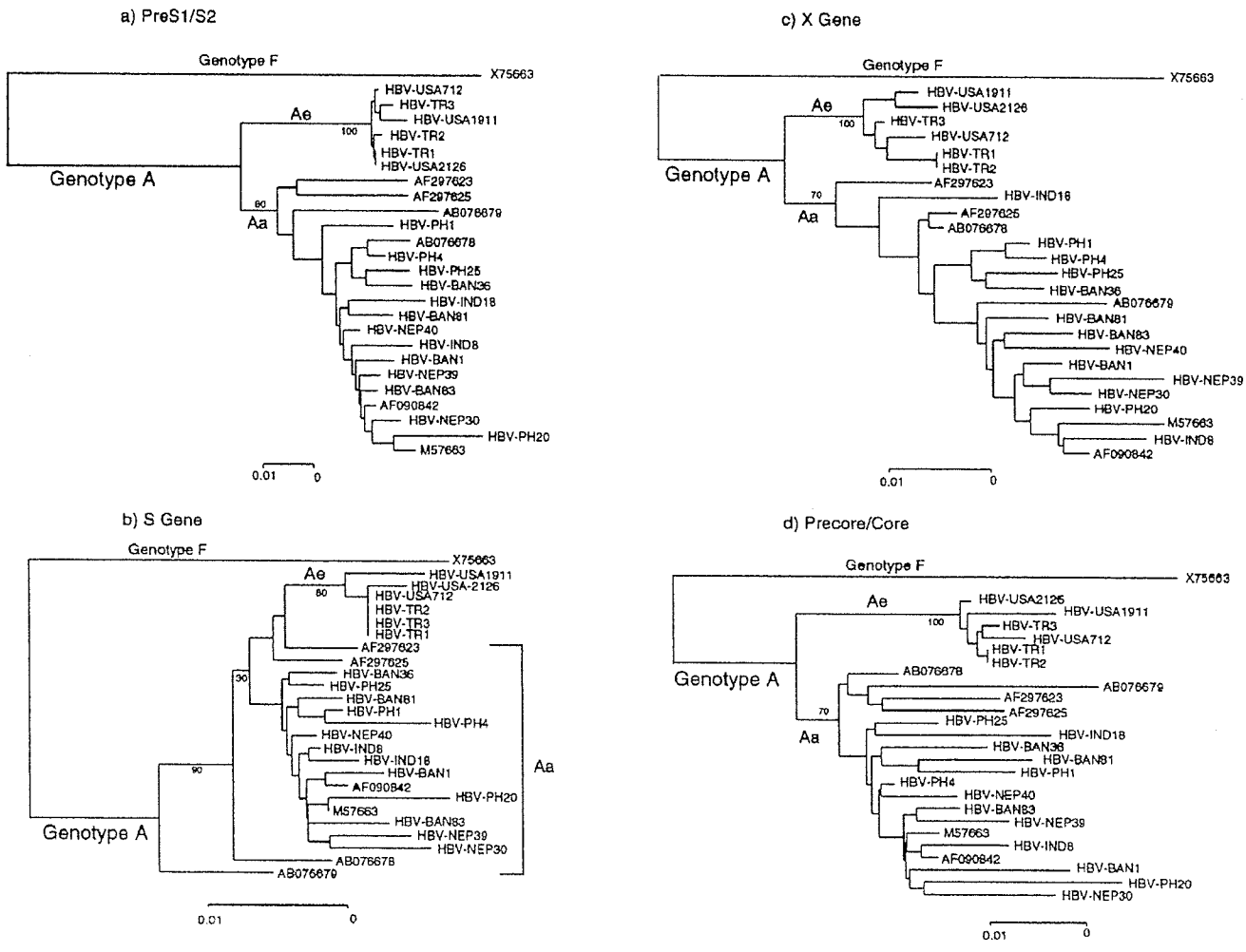

Fig. 2. Phylogenetic trees constructed using partial genome sequences of 25 HBV/A isolates. Nineteen full-length sequences were determined in the present study. Four trees representing (a) the preS1/preS2 region, (b) the S gene, (c) the X gene and (d) the precore region plus the core gene are shown with the sequence of genotype F serving as an outgroup. Genetic distance is indicated below each tree. Bootstrap values are shown at the nodes of the main branches.

the precore/core region. Phylogenetic trees constructed from S gene sequences, however, revealed no significant bootstrap values at the bifurcation of Ae and Aa.

With the availability of many sequences of the preS2 region and the S gene for genotype A isolates, 49 preS2/S sequences of genotype A were retrieved from the DNA databases, and a phylogenetic tree was constructed from them along with those of the 19 genotype A isolates sequenced in the present study (Fig. 3). Genotype A isolates from African countries (South Africa, Malawi and Zimbabwe) clustered with those from Asian countries (Bangladesh, India, The Philippines and Nepal) that were classified into subtype Aa, and they were separated from subtype Ae isolates from Western countries.

Pairwise genetic distances within 20 HBV/Ae and 19 HBV/Aa isolates, as well as between them, in the complete genome and each reading frame are shown in Table 1. The genetic divergence between HBV subtypes Ae and Aa was largest in the preS1/preS2 region amongst all the reading frames compared. Furthermore, HBV/Aa isolates had greater genetic divergence than HBV/Ae isolates in the complete genome as well as in all reading frames.

The serotype of hepatitis B surface antigen (HBsAg) was *adw* in all 20 HBV/Ae isolates and in 16 of the 19 HBV/Aa isolates. It was *ayw* in three HBV/Aa isolates including two (HBV-PH1 and HBV-PH4) from the present study and AB076678 from Malawi. Serotypes were deduced by codons 122 and 160 for either lysine or arginine (Okamoto *et al.*, 1987).

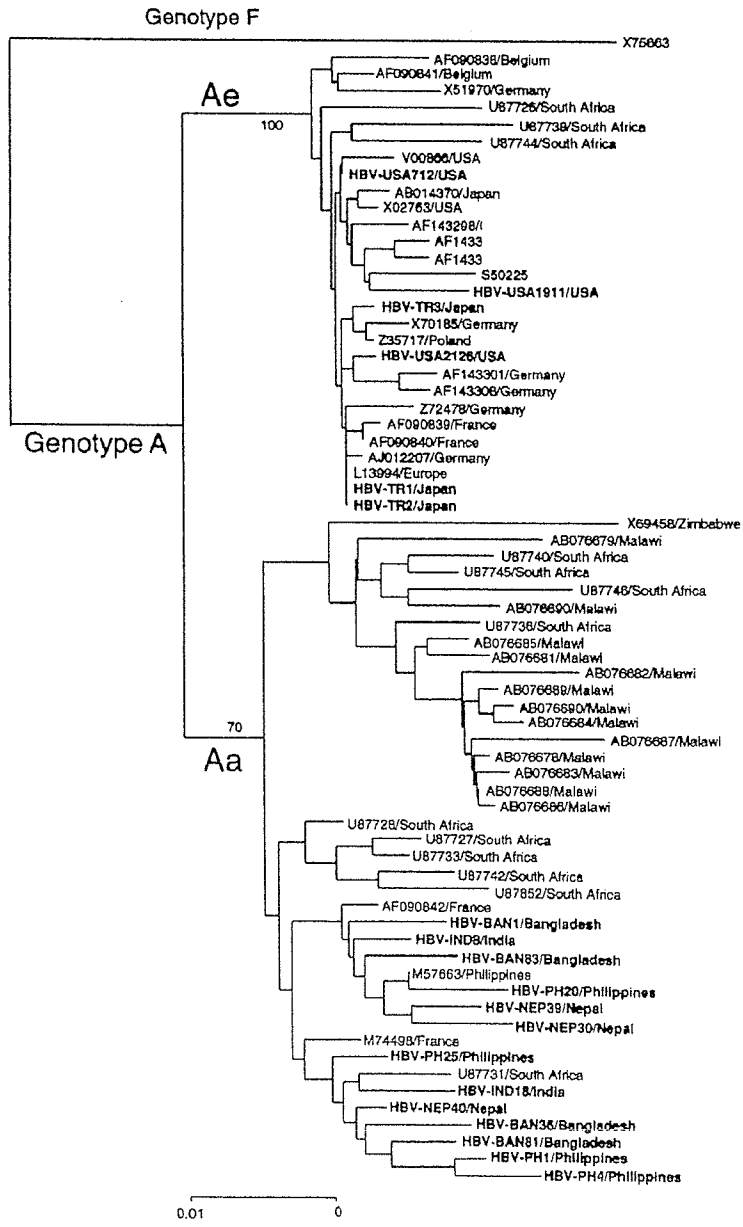

Fig. 3. Phylogenetic tree constructed using the preS2 and S gene sequences of 68 HBV/A isolates. They clustered on two separate branches, Ae (European genotype A) and Aa (African/Asian genotype A corresponding to A′ proposed by Bowyer *et al.*, 1997). The 19 HBV/A isolates, the sequences of which were determined in this study, are shown in boldface; accession numbers are given for sequences of the other 49 HBV isolates. The country of origin is indicated after a solidus for each HBV/A isolate. Genetic distance is indicated below the tree. Bootstrap values are shown at the nodes of the main branches.