Table 2. Number of Transarterial Chemoembolization Procedures Performed as a Function of Treatment with Nucleoside Analogues

No. Transarterial Chemoembolization Procedures	1	2	3	4	5	6	7	8
Nucleoside analogues (−) (n = 60)	28 (46.7%)	20 (33.3%)	7 (11.7%)	5 (8.3%)	0	0	0	0
Nucleoside analogues (+) (n = 21)	5 (23.8%)	4 (19.0%)	5 (23.8%)	0	3 (14.3%)	1 (4.8%)	1 (4.8%)	2 (9.5%)

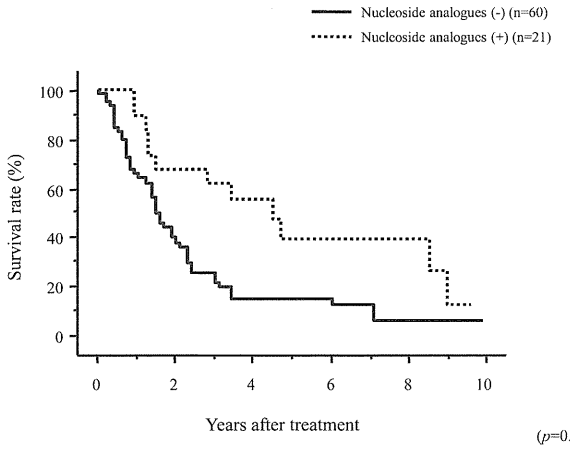

Figure 1. Plot of the Kaplan-Meier product-limit functions for survival after transarterial chemoembolization for initial HCC in the patients who did and did not receive nucleoside analogues.

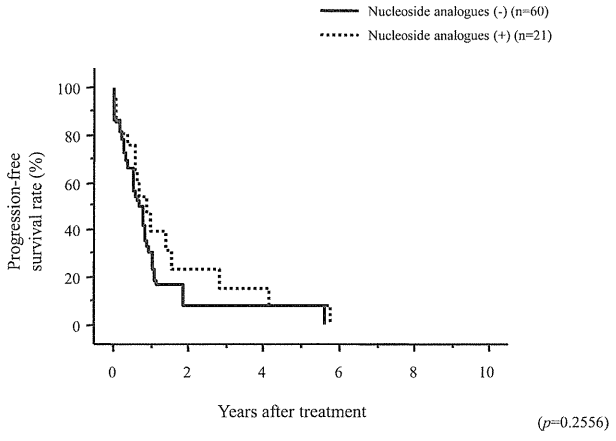

Figure 2. Plot of the Kaplan-Meier product-limit functions for progression-free survival after transarterial chemoembolization for initial HCC in the patients who did and did not receive nucleoside analogues.

patients with nucleoside analogues; $P = .3893$). The reasons for not offering further chemoembolization treatments to patients who did not receive nucleoside analogue therapy were emerging signs of liver failure (including ascites, jaundice, and hepatic coma) in 29 (48.3%) patients, progression to Child-Pugh C liver function in 18 (30.0%) patients, and progression of HCC (including extrahepatic metastases and invasion of the main portal vein trunks and left or right main portal vein) in 13 (21.7%) patients. The reasons for not offering further chemoembolization to the patients who did receive nucleoside analogue therapy were emerging signs of liver failure in 6 (28.6%) patients, progression to Child-Pugh C liver function in 4 (19.0%) patients, and HCC progression in 11 (52.4%) patients. Further chemoembolization was denied because of HCC progression more frequently in patients who were treated with nucleoside analogues ($P = .0174$).

Figure 1 shows the survival curves for the two patient groups. The 1-year, 3-year, and 5-year survival rates were 89.5%, 66.8%, and 40.5% in the patients treated with nucleoside analogues and 72.6%, 27.5%, and 14.3% in the patients who did not receive nucleoside analogues. The survival rate was significantly higher in the patients who were treated with nucleoside analogues ($P = .0051$). By contrast, there was no difference in the progression-free survival rates between the two groups ($P = .2556$) (**Fig 2**).

A multivariate analysis was performed to examine the factors that influenced survival after chemoembolization for the initial HCC (**Table 3**). Multiple tumors and portal vein invasion at the initial HCC diagnosis independently reduced the survival rate, and nucleoside analogue intake was an independent factor that increased the survival rate. When multivariate analysis included the number of chemoembolization treatments as an independent variable, the number of chemoembolization treatments was an independent factor associated with improved survival, and the statistical significance of nucleoside analogue intake disappeared (**Table E2**).

DISCUSSION

The results of the present study showed an association of nucleoside analogue therapy with longer survival in patients with HBV-associated HCC who were treated with chemoembolization for initial and recurrent disease. A multivariate analysis showed that nucleoside analogue intake was an independent factor that affected patient survival. However, the statistical significance of nucleoside analogue intake for improved survival disappeared when the multivariate analysis included the number of chemoembolization treatments as an independent variable, and the number of chemoembolization treatments was the factor that most affected survival. The patients who had received nucleoside analogues underwent a significantly greater number of chemoembolization treatments for HCC than the patients who were not treated with nucleoside analogues. Taken together, these results suggest that the association between nucleo-

Table 3. Multivariate Analyses of Factors Associated with Patient Survival

Factor		Parameter Estimate	Standard Error	Chi	Risk Ratio (95% Confidence Interval)	*P* Value
Age		−0.0188	0.0158	1.41	0.9814 (0.9512–1.0122)	.2342
Sex	Male				1	
	Female	0.0378	0.1804	0.04	1.0385 (0.7096–1.4504)	.8353
Child-Pugh class	A				1	
	B	0.1316	0.1428	0.84	1.1406 (0.8580–1.5057)	.3602
Tumor size	≤ 2 cm				1	
	> 2 cm and ≤ 5 cm	0.2868	0.1688	2.98	1.3322 (0.9625–1.8733)	.0842
	> 5 cm	0.0282	0.1939	0.02	1.0286 (0.7029–1.5113)	.8843
Tumor number	Single				1	
	Multiple	0.3492	0.1516	5.71	1.4179 (1.0631–1.9331)	.0169
Portal vein invasion	Absent				1	
	Present	0.3970	0.1852	4.31	1.4874 (1.0232-2.1235)	.0379
Nucleotide analogue	No				1	
	Yes	−0.4420	0.1727	7.46	0.6428 (0.4483–0.8871)	.0063

Note–Data on Child-Pugh class, tumor size, tumor number, and portal vein invasion refer to the status at initial diagnosis of hepatocellular carcinoma.

side analogue intake and improved patient survival was likely mediated by the increased number of chemoembolization treatments. The use of nucleoside analogues may have slowed the progressive decline in liver function that occurs even with repeated chemoembolization treatments, potentially allowing more sessions of chemoembolization treatment in patients who would otherwise have been excluded from chemoembolization treatment because of progressive liver dysfunction. Additional chemoembolization sessions may have explained the improved patient survival, although not the improved progression-free survival. Several groups have reported on the beneficial survival effects nucleoside analogues exert by preserving liver function in patients with HCC and HBV who undergo curative treatment (29,30). Our experience may suggest that this finding also applies to patients receiving chemoembolization as palliative therapy.

Although previous studies reported that nucleoside analogues can suppress the development of HCC (17,31), it has not been confirmed that nucleoside analogues can suppress HCC recurrence after treatment (30,32–34). Because the patients in the present study had been treated for both initial and recurrent HCC solely by chemoembolization, which is not a curative treatment, it is difficult to determine the extent to which nucleoside analogues prevent HCC progression or recurrence. Although there was no difference in the progression-free survival rate after the initial HCC treatment based on nucleoside analogue intake, further studies are needed to investigate whether the suppressive effects of nucleoside analogues on HCC recurrence or progression play a role in improving the survival of HBV-infected patients with HCC.

There are several limitations to this study. This was a retrospective study, and the patients were not randomly assigned to treatment arms. There may have been selection bias toward the patients who were administered nucleoside analogues. In addition, the data on liver function deterioration during the course of HCC recurrence and retreatment were insufficient, and the mechanisms behind the effect of nucleoside analogues on patients with HCC treated with chemoembolization were not elucidated. Additional studies are necessary to elucidate these mechanisms.

In conclusion, administering nucleoside analogues for chronic hepatitis B was associated with longer survival and more chemoembolization treatments in patients with HCC who were treated solely with chemoembolization. Additional studies are needed to examine these findings further and to clarify the mechanisms underlying this association.

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Table E1. Pretreatment Characteristics of Study Patients (n = 81)

Age (mean ± SD, y) (range)	60.6 ± 9.2 (37–81)
Sex ratio (female/male)	14 (17.3%)/67 (82.7%)
Child-Pugh class (A/B)	49 (60.5%)/32 (39.5%)
Albumin (mean ± SD, g/dL)	3.42 ± 0.73
Total bilirubin (mean ± SD, mg/dL)	1.04 ± 0.82
15-minute retention rate of ICG (%)*	20.0 ± 13.5
Prothrombin (%)	80.1 ± 20.0
Platelet count (× 1,000/mL)	135 ± 77
Tumor size (mean ± SD, cm) (range)	4.38 ± 3.15 (1.0–15.9)
Tumor size (≤ 2 cm/> 2 cm and ≤ 5 cm/> 5 cm)	21 (25.9%)/36 (44.5%)/24 (29.6%)
Tumor number (single/multiple)	29 (35.8%)/52 (64.2%)
Portal vein invasion (absent/present)	63 (77.8%)/18 (22.2%)
AFP (median, ng/mL) (range)	61.4 (0.8–1,304,200)
AFP (≥ 20 ng/mL/< 20 ng/mL)	48 (59.3%)/33 (40.7%)
AFP-L3 (median, %) (range)	6.1 (0–64.0)
AFP-L3 (≥ 10%/< 10%)	31 (38.3%)/50 (61.7%)
DCP (median, mAU/mL) (range)	62.0 (10–75,000)
DCP (≥ 40 mAU/mL/< 40 mAU/mL)	54 (66.7%)/27 (33.3%)

AFP = alpha-fetoprotein; AFP-L3 = *Lens culinaris* agglutinin-reactive AFP; DCP = des-gamma-carboxy prothrombin; ICG = indocyanine green test.
* ICG test was not performed in 14 patients.

Table E2. Multivariate Analyses of Factors Associated with Patient Survival (including Number of Chemoembolization Treatments)

Factor		Parameter Estimate	Standard Error	Chi	Risk ratio (95% Confidence Interval)	*P* Value
Age		−0.0250	0.0150	2.79	0.9753 (0.9469–1.0047)	.0949
Sex	Male				1	
	Female	−0.0013	0.1794	0.00	0.9987 (0.6836–1.3912)	.9943
Child-Pugh class	A				1	
	B	−0.0173	0.1476	0.01	0.9828 (0.7329–1.3106)	.9064
Tumor size	≤ 2 cm				1	
	> 2 cm and ≤ 5 cm	0.2361	0.1668	2.06	1.2662 (0.9183–1.7740)	.1512
	> 5 cm	0.0940	0.1920	0.24	1.0986 (0.7529–1.6069)	.6242
Tumor number	Single				1	
	Multiple	0.4285	0.1562	8.23	1.5350 (1.1415–2.1140)	.0041
Portal vein invasion	Absent				1	
	Present	0.3841	0.1843	4.05	1.4683 (1.0107–2.0898)	.0440
Nucleotide analogue	No				1	
	Yes	−0.1040	0.1903	0.31	0.9013 (0.6067–1.2859)	.5793
No. chemoembolization procedures		−0.3658	0.1194	10.00	0.6936 (0.5450–0.8720)	.0016

Note–Data on Child-Pugh class, tumor size, tumor number, and portal vein invasion refer to the status at initial diagnosis of hepatocellular carcinoma.

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特集II　B型肝炎に対する新治療戦略

肝発癌を視野に入れたB型肝炎の治療戦略*

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Key Words: hepatocarcinogenesis, hepatitis B, nucleos(t)ides, HBV DNA

はじめに

近年，B型肝炎ウイルス(HBV)に対する核酸アナログ製剤が3種類(ラミブジン，アデフォビル，エンテカビル)登場し，治療環境が大きく変化している．核酸アナログ製剤は以前使用されることの多かったインターフェロン(IFN)と比べ副作用が少なく，抗ウイルス効果の高い治療として幅広く使用されるようになってきた．また，核酸アナログ製剤は肝機能に関係なく投与可能であり，その点に関してもIFNを凌駕している．核酸アナログ製剤が臨床的に使用されるようになったのは2000年にラミブジンが認可されたことからはじまり，長期投与により生じることが多いラミブジン耐性株に対してアデフォビルが2004年に認可された．さらに，アデフォビルより変異株出現率が低いエンテカビルが2006年に認可された．また，近年は抗癌剤，生物学的製剤，免疫抑制剤使用によるHBVの再活性化の問題[1]もあり，核酸アナログ製剤を使用する機会も増えている．WHO(World Health Organization)がまとめた報告によると，3億5千万人が持続感染者と推測されており，世界人口の3/4が高度感染地域で暮らしている状態で，年間60～100万人がB型肝炎に起因する慢性肝炎，肝硬変，肝癌で死亡していると推定されている[2]．今後，慢性B型肝炎治療において，肝発癌も視野に入れた治療が重要となる．今回われわれは当院で経験したB型肝炎の核酸アナログ製剤を中心とした発癌との関連性に関して検討した．

対象および方法

1998～2008年の間に大垣市民病院で経験したB型肝炎患者1,973例中，①HBs抗原が6か月以上陽性，②経過観察開始から3年以上経過，③ALTを年2回以上測定，④経過観察開始時にHBe抗原，HBVDNA量測定，そして発癌例では⑤経過観察開始後1年以降で発癌，核酸アナログ服用例では⑥1年以上服薬し，かつ服薬開始1年以降に発癌を満たす785例を対象とした．785例中核酸アナログ投与群は148例，非投与群は637例であった．核酸アナログ製剤の使用に関して，ラミブジン不応に対してアデフォビルを併用し，2006年からは主にエンテカビルを投与している．核酸アナログ製剤使用148例の内訳はラミブジン単独使用21例(14%)，ラミブジンとアデフォビル併用36例(24%)，ラミブジンからエンテカビ

* Treatment strategy of hepatitis B in consideration of hepatocarcinogenesis.

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表1 肝発癌に関与する因子(多変量解析，n=785)

		ハザード比(95% CI)	P
年齢(歳)	≦40	1	
	>40	5.181(2.187～12.271)	<0.001
性	女	1	
	男	3.268(1.712～6.239)	<0.001
血小板数(×10⁴/m³)	≦15	1	
	>15	1.693(1.132～4.364)	0.0366
γ-GTP(IU/l)	≦56	1	
	>56	1.913(1.116～3.278)	0.018
HBV-DNA(log copies/ml)	≦5.0	1	
	>5.0	5.626(3.380～9.362)	<0.001
ALB(g/l)	≦3.5	1	
	>3.5	2.507(1.297～4.845)	=0.002
AFP(ng/ml)	≦10	1	
	>10	2.777(1.579～4.883)	<0.001

785例全体で多変量解析(Cox比例ハザードモデル，変数増加法)を用いて，肝発癌に関与する因子を検討した．投入因子は年齢，性，核酸アナログ投与の有無，血小板，HBe抗原，HBVDNA量，ALT，γ-GTP，T. BIL，ALP，ALB，AFPの12因子である．投入因子：年齢，性，核酸アナログ投与の有無，血小板，HBe抗原，HBVDNA，ALT，γ-GTP，T. BIL，ALP，ALB，AFP

ルへの切り替え症例55例(37%)，当初からエンテカビル使用36例(24%)であった．

まず，785例全体で多変量解析(Cox比例ハザードモデル，変数増加法)を用いて，肝発癌に関与する因子を検討した．一方，propensity matching法を用いて背景因子(経過観察開始時年齢，性別，alanine aminotransferase(ALT)，血小板数，HBV-DNA量，HBe抗原，Child-Pugh分類)をそろえ核酸アナログ製剤使用例と非使用例を抽出し，各種肝機能に対する効果(核酸アナログ使用例では使用開始時から，核酸アナログ非使用例では経過観察開始時から)と肝発癌との関連を検討した．検討した項目は，血小板，ALT，gamma glutamyltranspeptidase(γ-GTP)，total bilirubin(T. BIL)，alkaline phosphatase(ALP)，albumin(ALB)，alpha-fetoprotein(AFP)で，われわれが以前から提唱している時間軸を考慮に入れた積分平均値[3]を求めて検討した．ただし発癌例では発癌1年前までの値を使用した．HBコア関連抗原(HBcrAg)の測定を行うことができた一部の症例ではHBcrAgとの発癌の関連性に関しても検討を行った．

統計は，StatFlex6.0(for Windows，Artech Co.,Ltd)を用いて解析を行った．

結　果

観察中央値は8.1年(1.0～16.2年)であった．発癌に関与する因子として，変数増加法により多変量解析(Cox比例ハザードモデル)を行ったところ，肝発癌に関与する因子として高齢(≦40歳に対して>40歳の場合はhazard ratio(HR)5.181，95% CI：2.187～12.271，$P<0.001$)，男性(女性に対して男性はHR3.268，95% CI：1.712～6.239，$P<0.001$)，HBV-DNA高値(≦5 log copies/mlに対して>5 log copies/mlの場合はHR5.626，95% CI：3.380～9.362，$P<0.001$)，血小板数低値(>15万/m³に対して≦15万/m³の場合はHR1.693，95% CI：1.132～4.364，$P=0.0366$)，γ-GTP高値(≦56IU/lに対して>56IU/lの場合はHR1.913，95% CI：1.116～3.278，$P=0.018$)，ALB低値(>3.5 g/lに対して≦3.5 g/lの場合はHR2.507，95% CI：1.297～4.845，$P=0.002$)，AFP高値(≦10ng/mlに対して>10ng/mlの場合はHR2.777，95% CI：1.579～4.883，$P<0.001$)の7因子が選択された(表1)(投入因子は年齢，性，核酸アナログ投与の有無，血小板数，HBe抗原，HBV-DNA量，ALT，γ-GTP，T. BIL，ALP，Alb，AFPの12因子である)．

一方，核酸アナログ製剤使用例と非使用例で，

表2　経過観察時間開始後の血液生化学値(積分平均値)

	投与群 (n=117)	非投与群 (n=117)	*P* value
血小板数(×10⁴/m³)	17.0(3.3～37.2)	14.8(3.3～39.1)	0.006
ALT(IU/l)	28.2(8.5～88.9)	39.1(8.5～88.9)	<0.001
γ-GTP(IU/l)	27.0(10.9～267)	36.2(9.5～269)	0.0427
T. bil(mg/dl)	0.70(0.3～2.0)	0.70(0.3～2.6)	0.155
ALP(IU/l)	242(113～1028)	265(140～1247)	<0.001
ALB(g/l)	4.4(3.0～5.0)	4.0(0.9～73)	<0.001
AFP(ng/ml)	2.15(0.9～285)	4.50(0.9～723)	<0.001

Propensity matching法を用いて背景因子(初診時年齢，性別，ALT, 血小板数，HBV-DNA量，HBe抗原，Child-Pugh分類)をそろえ核酸アナログ製剤使用例と非使用例を抽出し，各種肝機能に対する効果(核酸アナログ使用例では使用開始時から，核酸アナログ非使用例では経過観察開始時から)と肝発癌との関連を検討した．検討した項目は，血小板，ALT, γ-GTP, T. BIL, ALP, ALB, AFPの7項目である．それぞれ積分平均値で評価した．

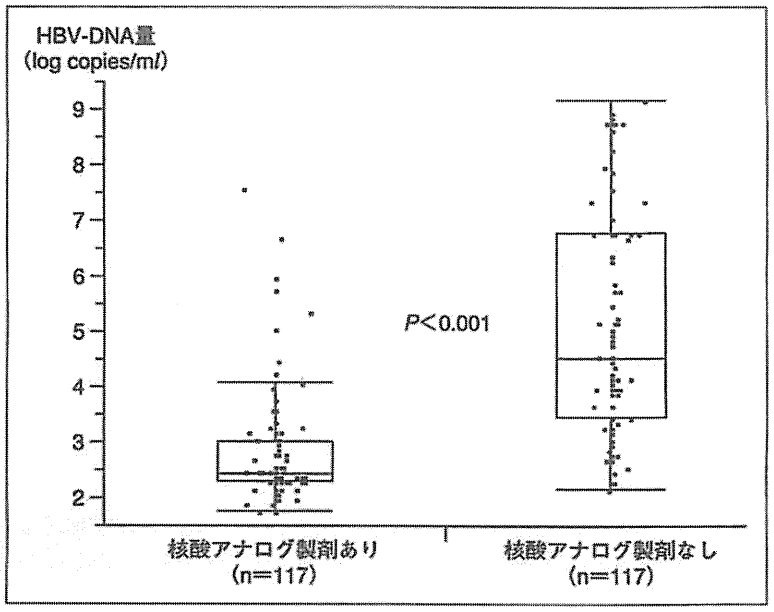

図1　核酸アナログ製剤使用の有無による，観察期間中のHBV-DNA量の比較
Propensity matching法を用いて背景因子をそろえた核酸アナログ製剤使用例と非使用例各112例でのHBV-DNA量を積分平均値で評価した．

propensity scoreを用いて治療開始時もしくは経過観察時の前述の7因子でマッチングさせたところ，核酸アナログ製剤使用例117例，核酸アナログ製剤非使用例117例が選択された．核酸アナログ製剤投与群は非投与群と比較し，ALTが低値($P<0.001$)，ALBが高値($P<0.001$)，γ-GTPが低値($P=0.042$)，ALPが低値($P=0.042$)，血小板が高値($P=0.006$)，AFPが低値($P<0.001$)となり，肝機能は明らかに改善した(表2)．HBV-DNA量に関しても同様に核酸アナログ製剤使用例で有意な低下がみられた(図1)．

核酸アナログ製剤の使用の有無により累積発癌率を検討したところ3年，5年，10年でそれぞれ，核酸アナログ製剤投与例では2.1%，7.2%，12.1%であったのに対し，核酸アナログ製剤非投与例では6%，12.4%，43.9%を示し(HR0.419，95%CI：0.1821～0.9637，$P=0.041$)と前者が有意に累積発癌率が低値であった(図2)．

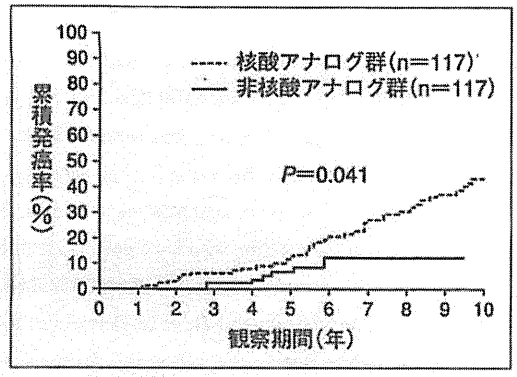

図2　核酸アナログ製剤投与と発癌率
Propensity matching法を用いて背景因子をそろえた核酸アナログ製剤使用例と非使用例各112例での累積発癌率を示す．

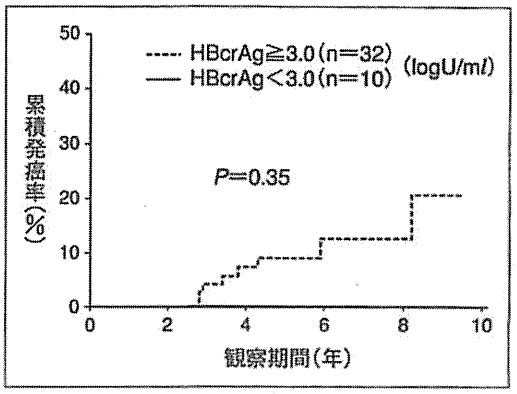

図3　核酸アナログ製剤使用例におけるHBcrAgと発癌率
核酸アナログ製剤使用例でHBコア関連抗原を測定可能であった42例でHBコア関連抗原量が3 logU/ml以上(n=32)と3 logU/ml未満(n=10)での累積発癌率を示す．

また，核酸アナログ製剤使用例の42例でHBコア関連抗原が測定され，HBコア関連抗原量が3 logU/ml以上(n=32)と3 logU/ml未満(n=10)では累積発癌率に関して3 logU/mlが有意差はないものの，肝発癌がみられたのはHBコア関連抗原量が3 logU/ml以上の症例のみであった(図3)．

考　察

今回のわれわれの検討において，肝発癌に関与する因子として抽出されたものは高齢，男性，HBV-DNA量高値，血小板数低値，γ-GTP値高値，ALB低値，AFP高値の7因子であった．HBVキャリアに関して肝発癌に関与する因子については数多く報告がなされている．Chen[4]らはHBe抗原陰性，HBV-DNA量＜4 log copies/ml，であり肝硬変，肝癌，ALT高値の見られない患者を非活動性キャリアとして1,932人とそれに対するコントロール群18,137人を平均13.1年間フォローした結果，年間発癌率はキャリア群で0.06%，コントロール群では0.02%であり，キャリア群の多変量補正された肝発癌のハザード比は4.6(95%CI：2.5～8.3)，肝関連死に関しては2.1(95%CI：1.1～4.1)と非活動性キャリアは肝発癌，肝関連死において有意に高率であると結論している．また，高齢，飲酒も独立した発癌の寄与因子であるとも報告している[4]．背景肝による年間の発癌リスクは，非活動性キャリアからの発癌は0.2%未満，慢性B型肝炎から1%未満，代償性肝硬変から2～3%，非代償性肝硬変からは7～8%とされておりその差は明らかである[5]．また，宿主因子は肝硬変，糖尿病，肥満，アルコール摂取，高齢，男性，家族歴，人種(アジア・アフリカ人)，ウイルス因子はHBVDNA量，HBe抗原陽性，genotype，プレコアやコアプロモーター変異，C型肝炎やHIVとの合併が関与しているとの報告もされている[6]．自験例でγ-GTP高値が肝発癌寄与因子となっているのは飲酒との関連性も推定される．また，血小板低値，ALB低値は背景肝が肝硬変であることの交絡因子とも考えられる．特にウイルス側の因子として近年注目されているのはHBV-DNA量である．台湾からの前向きのコホート研究(REVEAL-HBV Study)では，3,653人のHBs抗原陽性例の発癌を検討したところ(観察中央値11.4年)，性別，年齢，喫煙，アルコール摂取，HBe抗原，ALT値，肝硬変など因子とは独立して，HBV-DNA量が増加するほど累積発癌も増加する結果となった[7]．国内ではIshikawaらが，65例のB型肝硬変患者から発癌の検討ではHBV-DNAが3.7LGE/ml以上の症例で多いと報告している[8]．このように肝発癌には高ウイルス量が強く寄与する結果との報告が多く認められる．また，KumadaらのALT正常例(3年以上のフォローが可能でありALT積分平均値が40IU/l未満371例)に絞っての発癌に関する検討では，HBV-DNA高値(≧5.0 log copies/ml)と血小板数低値(＜15.0×10^4/mm^3)で高率に発癌すると述べている[3]．全

症例を含めた今回の検討でも同様の結果であり，HBV-DNA高値が発癌に関与する因子と考えられ，ウイルス量を減少させることが肝発癌の抑制につながると期待される．Laiwらは大規模なRCT (the Cirrhosis Asia Lamivudine study)でラミブジンによる肝発癌抑制効果と肝関連死の低減効果を証明している[9]．国内では，Matsumotoらによる後ろ向き研究で，肝発癌に対する有用性が検討され，ラミブジンの発癌抑制効果が示されている[10]．今回のわれわれの検討でも核酸アナログ製剤による肝発癌抑制効果は有意であった．同時に，核酸アナログ製剤使用例ではALB値が有意に高くなり，血小板値も高くなるなど肝機能改善効果もみられ，肝細胞癌を治療する上で核酸アナログ製剤は欠かせない存在であると思われる．投与薬剤および方法はラミブジン単独，ラミブジンとアデフォビルの併用，エンテカビル単独，ラミブジンからエンテカビルへ切り替え例と多岐にわたるが，変異株出現に注意しながらいかにウイルス量を低く抑えるかが重要と考えられる．

一方，最近注目されているHBcrAgは肝組織中のcovalently closed circular DNA(cccDNA)量を反映していると考えられている[11]が，核酸アナログ使用中の症例において，肝細胞癌の再発はHBコア関連抗原量が多い症例に有意に高率であったとの報告も認められる[12]．自験例では，核酸アナログ製剤使用例のうちHBcrAgが3 logU/ml未満の症例では発癌例はみられず，3 logU/ml以上の症例での発癌のみであったが有意の差は認めなかった．症例数が少ないためとも考えられ，さらなる症例の集積が必要と思われる．

おわりに

今回の検討では，核酸アナログ製剤の使用が肝発癌抑制効果をもたらすのみならず，肝機能改善に有効であることが示された．変異株の出現に十分注意しながら，核酸アナログを使用し，ウイルス量を低値に保つことが肝発癌の抑制，肝機能の改善につながると考えられる．

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ORIGINAL ARTICLE

Hepatitis B virus strains of subgenotype A2 with an identical sequence spreading rapidly from the capital region to all over Japan in patients with acute hepatitis B

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ABSTRACT

Objective To examine recent trends of acute infection with hepatitis B virus (HBV) in Japan by nationwide surveillance and phylogenetic analyses.

Methods During 1991 through 2009, a sentinel surveillance was conducted in 28 national hospitals in a prospective cohort study. Genotypes of HBV were determined in 547 patients with acute hepatitis B. Nucleotide sequences in the preS1/S2/S gene of genotype A and B isolates were determined for phylogenetic analyses.

Results HBV genotype A was detected in 137 (25% (accompanied by genotype G in one)) patients, B in 48 (9%), C in 359 (66%), and other genotypes in the remaining three (0.5%). HBV persisted in five with genotype A including the one accompanied by genotype G; another was co-infected with HIV type 1. The genotype was A in 4.8% of patients during 1991–1996, 29.3% during 1997–2002, and 50.0% during 2003–2008 in the capital region, as against 6.5%, 8.5% and 33.1%, respectively, in other regions. Of the 114 genotype A isolates, 13 (11.4%) were subgenotype A1, and 101 (88.6%) were A2, whereas of the 43 genotype B isolates, 10 (23.3%) were subgenotype B1, 28 (65.1%) were B2, two (4.7%) were B3, and three (7.0%) were B4. Sequences of 65 (64%) isolates of A2 were identical, as were three (23%) of A1, and five (18%) of B2, but none of the B1, B3 and B4 isolates shared a sequence.

Conclusions Acute infection with HBV of genotype A, subgenotype A2 in particular, appear to be increasing, mainly through sexual contact, and spreading from the capital region to other regions in Japan nationwide. Infection persisted in 4% of the patients with genotype A, and HBV strains with an identical sequence prevailed in subgenotype A2 infections. This study indicates the need for universal vaccination of young people to prevent increases in HBV infection in Japan.

Significance of this study

What is already known about this subject?

- In Japan, a national prevention programme was started in 1986 with selective vaccination of babies born to mothers who carry hepatitis B virus (HBV). Since then, the prevalence of hepatitis B surface antigen among younger generations has decreased sharply.
- However, retrospective studies indicate that the frequency of HBV genotype A is increasing among patients with acute hepatitis B (AHB) within the capital region of Japan.
- Infection with genotype A more often persists than infection with other genotypes.
- Because there is no reliable and comprehensive surveillance system for AHB in Japan, the incidence of AHB and factors responsible for changes over many years are not known.

What are the new findings?

- This is a prospective cohort study for surveillance of AHB throughout Japan in a national research programme.
- The incidence of AHB in Japan has not decreased, because genotype A infections have increased over time.
- Genotype A infections started to increase in the capital region of Japan, and then spread to other regions 5–6 years later.
- About 90% of genotype A found in AHB patients in Japan is subgenotype A2.
- Subgenotype A2 isolates from patients with AHB tend to preserve sequence identity over time, indicating that particular subgenotype A2 strains have been transmitted without undergoing mutations.

Hepatitis B virus (HBV) has been classified into 10 genotypes, designated A–J, based on a >8% divergence in the full-genome sequence.[1–7] Different genotypes are associated with distinct clinical manifestations, such as severity and progression of liver disease, as well as response to antiviral treatments.[8–10] Some genotypes are subclassified: genotype A into at least two subgenotypes, A1 (Asian/African type) and A2 (European type)[11–13];

Significance of this study

How might it impact on clinical practice in the foreseeable future?

- It needs to be noted that subgenotype A2 infections are spreading among sexually active generations in Japan.
- Although selective vaccination has prevented mother-to-baby transmission of HBV since 1986, it does not contain sporadic infections in Japan.
- Herd vaccination of younger generations needs to be considered in Japan.

B into B1 (Japanese type) and B2 (Asian type)[14 15]; and C into C1 (Southeast-Asian type) and C2 (East-Asian type).[16] Subgenotypes also influence the replication of HBV and clinical manifestation.[15 17 18]

According to a report from Japan in 2001,[19] genotype C was the most prevalent (84.7%), followed by genotype B (12.2%) and A (1.7%), among patients with chronic hepatitis B. In 2002, genotype A became the most prevalent in patients with acute hepatitis B (AHB) around Tokyo, the capital region of Japan.[20 21] Several reports have shown that infection with HBV genotype A is associated with particular sexual behaviours, such as homosexual activity and promiscuous sexual contacts, and tends to persist longer than that with HBV genotype C.[22 23] These reports have raised concerns about the horizontal HBV infection in adults, which, in general, is considered to resolve spontaneously. However, adult-acquired HBV infection may result in chronic HBV infection in some instances.

Information on changes in genotype distribution over time, as well as genotype-specific clinical manifestations, may help in planning preventive measures and antiviral therapy strategies. Therefore it is important to examine how genotype A infection has spread in Japan, and what clinical and virological characteristics it possesses.

We have been conducting a nationwide, sentinel surveillance on acute viral hepatitis for more than 30 years. As part of this surveillance, a prospective cohort study has been conducted on 547 patients with AHB in 28 medical centres over the 19 years from 1991 to 2009. Geographical and longitudinal distributions of HBV genotypes/subgenotypes were surveyed, and their influence on clinical outcome was evaluated.

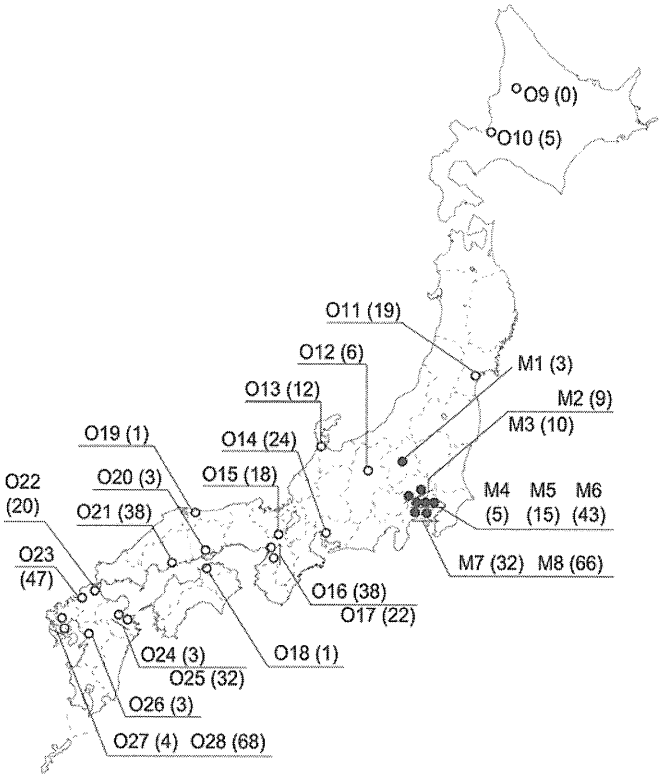

Figure 1 Locations of participating hospitals in Japan. Hospitals in the capital region (M1–M8) are indicated by eight closed circles, and those in other regions (O9–O28) by 20 open circles. Numbers in parentheses indicate the total number of enrolled subjects for each site. The hospitals are: M1, Nishigunma Hospital, Gunma; M2, Nishisaitama-Chuo Hospital, Saitama; M3, National Disaster Medical Center, Tokyo; M4, Tokyo Hospital, Tokyo; M5, Tokyo Medical Center, Tokyo; M6, National Center for Global Health and Medicine, Tokyo; M7, Sagamihara Hospital, Kanagawa; M8, Yokohama Medical Center, Kanagawa; O9, Asahikawa Medical Center, Hokkaido; O10, Hokkaido Medical Center, Hokkaido; O11, Sendai Medical Center, Miyagi; O12, Matsumoto Medical Center, Nagano; O13, Kanazawa Medical Center, Ishikawa; O14, Nagoya Medical Center, Aichi; O15, Kyoto Medical Center, Kyoto; O16, Osaka National Hospital, Osaka; O17, Osaka-Minami Medical Center, Osaka; O18, Zentsuji Hospital, Kagawa; O19, Yonago Medical Center, Tottori; O20, Okayama Medical Center, Okayama; O21, Kure Medical Center and Chugoku Cancer Center, Hiroshima; O22, Kokura Medical Center, Fukuoka; O23, Kyushu Medical Center, Fukuoka; O24, Beppu Medical Center, Oita; O25, Oita Medical Center, Oita; O26, Kumamoto Medical Center, Kumamoto; O27, Ureshino Medical Center, Saga; and O28, Nagasaki Medical Center, Nagasaki.

PATIENTS AND METHODS

Patients

A total of 681 patients with sporadic AHB were enrolled consecutively in a survey carried out by the Japan National Hospital Acute Hepatitis Study Group (JNHAHSG). They were admitted to 28 national hospitals from January 1991 to the end of December 2009. They were grouped geographically into two areas: the capital region (Gunma, Saitama, Tokyo and Kanagawa) and other regions (figure 1). Patients were also longitudinally categorised into three periods: 1st (1991–1996), 2nd (1997–2002) and 3rd (2003–2008). In addition, the year 2009 provided the most recent data. Of the 681 patients, 547 (80.3%) entered the study, for whom serum samples were available on admission and had been stored at −20°C.

The diagnosis of AHB was based on the following criteria: (1) acute onset of liver injury without a history of liver dysfunction; (2) detection of hepatitis B surface antigen (HBsAg) in the serum; (3) positivity for IgM antibody to HBV-core antigen (IgM anti-HBc) in high titres (detectable in sera diluted 10-fold); and (4) absence of past or family history of chronic HBV infection. Severe acute hepatitis (SAH) was defined as prothrombin time (PT) ≤40% and hepatic encephalopathy of grade ≤I. Fulminant hepatitis (FH) was diagnosed from PT ≤40% and hepatic encephalopathy of grade ≥II. Patients in whom HBsAg remained in the serum for >6 months after onset were considered to have acquired chronic HBV infection. The following information was collected from each patient: year and age at onset, gender, residential area, HBsAg, IgM anti-HBc, alanine aminotransferase, total bilirubin, PT, severity of liver disease, mortality, routes of transmission, sexual behaviours, travelling abroad in recent past, HBV genotype, mutations in precore (PreC) and core promoter (CP) regions, and RNA of hepatitis D virus. Antibody to HIV type 1 (anti-HIV) was

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determined in patients who were at high risk and gave consent to testing.

Informed consent was obtained from each patient. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and the Ministry of Education, Culture, Sports Science and Technology of Japan, and was approved by the ethics committee of each institution.

Extraction of HBV DNA

HBV DNA was extracted from serum (100 μl) by the SMITEST EX-R&D Nucleic Acid Extraction Kit (MBL Co, Nagoya, Japan) and used for genotyping/subgenotyping and detecting mutations in PreC and CP regions.

HBV genotypes

Genotypes were determined in Nagasaki Medical Center with the SMITEST HBV Genotyping Kit (MBL) by hybridisation with type-specific probes immobilised on a solid-phase support.[24]

Determination of HBV subgenotypes

For subgenotyping, HBV DNA was amplified by PCR with TaKaRa Ex Taq (Takara Bio, Shiga, Japan). PCR was performed with appropriate nested primers to amplify a ~1.2 kb sequence in the preS1/S2/S gene (nucleotides 2854–835 in the reference isolate (AB116077)). PCR products were purified, subjected to cycle sequencing reaction with the BigDye Terminator v1.1 (Applied Biosystems, Tokyo, Japan), and applied to the DNA sequencer (3100-Avant; Applied Biosystems).

Mutations in the PreC and CP regions

The A1896 mutation in the PreC region was detected by the enzyme-linked minisequence assay (SMITEST HBV PreC ELMA; Roche Diagnostics, Tokyo, Japan), and mutations in the CP region for T1762/A1764 by the enzyme-linked specific probe assay (SMITEST HBV Core Promoter Mutation Detection Kit; Roche Diagnostics). The results were recorded as 'wild-type' and 'mutant types' dominantly expressed by HBV isolates.[25]

Phylogenetic analyses

Nucleotide sequences were aligned, and phylogenetic trees were constructed by the CLUSTAL W program v1.83 (DDBJ homepage: http://clustalw.ddbj.nig.ac.jp/top-j.html). The statistical validity was assessed by bootstrap resampling with 1000 replicates. Reference HBV strains were retrieved from the GenBank database.

Statistical analysis

Results were expressed as percentage or mean±SD. Statistical differences were evaluated by χ^2 and Fisher exact tests for categorical variables, and analysis of variance and Scheffe's test for quantitative variables, using the SPSS software. The 95% CI, for the difference in means, was calculated in analyses for quantitative variables. $p<0.05$ was considered significant.

RESULTS

Distribution of HV genotypes

HBV genotypes were determined in the 547 patients with AHB. The genotype was A in 137 (25.0%) patients (accompanied by G in one (0.2%)), B in 48 (8.8%), C in 359 (65.6%), D in one (0.2%), E in one (0.2%), and H in one (0.2%). Because HBV genotype G is a defective virus and cannot replicate by itself,[26 27] the single patient with mixed genotypes A and G was included in the 137 patients with genotype A in further analyses. RNA of hepatitis D virus was detected in three of the 453 (0.7%) patients. Anti-HIV was examined in patients at high risk of infection and detected in 14 of the 53 (26.4%) who gave consent to testing.

Demographic and clinical differences among patients infected with HBV of distinct genotypes

Demographic and clinical characteristics of patients with different genotypes are compared in table 1. There was no difference in mean age among patients with genotypes A, B and C. The proportion of men was higher in patients with genotype A than B or C (94.2% vs 79.2%, $p<0.05$; or 56.0%, $p<0.0001$), and in those with genotype B than C (79.2% vs 56.0%, $p<0.05$).

Maximum levels of total bilirubin were higher in patients with genotype A than C (9.6±7.6 vs 7.1±6.2 mg/dl, $p<0.05$), with a difference of 2.5 mg/dl (95% CI 0.93 to 4.08), whereas the highest alanine aminotransferase activity and lowest PT values did not differ among patients with distinct genotypes.

SAH developed in four (2.9%) patients with genotype A, four (8.3%) with genotype B, and 26 (7.2%) with genotype C. FH developed in one (2.1%) patient with genotype B and eight (2.2%) with genotype C; no patients with genotype A developed FH. Eight (1.5%) patients died, including one with genotype B and seven with genotype C. There were no significant differences among patients with different genotypes in the frequency of SAH or FH or mortality.

The outcome of AHB was traceable in 514 of the 547 (94.0%) patients. Chronic infection with persistence of HBsAg for >6 months developed in five of the 123 (4.1%) patients with genotype A (including the one accompanied by genotype G), none of the 46 (0%) with genotype B, and none of the 342 (0%) with genotype C; it was more common in patients with genotype A than C ($p<0.05$). HBV infection persisted exclusively in the patients with genotype A, either alone (four patients) or together with genotype G (one).

Among the five patients who acquired chronic HBV infection, four (three with genotype A and one with mixed genotypes A and G) were examined for anti-HIV, and one with genotype A was found to be positive. HBV infection persisted in three (including the one with anti-HIV) of the five patients for >1 year after the onset, and the remaining two (both without anti-HIV) cleared HBsAg from the serum after retaining it for >6 months.

Mutations in the PreC and/or CP region were detected in 3.7% (4/109) of patients with genotype A, 15.4% (6/39) of those with genotype B, and 25.5% (79/310) of those with genotype C. They were significantly less common in patients with genotype A than B or C (A vs B, $p<0.05$; A vs C, $p<0.0001$). The only patient with genotype A who had the PreC mutation was simultaneously infected with genotype G.

Routes of transmission were identifiable in 275 of the 547 (50%) patients, and the main route was heterosexual contacts; those in the remaining patients could not be disclosed. The frequency of heterosexual activity did not differ among patients with distinct genotypes. However, homosexual activity was more common in patients with genotype A than B or C (21.2%, 0% and 0.8%, respectively (A vs B, $p<0.001$; A vs C, $p<0.0001$)). Among the 32 homosexual men, HBV genotype A was detected in 29 (91%). Consent to anti-HIV testing was given by 10 of the 29 patients, and four of these (40%) were positive.

Longitudinal changes in the distribution of genotypes

Figure 2 illustrates changes in the distribution of HBV genotypes through three 6-year periods over 18 years (1991–2008). In addition, data from 2009 are shown. HBV genotype A accounted

Table 1 Demographic and clinical characteristics of patients with acute hepatitis who were infected with HBV of different genotypes (1991–2009)

Feature	Total (n=547)	HBV genotypes			
		A (n=137)† (25.0%)	B (n=48) (8.8%)	C (n=359) (65.6%)	Others (n=3)‡ (0.5%)
Age (years)	35.6±14.8	35.2±12.2	39.6±15.6	35.1±15.5	49.7±13.6
Male	367 (67.1%)	129 (94.2%)¶ * †† ***	38 (79.2%)†† *	201 (56.0%)	3 (100%)
ALT (IU/l)§	2553±1563	2289±1069	2557±1412	2342±1728	3333±2406
T-Bil (mg/dl)§	7.8±6.7	9.6±7.6†† *	7.7±7.4	7.1±6.2	9.0±2.5
PT (%)§	74.6±22.6	75.2±15.9	73.8±24.5	74.7±24.5	15.8‡‡
Severe hepatitis	34 (6.2%)	4 (2.9%)	4 (8.3%)	26 (7.2%)	0 (0.0%)
Fulminant hepatitis	10 (1.8%)	0 (0.0%)	1 (2.1%)	8 (2.2%)	1 (33.3%)
Mortality	8 (1.5%)	0 (0.0%)	1 (2.1%)	7 (1.9%)	0 (0.0%)
HBsAg persisting >6 months	5/514 (1.0%)	5/123 (4.1%)†† *	0/46 (0.0%)	0/342 (0%)	0/3 (0.0%)
PreC/CP mutations					
PreC	43/461 (9.3%)	1/109 (0.9%)¶ * †† *	6/39 (15.4%)	34/310 (11.0%)	2/3 (66.7%)
CP	69/461 (15.0%)	3/109 (2.8%)†† ***	0/39 (0.0%)†† *	63/310 (20.3%)	3/3 (100%)
PreC and/or CP	92/461 (20.0%)	4/109 (3.7%)¶ * †† ***	6/39 (15.4%)	79/310 (25.5%)	3/3 (100%)
Transmission route					
Homosexual	32 (5.9%)	29 (21.2%)¶ ** †† ***	0 (0.0%)	3 (0.8%)	0 (0.0%)
Heterosexual	217 (39.5%)	52 (38.0%)	25 (52.1%)	139 (39.6%)	1 (33.3%)
Medical procedure	16 (2.9%)	2 (1.5%)	2 (4.2%)	12 (3.3%)	0 (0.0%)
Other	10 (1.8%)	1 (0.7%)	1 (2.1%)	7 (1.9%)	1 (33.3%)
Undetermined	272 (49.7%)	53 (38.7%)†† *	20 (41.7%)	198 (55.2%)	1 (33.3%)
Anti-HIV	14/53 (26.4%)	11/35 (31.4%)	0/3 (0.0%)	3/15 (20.0%)	0/0

Values are mean±SD or number (%).
†One patient with genotype A was simultaneously infected with genotype G.
‡Each patient was infected with genotype D, E or H.
§Highest values during the clinical course are shown for ALT and T-Bil, and lowest values for PT.
Statistical analysis was performed to compare genotypes A, B and C.
¶Significantly different compared with genotype B.
††Significantly different compared with genotype C.
*p<0.05, **p<0.001, ***p<0.0001.
‡‡Data from the patient with genotype E only.
ALT, alanine aminotransferase; CP, core promoter; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; PreC, precore; PT, prothrombin time; T-Bil, total bilirubin.

for 6% (9/150) in the 1st period, 15.4% (19/123) in the 2nd, and 39.4% (89/226) in the 3rd, with significant differences between 1st and 2nd (p<0.05), 2nd and 3rd (p<0.0001), and 1st and 3rd (p<0.0001). Conversely, AHB associated with genotype C decreased through three periods with significant differences, while AHB associated with genotype B did not change appreciably.

On the basis of these results, the yearly incidence in each of the three 6-year periods is calculated to be: 25.0 cases including 1.5 with genotype A in the 1st period; 20.5 cases including 3.2 with genotype A in the 2nd; and 37.7 cases including 14.8 with genotype A in the 3rd. Hence, the incidence of AHB had not changed markedly over the 12 years from 1991 to 2002, but increased thereafter until 2008. Of the increment in the 3rd period of 17.2 (37.7 minus 20.5) cases, there were 11.6 (14.8 minus 3.2) with genotype A; they accounted for 67% (11.6/17.2) of the recent increase in AHB.

Regional distributions and longitudinal changes in genotype A

Among the 183 patients from the capital region, the genotype was A in 65 (35.5%), B in 22 (12.0%), C in 94 (51.4%), E in one (0.5%), and H in one (0.5%) (table 2). Of the remaining 364 (66.5%) patients from other regions, by contrast, the genotype was A in 72 (19.8%), B in 26 (7.1%), C in 265 (72.8%), and D in one (0.3%). Genotype A was significantly more common in the capital than in other regions (35.5% vs 19.8%, p<0.0001). In the capital region, genotype A accounted for 4.8% (2/42) in the 1st period, 29.3% (12/41) in the 2nd, and 50.0% (42/84) in the 3rd. There were significant differences between the 1st and 2nd periods (p<0.05), 2nd and 3rd (p<0.05), and 1st and 3rd (p<0.0001). In other regions, by contrast, genotype A accounted for 6.5% (7/108) in the 1st period, 8.5% (7/182) in the 2nd, and 33.1% (47/142) in the 3rd. For the first time in other regions, genotype A increased in the 3rd period, in comparison with the 1st and 2nd (1st vs 3rd, p<0.0001; 2nd vs 3rd, p<0.0001).

Subgenotypes of genotype A

Of the 137 genotype A isolates, amplification and sequencing of HBV DNA were feasible in 114 (83.2%); the isolate from the single patient with genotypes A and G was excluded. A phylogenetic tree was constructed, on the entire preS1/S2/S genes of ~1.2 kb, for these 114 isolates along with 34 genotype A isolates retrieved from the database (figure 3).

Of the 114 isolates in this study, 101 (88.6%) were subgenotype A2, and the remaining 13 (11.4%) were subgenotype A1. In a pair-wise comparison, the sequence divergence among the 101 subgenotype A2 isolates was 0–1.3%, and that among the 13 subgenotype A1 isolates spanned 0% to 2.3%. The sequence divergence between subgenotype A2 and A1 isolates ranged from 2.6% to 4.7%.

A sequence of 1203 nucleotides was possessed in common by three of the 101 (3%) isolates of subgenotype A2. For convenience, the group comprising these three isolates was labelled 'identical group I'. Likewise, an additional six 'identical groups' were found, and numbered from 'II' to 'VII'. They comprised 35 (35%), seven (7%), two (2%), three (3%), 12 (12%) and three (3%) of the 101 isolates of subgenotype A2. In contrast, only one identical group, designated 'VIII', was constructed by three of the 13 (23%) isolates of subgenotype A1.

Some isolates of subgenotype A1 and A2 were obtained from patients who had travelled to foreign countries in the recent past (5/13 (38.5%) patients with A1 to Africa, Philippines, Myanmar and China; and 5/101 (5.0%) patients with A2 to Europe, Thailand, Brazil and the USA).

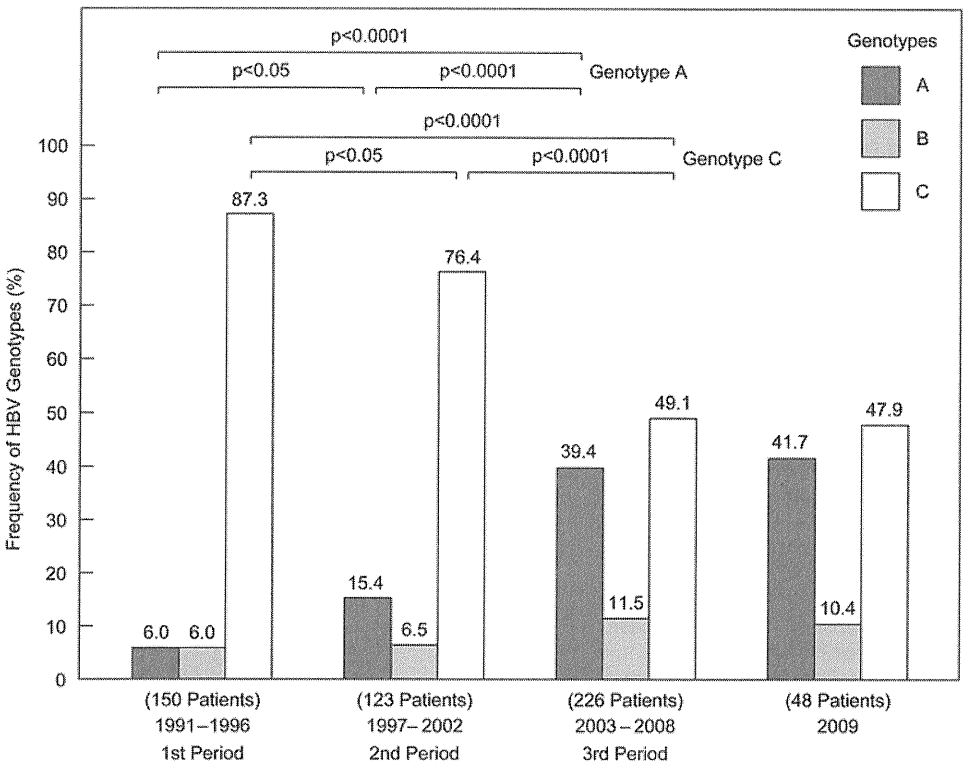

Figure 2 Distribution of hepatitis B virus (HBV) genotypes in three periods.

Subgenotypes of genotype B

Of the 48 isolates of genotype B, subgenotyping was feasible in 43 (90.0%). A phylogenetic tree was constructed on preS1/S2/S-gene sequences from these 43 isolates, along with those from 25 isolates of genotype B retrieved from the database (figure 4). Of the 43 isolates in this study, 10 (23.3%) were subgenotype B1, 28 (65.1%) were B2, two (4.7%) were B3, and three (7.0%) were B4. In a pair-wise comparison, the sequence divergence among 10 subgenotype B1 isolates ranged from 0.4% to 1.4%, and that among 28, two and three isolates of subgenotypes B2, B3 and B4 spanned 0–1.7%, 0.5% and 0.6–0.8%, respectively. The inter-subgenotype divergence among B1–B4 ranged from 0.6% to 4.4%.

One 'identical group' made up of five isolates was detected among the 28 of subgenotype B2; it was named 'IX'. In contrast, no 'identical group' was found in 10, two or three isolates of subgenotype B1, B3 or B4.

Some isolates of subgenotypes B2, B3 and B4 were obtained from patients who had travelled to foreign countries in the recent past (7/28 (25.0%) patients with B2 to China and other countries; 1/2 (50.0%) patients with B3 to a country unknown; and 1/3 (33.3%) patients with B4 to Vietnam). However, none of the 10 subgenotype B1 isolates was associated with travel to foreign countries.

Identical groups

The proportion of isolates that shared a sequence in identical groups was higher for subgenotype A2 (64.4%) than for A1, B1, B2, B3 or B4 (23.1%, 0%, 17.9%, 0% or 0%, respectively (A2 vs A1, $p<0.001$; A2 vs B1, $p<0.0001$; A2 vs B2, $p<0.0001$)).

Homosexual activity was more common in patients belonging to the seven identical groups than the non-identical group of subgenotype A2 (17/65 (26.2%) vs 3/36 (8.3%), $p<0.05$). Among the isolates in the seven identical groups of subgenotype A2, those in groups I, III and VII clustered locally during short periods of 2–7 years. In contrast, subgenotype A2 isolates in groups II and VI were scattered widely over longer periods of 11–16 years.

DISCUSSION

In Japan, as in most Asian countries, the persistent HBV carrier state had been established mainly through perinatal transmission from mother to baby and horizontal infection during infancy. In 1986, a national prevention programme was launched in Japan with selective vaccination of babies born to carrier mothers with hepatitis B e antigen (HBeAg). In 1995, this was extended to babies born to HBeAg-negative carrier mothers. As a result, the prevalence of HBsAg among younger people born since 1986 has decreased dramatically.[28 29] However, there are an

Table 2 Changes in the distribution of genotype A compared between the capital region and other regions over three periods

Area	n	1st Period (1991–1996)	2nd Period (1997–2002)	3rd Period (2003–2008)	2009
Capital region	65/183 (35.5%)†***	2/42 (4.8%)‡* §***	12/41 (29.3%)†* §*	42/84 (50.0%)†*	9/16 (56.3%)
Other regions	72/364 (19.8%)	7/108 (6.5%)§***	7/82 (8.5%)§***	47/142 (33.1%)	11/32 (34.4%)
Total	137/547 (25.0%)	9/150 (6.0%)‡* §***	19/123 (15.4%)§***	89/226 (39.4%)	20/48 (41.7%)

Statistical analysis of the differences between the capital and other regions was performed, as well as through the 1st, 2nd and 3rd periods.
†Significantly different compared with other regions.
‡Significantly different compared with the 2nd period.
§Significantly different compared with the 3rd period.
*$p<0.05$, ***$p<0.0001$.

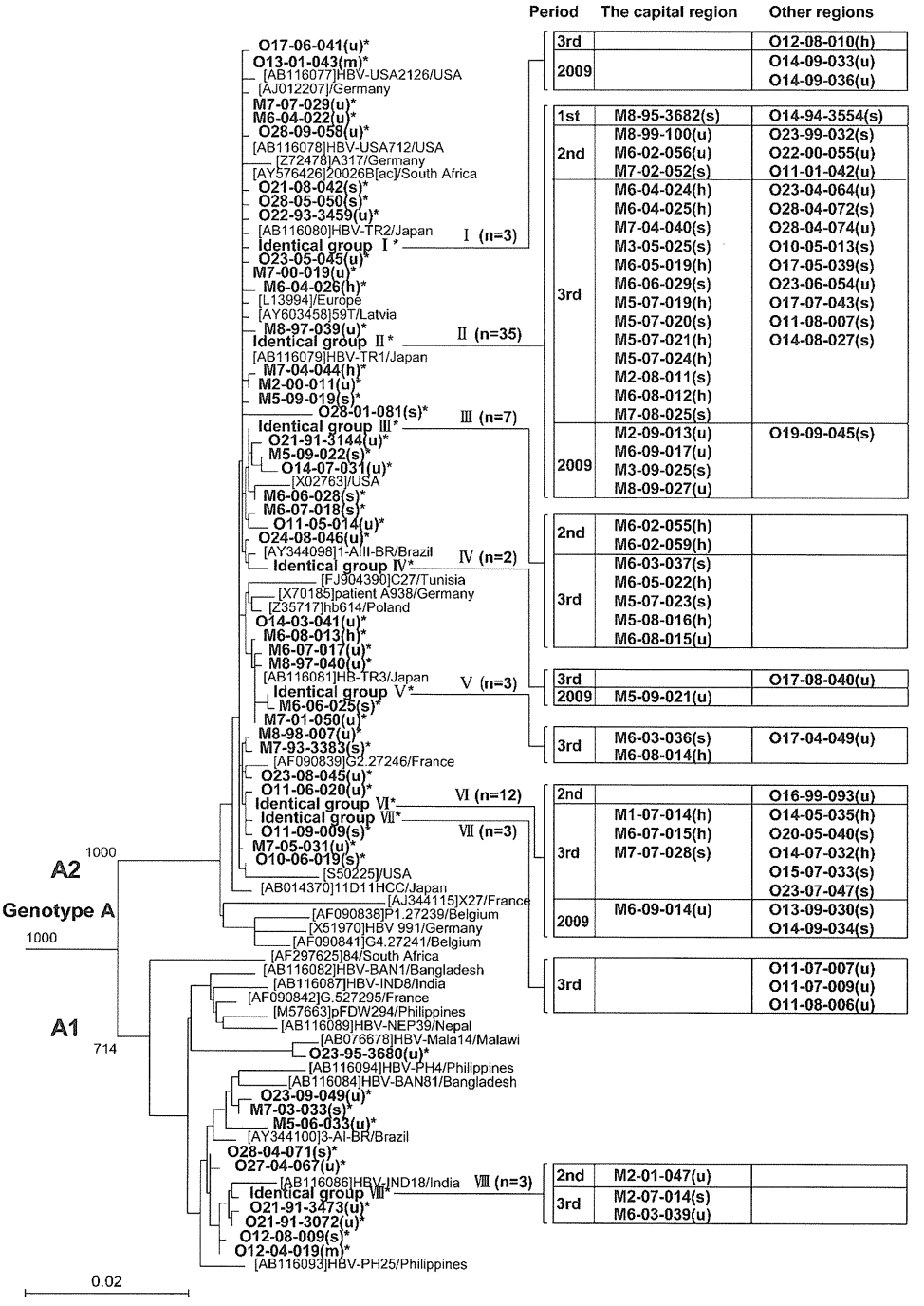

Figure 3 Phylogenetic analysis of genotype A strains by the neighbour-joining method. Isolates obtained in this study are shown in bold with asterisks. Hospitals in the capital region are labelled M1—M8 and those in other regions O9—O28 (corresponding to those in figure 1). Year of onset is indicated by the last two digits after the first hyphen. Numbers after the second hyphen represent the identification numbers of patients in each year (not always consecutive). Transmission routes are shown in lower-case letters in parentheses: h, homosexual; s, heterosexual; m, medical procedure; o, others; and u, undetermined. Isolates with identical sequences are bracketed in 'Identical groups I through VIII' on the tree. Each bracket is divided by areas and periods. Reference hepatitis B virus (HBV) isolates, including 12 of subgenotype A1 and 22 of subgenotype A2, were obtained from the database and specified by their accession numbers, isolate names and countries of origin. Bootstrap values are indicated on major phylogenetic branches.

estimated one million HBV carriers in Japan at present.[30] Furthermore, many Japanese remain at increased risk of horizontal infection with HBV, because they have not received selective vaccination and therefore do not have the antibody to HBsAg. Because AHB is extremely under-reported and no national surveillance data are available in Japan, the incidence has not been determined accurately. In the USA, the incidence of AHB has decreased markedly since the adoption of a comprehensive immunisation strategy in 1991.[31 32]

In the present study over 1991—2009, we conducted a nationwide, sentinel surveillance on AHB in Japan. In the 547 patients recruited over 19 years, genotype C was the most prevalent (65.6%), followed by genotype A (25.0%) and genotype B (8.8%). Demographic and clinical differences were observed among patients with genotypes A, B and C (table 1). The proportion of men reached 94.2% for genotype A infection, higher than that for genotype B (79.2%) or C (56.0%) infection. In the analysis of the route of transmission, homosexual activity was reported by 21.2% of patients with genotype A; all were male. In general, sexual activity tends to be higher in men than women. The predominance of genotype A in men may be attributable to a high frequency of homosexual activity among men.

Although adult-acquired HBV infection persists at a high frequency of ~10% in European countries and the USA,[33] it rarely, if ever, becomes chronic in Japan. Recent studies suggest that the chance of a chronic outcome of AHB may differ by HBV genotype[21 34]; it is more common for genotype A than other genotypes.[22 35 36] In the present study, HBV infection persisted in 4.1% of patients with genotype A, in comparison with 0% of

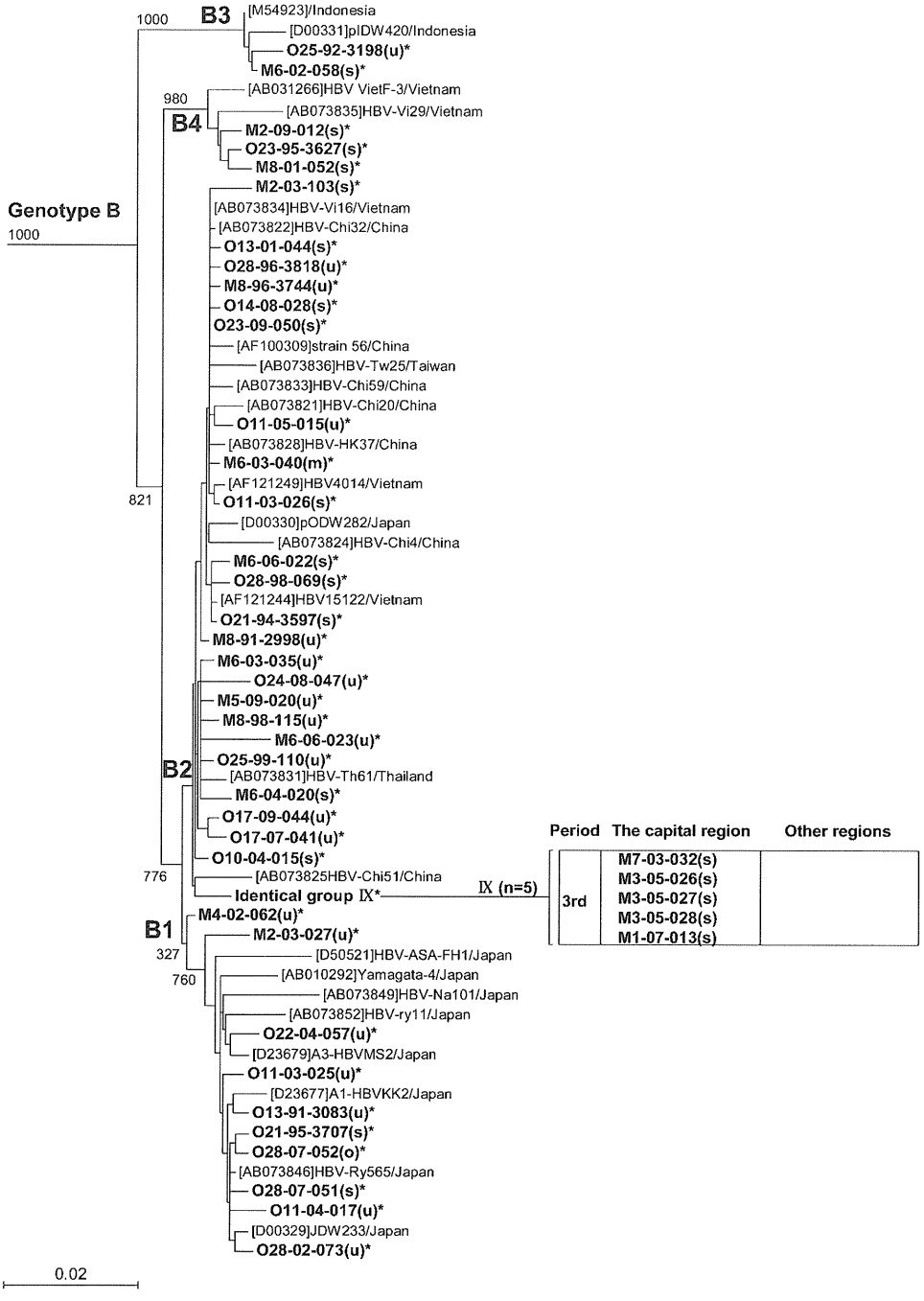

Figure 4 Phylogenetic analysis of genotype B strains by the neighbour-joining method. Hepatitis B virus (HBV) isolates obtained in the present study are specified in the same manner as in figure 3, and isolates with an identical sequence are bracketed in 'Identical group IX' on the tree. Of them, 10 reference isolates of subgenotype B1 and 13, two and two of those of B2, B3 and B4, respectively, were retrieved from the database; they are specified as in figure 3.

those with genotype C. Remarkably, all five patients with AHB who acquired chronic infection possessed HBV genotype A, either alone (four patients) or together with HBV genotype G (one). Increasing genotype A infections may have changed the genotype distribution in patients with AHB and those with chronic HBV infection. In Japanese patients with chronic hepatitis B, the proportion of genotype A has doubled, from 1.7% in 1999–2000 to 3.5% in 2005–2006.[37]

The genotype was A in 29 of the 32 (91%) homosexual men. Of the 29 homosexuals with genotype A, 10 gave consent to anti-HIV testing, and four of these (40%) were found to be positive. Of the five patients who acquired chronic HBV infection, anti-HIV was tested in four (three with genotype A and one with genotypes A and G), and one with genotype A was found to be positive. There is a possibility that co-infecting HIV in this patient with genotype A may have promoted chronic HBV infection; HIV is known to prolong and aggravate HBV infection by compromising immune responses.[38]

Patients with FH in this study were infected with either HBV genotype B (1/48 (2.1%)) or C (8/359 (2.2%)); no patients with genotype A developed FH. PreC and/or CP mutations were significantly less common in genotype A (1/109 (3.7%)) than B (6/39 (15.4%)) or C (279/310 (5.5%)) infection. The single patient with genotype A who had PreC mutation was simultaneously infected with HBV genotype G. There is a possibility that the PreC mutation in this patient was from HBV genotype G.[26] FH did not develop in any patients with genotype A, which may be attributable, at least in part, to the lack of PreC mutation in genotype A infections.[39]

Previous reports have shown that genotype A is common in patients with AHB in Metropolitan Tokyo,[20 21 40] as well as around Aichi located in the middle of Mainland Japan.[22]

Yotsuyanagi *et al*[23] reported that genotype A is more common in patients with AHB in the metropolitan region than in other regions. Sugauchi *et al*[41] found that, in patients with AHB, the proportion with genotype A has increased over time. The present study indicates that the number of patients with AHB in Japan would not have decreased. We found that the proportion of patients with genotype A infection is increasing in the 28 national hospitals in Japan (6.0% in the 1st period, 15.4% in the 2nd, and 39.4% in the 3rd (figure 2)), with the prevalence much higher in the capital than other regions (35.5% vs 19.8% (table 2)).

In this study, there was a time lag in the increase in genotype A infection between the capital region and other regions of Japan (table 2). In the capital region, the prevalence of genotype A started to increase in the late 1990s, and kept increasing through the early 2000s (4.8% in the 1st period, 29.3% in the 2nd, 50.0% in the 3rd, and 56.3% in 2009). In other regions, by contrast, the frequency of genotype A did not change during the late 1990s, and increased significantly in the 2000s (6.5% in the 1st period, 8.5% in the 2nd, 33.1% in the 3rd, and 34.4% in 2009). Thus infiltration of genotype A infection into other regions occurred 5–6 years behind the epidemic in the capital region. This indicates that genotype A infection originated in the capital region and then spread to other areas of Japan.

Some genotypes are classified into several subgenotypes, and they have distinct geographical distributions.[42] Hence, subgenotypes are useful in tracing the route of HBV infection. By phylogenetic analysis (figures 3 and 4), 88.6% of genotype A isolates had the European–American type (A2), and the remaining 11.4% possessed the Asian–African type (A1). Likewise, 76.7% of genotype B isolates had Asian types (B2–B4), and the remaining 23.3% possessed the type endemic to Japan (B1). Of the 157 HBV isolates of genotype A or B, 147 (93.6%) had subgenotypes foreign to Japan. They are thought to have been transmitted from foreign sex workers, and spread among certain populations who share particular sexual behaviours in Japan.[41]

Of note, some HBV isolates of distinct subgenotypes possessed an identical sequence in the preS1/S2/S gene. The isolates of subgenotype A2 were prominent in this regard, and more often had the same sequence than those of other subgenotypes, such as A1, B1 and B2. The high prevalence of subgenotype A2 isolates with an identical sequence would not have been caused by cross-contamination. If cross-contamination had occurred, it would have affected isolates of all subgenotypes, and not influenced subgenotype A2 isolates preferentially. As many as 35% of subgenotype A2 isolates had an identical sequence, and those with the same sequence increased to 56.3% in the recent 2009 survey in Metropolitan Tokyo. Furthermore, some subgenotype A2 isolates in groups I, III and VII clustered locally within short periods, whereas others in groups II and VI were scattered widely over a long period of time. On the basis of these results, it is tempting to speculate that some subgenotype A2 strains would have been transmitted from person to person without undergoing mutations for many years.

In summary, the present study indicates the following. (1) AHB in the 28 national hospitals in Japan has not decreased, because genotype A infections are increasing. (2) Genotype A infections started to increase in the capital region, and then spread to local areas 5–6 years later. (3) Approximately 90% of genotype A in patients with AHB is subgenotype A2. (4) Subgenotype A2 strains with an identical sequence are spreading among younger generations with high sexual activity. (5) On the basis of the results obtained, AHB in Japan is not decreasing, because HBV of subgenotype A2 is prevailing in particular subpopulations at high risk. Finally, in order to prevent further increases in AHB in Japan, universal vaccination of young people deserves consideration.

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Contributors YT, HY and HI designed data collection tools, monitored data collection for the whole study, wrote the statistical analysis plan, cleaned and analysed the data. YT, HY and YM drafted and revised the paper. HY, NM, MN, EM, TK, YW, TM, MS, TH, TS, YM, TK, MT, HK, HO, SH and SA collaborated in data and sample collection.

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Hepatitis B virus strains of subgenotype A2 with an identical sequence spreading rapidly from the capital region to all over Japan in patients with acute hepatitis B

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INTERNAL MEDICINE

□ CASE REPORT □

An Adult Patient with Acute Infection with Hepatitis B Virus Genotype C that Progressed to Chronic Infection

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Abstract

In 2008, a 28-year-old woman consulted our hospital due to general fatigue. Her ALT level was within normal range but she was positive for hepatitis B surface antigen (HBsAg). Her ALT level was nearly within normal range thereafter and she was consistently positive for HBeAg. Later, it was proven that she was negative for HBsAg in 1999. She had been a sex worker in 2007-2008. Complete genome sequencing revealed that her HBV was genotype C. The present case may indicate that it is possible for acute infection with HBV genotype C to progress to chronic infection in adults.

Key words: hepatitis B virus, acute hepatitis, genotype, chronic hepatitis

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Introduction

Hepatitis B virus (HBV) is widely distributed worldwide. Approximately 350 million people are chronically infected and 2 billion people have been exposed to HBV. The clinical outcome in patients with acute hepatitis B varies widely. HBV is one of the most important causes of liver cirrhosis and hepatocellular carcinoma (1). Hepatitis is self-limited in most patients, whereas 1% or 2% of patients progress to fulminant hepatic failure, and some progress to chronic infection. It has been reported that the rate of progression from acute to chronic HBV infection is 90% in newborns and 5-10% in adults (2, 3).

There are at least 8 HBV genotypes, each defined by a sequence divergence greater than 8% of the entire HBV genome, which consists of approximately 3,200 nucleotides (4), and is designated by the capital letters A-H in the order of their discovery. There are some differences in infectious routes and clinical features among genotypes. The main infectious route in chronically infected patients is horizontal for genotypes A and D, whereas it is vertical for genotypes B and C. It has been established that the rate of chronicity from acute infection differs between HBV genotypes; the rate of chronicity is high in genotype A and low in other genotypes. The rates of chronicity of genotype A and genotype C infections have been reported to be 3-23% and 0-1%, respectively (5-8).

In the present report, we describe an adult patient who progressed to chronic infection after infection with HBV genotype C.

Case Report

A 28-year-old woman consulted our hospital in August 2008 due to general fatigue that had persisted for 1 month. She was worried about sexually transmitted disease (STD) because she had been a sex worker in 2007-2008 before approaching our hospital. Laboratory examination revealed that hemogram and liver function tests were within normal range. She was negative for *Chlamydia. trachomatis* antigen, Treponema pallidum Latex Agglutination (TPHA), gonococcal DNA, human immunodeficiency virus (HIV) antigen and antibody, and anti-human leukemia virus 1 (HTLV-1), but

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