厚生労働科学研究費補助金（難病・がん等の疾患分野の医療の実用化研究事業）
分担研究報告書

肝癌症例における肝炎ウイルス性肝癌と非ウイルス性肝癌の比較

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研究要旨　【目的】NBNC症例の背景肝組織及び肝癌組織の特徴について肝炎ウイルス性肝癌との比較検討を行った。【対象・方法】1999年より2009年までの期間、長崎肝疾患研究会（NASLD）参加施設において肝癌と登録された3204例の中で、HBsAg、HCVAb、AFP、PIVKA-II未測定例、TNM stage不明例を除外し、背景肝組織像及びが確認できた320例を対象とした。【結果】景肝組織像及びが確認できた320例で検討したところ、HBV、HCV、B+C、NBNCのstage2以下の症例は、各々23%、21%、14%、39%と有意にNBNC症例のstageが軽度であり、grade2以下の症例は各々55%、31%、29%、57%とHCV症例と比較し有意にgrade軽微であった。肝癌の分化度は、肝癌の分化度について検討した結果、 HBV及びNBNCは、HCV症例より低分化な症例が有意に多くみられた。【結論】NBNCでは、肝炎ウイルス関連肝癌と比較し肝組織において肝線維化及び活動性は軽度であった。一方、肝炎ウイルス関連肝癌と比較してNBNC肝癌は、TNM stageが進行した状態で診断されている。

共同研究者
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A．研究目的

非B非C型肝癌（NBNC）は九州地区において1996年は7.6%であったのに対し2008年21.6%と著明に増加傾向を示している。しかし、NBNCに対するサーベランスは確立されてなくTNM stageが進行した状態で診断されているのが現状である。その一方、早期にNBNC肝癌を診断し治療を行った場合、肝炎ウイルス関連肝癌より予後が良いと報告されているが、その詳細については検討されていない。本研究では、NBNC症例の背景肝組織及び肝癌組織の特徴について肝炎ウイルス性肝癌との比較検討を行った。

B．研究方法

1999年より2009年までの期間、長崎肝疾患研究会（NASLD）参加施設において肝癌と登録された3204例の中で、HBsAg、HCVAb、AFP、PIVKA-II未測定例、TNM stage不明例を除外したB型肝炎関連肝癌（HBV）474例（18%）、C型肝炎関連肝癌（HCV）1533例（58%）、HBs抗原陽性/HCV抗体陽性例（B+C）40例（2%）、NBNC591例（22%）の2638例を対象とした。

（倫理面への配慮）

本研究はretrospective studyのため、症例に対して診療上の必要性のため行った臨床検査の結果をもとに検討を行った。解析の際は、氏名・IDを暗号化し、個人を特定できないように配慮した。尚、この研究については長崎大学病院倫理委員会にて了承を得ている。

C．研究結果

NBNCに寄与する因子についてロジステック回帰分析による多変量解析で検討したところ、年齢（70才以上: p=0.001 RR1.63)、性別（女性: 0.002 RR1.73)、BMI（25以上: ＜0.001 RR 2.12)、飲酒歴（多飲: p=＜0.001 RR14.73)、糖尿病（あり: p=＜0.001 RR 2.42)、AST（56IU/l未満: 0.035 RR 1.47)、ALT（46IU/l未満: p=＜0.001 RR2.08)、AFP（20-199ng/ml: p=0.005 RR0.60； 200 ng/ml以上: p=0.018 RR0.63)、PIVKA-II（40-199mAU/ml: p=0.010 RR1.64； 200 mAU/ml以上: p=＜0.001 RR2.08)、TNM stage（II: p=0.011 RR1.67； III: p=0.007 RR1.88； IV以上: p=0.004 RR2.40）であった。背景肝組織像及びが確認できた320例で検討したところ、HBV、HCV、B+C、NBNCのstage2以下の症例は、各々23%、21%、14%、39%と有意にNBNC症例のstageが軽度であり、grade2以下の症例は各々55%、31%、29%、57%とHCV症例と比較し有意にgrade軽微であった。肝癌の分化度は、肝癌の分化度について検討した結果、 HBV及びNBNCは、HCV症例より低分化な症例が有意に多くみられた。

D．考察

NBNCでは、肝炎ウイルス関連肝癌と比較し肝組織において肝線維化及び活動性は軽度であった。一方、肝炎ウイルス関連肝癌と比較してNBNC肝癌は、TNM stageが進行した状態で診断されている。

E．結論

NBNCでは肝予備能が良好であり早期の段階で診断することにより根治的治療が期待できると考えられる。

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2. 学会発表

なし。

G. 知的財産権の出願・登録状況

なし。

厚生労働科学研究費補助金（難病・がん等の疾患分野の医療の実用化研究事業）
分担研究報告書

ME3738の肝癌細胞に対する抗腫瘍効果など

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研究要旨　トリテルペン誘導体ME3738は、肝細胞癌細胞株を用いたC型肝炎ウイルス（HCV）のreplicationシステムにおいて濃度依存性にHCV-RNAとHCVコア蛋白の量を減少させ、さらにIFN-αとの併用で相乗的なHCV-RNAの減少を誘導することが報告されている。今回、ME3738の肝癌細胞に対する抗腫瘍効果についてin vitroとin vivoで検討を行った。その結果、ME3738は、肝癌細胞に対して種々の程度に直接的増殖抑制効果を示し、13種類の肝癌細胞株の50%増殖抑制濃度は、0.8～2.4μMであった。アポトーシス誘導は見られず、細胞周期の進行停止を2株で確認した。IFNとの併用では、相加作用は認めたが、相乗的な増殖抑制作用は認めなかった。肝細胞癌株HAK-1Bのヌードマウス皮下に移植腫瘍を使用したin vivoの実験では、ME3738単独群は、コントロールに比べ約30%腫瘍が減少した。しかし、IFNとME3738の併用群はIFN単独群と差は見られなかった。ME3738のC型慢性肝炎患者への投与は、ウイルス除去と抗腫瘍効果を発揮し、肝癌の発癌・再発予防に有効である可能性が推察される。

A．研究目的

日本における肝細胞癌の70%は、C型肝炎ウイルスの感染による慢性肝炎、肝硬変を背景に発症する。トリテルペン誘導体ME3738は、肝細胞癌細胞株を用いたC型肝炎ウイルス（HCV）のreplicationシステムにおいて濃度依存性にHCV-RNAとHCVコア蛋白の量を減少させ、さらにIFN-αとの併用で相乗的なHCV-RNAの減少を誘導することが報告されている。今回、ME3738の肝癌細胞に対する抗腫瘍効果についてin vitroとin vivoで検討を行った。

B．研究方法

A．ME3738単独あるいはIFNとの併用投与によるin vitroでの肝癌細胞の増殖抑制効果に関する検討：ME3738は、Meiji Seikaファルマ株式会社により供与された。pegylated IFN-α 2b（1,000 IU/mL、PEGIntron®、MSD）単独、ME3738（0.5～1μM）単独、両者併用による肝癌細胞増殖抑制効果について24時間、48時間、72時間後にそれぞれMTT assayで生細胞数を同定した。肝癌細胞株は、当教室で樹立した11種類の肝癌細胞株を使用した。アポトーシスの誘導の有無をannexin-Vとフローサイトメトリーを用いて検討した。また、ME3738の細胞周期への作用を2株で検討した。

B．ME3738単独あるいはIFNとの併用投与によるin vivoでの肝癌細胞の増殖抑制効果に関する検討：HAK-1B肝癌細胞を1,000万個ヌードマウスの皮下に接種し、約1週間後腫瘍径が1cm前後になった時点で実験を開始した。IFN単独投与群（1,920 IU/mouse：C型慢性肝炎の治療に使用され

るPEGIntron®の臨床投与量に相当、週2回皮下投与)、ME3738単独投与群（1.5mg/g含有餌)、IFNとME3738併用投与群（IFN、ME3738共に上記の量)、コントロール群(培養液皮下投与、通常の餌摂取）を作成し実験を行った。15日目にsacrificeした。また、腫瘍の短径と長径を2、3日に一度行い、(短径) 2 x 長径 x 1/2の式に当てはめて推定体積を求めてグループ間で比較した。

C．研究結果

A．In vitroでは、ME3738は、直接的にある一定濃度までは濃度依存性に増殖抑制作用を示した。24、48、72時間の接触時間の中で、24時間での増殖抑制効果が高く、11株の50%増殖抑制濃度（IC50）は、0.8～2.4 μMの範囲内であった。アポトーシスは認められなかったが、細胞周期の検討で、2株で、S期細胞周期進行停止が認められた。IFNと併用投与した場合、細胞株やME3738の濃度によりその反応性は異なったが、概して相加的な作用が見られた。

B．In vivoにおいては、IFN単独群とIFN＋ME3738併用投与群が、同程度の増殖抑制効果を示し、コントロールの約30%まで腫瘍体積が減少した。ME3738単独投与では、コントロールの69%の腫瘍体積を示した。IFNとME3738の相加・相乗作用は見られなかった。

D．考察

In vitroにおいて、ME3738は、肝癌細胞株に対して接触早期から単独でも抗腫瘍作用を示し、IFN-αとの併用により相加的な抗腫瘍作用も認めた。IFNとの併用では、明らかな相乗的増殖抑制効果は見られなかったが、相加的な増殖抑制作用は大部分で認められた。IFNの増殖抑制作用に抵抗性の細胞株の中でME3738に対しては感受性の細胞株が見られることから、併用により、より多くの肝癌細胞に増殖抑制作用が誘導できると思われる。ME3738は、全ての細胞株でアポトーシスの誘導は認めなかったが、2株でS期細胞周期進行停止が認められたため、細胞周期に作用して肝癌細胞の増殖を抑制している可能性があるが、さらに多くの細胞株で検討する必要がある。今後の課題である。

ME3738は、in vivoにおいて、IFNとの併用で、相加・相乗効果は明らかではなかったが、単独で抗腫瘍効果を示した事は、非常に興味深い。今後、腫瘍の組織学的解析により増殖抑制のメカニズムの解析が必要である。

以上より、ME3738のC型慢性肝炎患者への投与は、ウイルス除去と抗腫瘍効果を発揮し、肝癌の発癌・再発予防に有効である可能性が推察された。

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Ｆ．知的財産権の出願・登録状況
（予定を含む。）

1. 特許取得

あり（出願済み）。

2. 実用新案登録

なし。

3. その他

なし。

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難病・がん等の疾患分野の医療の実用化研究事業

病態別の患者の実態把握のための調査および肝炎患者の病態に即した相談に対応できる相談員育成のための研修プログラム策定に関する研究

平成23年度

総括研究報告書
分担研究報告書
（2／2）

論　　文　　集

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論　　文　　集

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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.

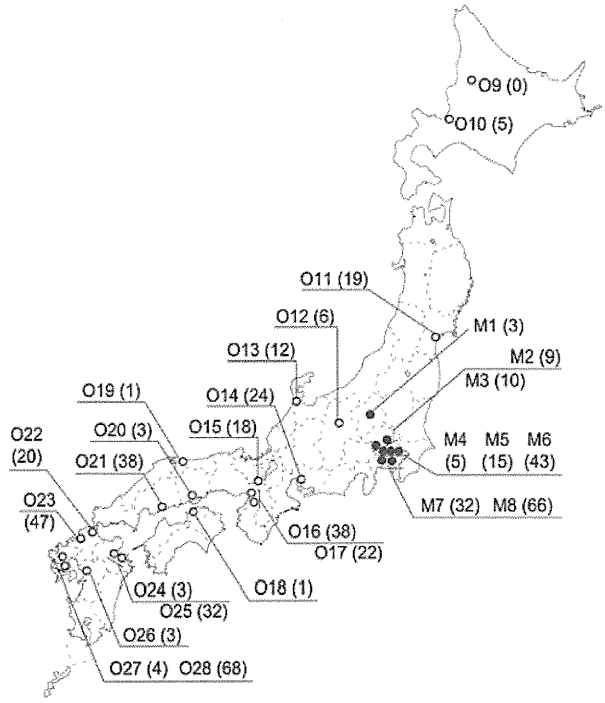

Figure 1 Locations of participating hospitals in Japan. Hospitals in the capital region (M1–M8) are indicated by eight closed circles, and those in other regions (O9–O28) by 20 open circles. Numbers in parentheses indicate the total number of enrolled subjects for each site. The hospitals are: M1, Nishigunma 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).

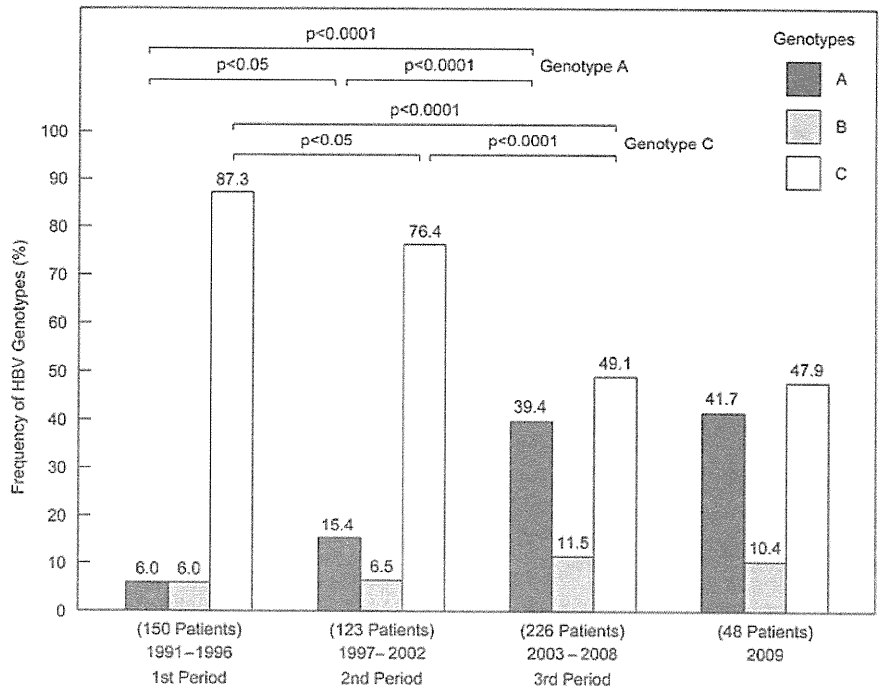

Figure 2 Distribution of hepatitis B virus (HBV) genotypes in three periods.

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.
*$p<0.05$, ***$p<0.0001$.

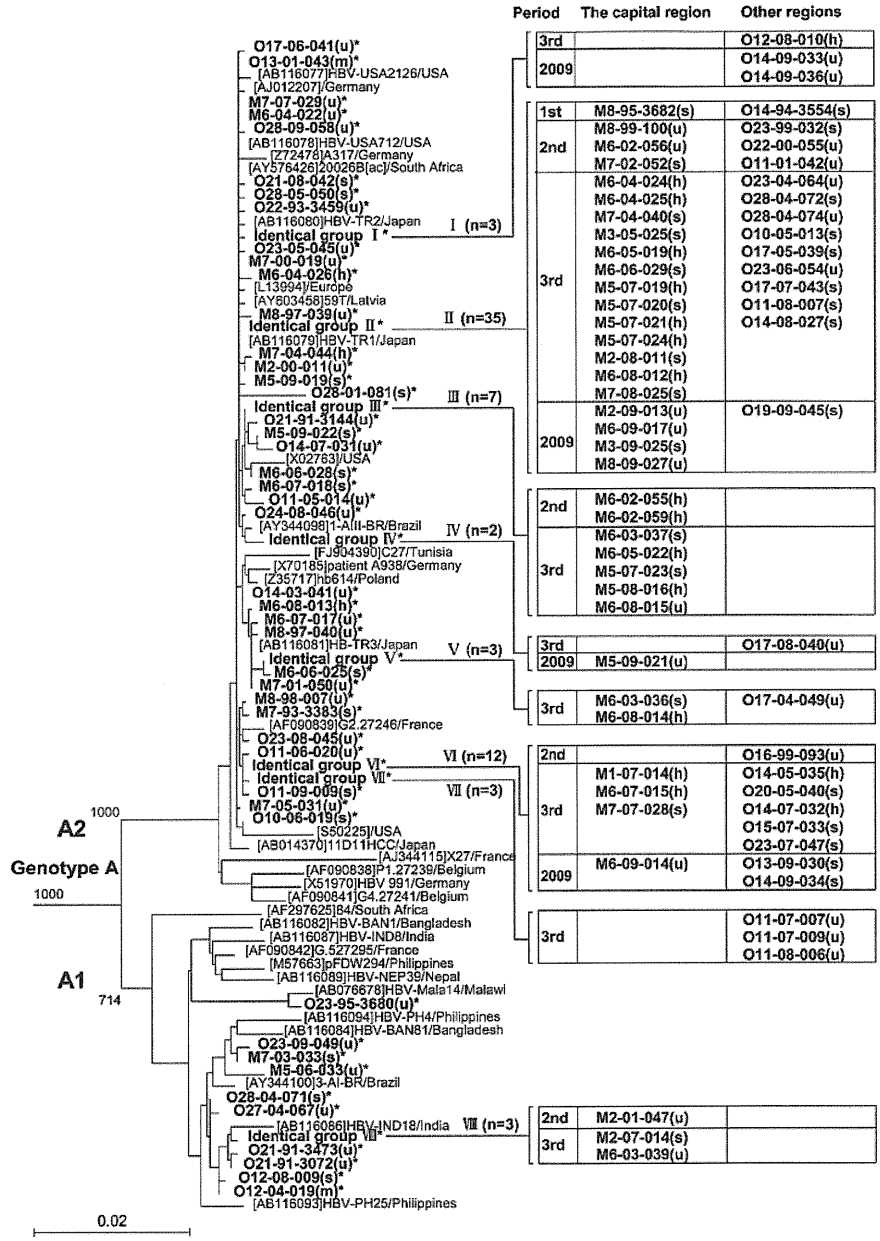

Figure 3 Phylogenetic analysis of genotype A strains by the neighbour-joining method. Isolates obtained in this study are shown in bold with asterisks. Hospitals in the capital region are labelled M1–M8 and those in other regions O9–O28 (corresponding to those in figure 1). Year of onset is indicated 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

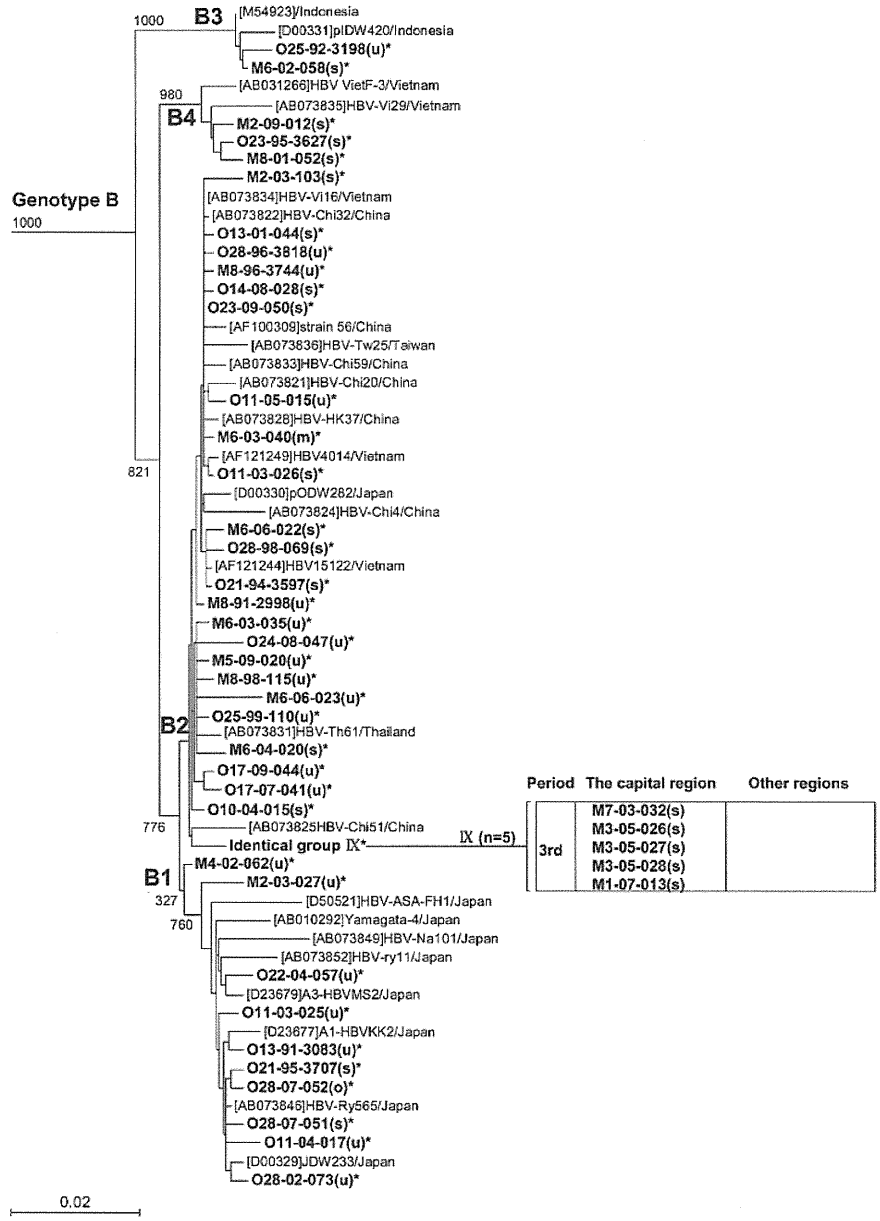

Figure 4 Phylogenetic analysis of genotype B strains by the neighbour-joining method. Hepatitis B virus (HBV) isolates obtained in the present study are specified in the same manner as in figure 3, and isolates with an identical sequence are bracketed in 'Identical group IX' on the tree. Of them, 10 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]