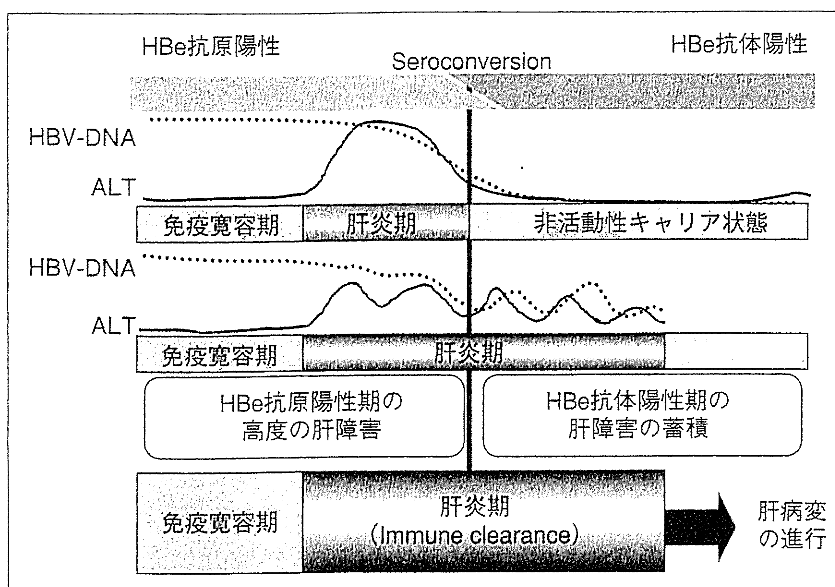

図１ Ｂ型肝炎の経過図

イルス性肝疾患の治療の標準化に関する研究」班によりＢ型肝炎の治療ガイドラインが策定され，毎年，更新されている[2)].

Ｂ型肝炎に対する治療薬

現在，わが国で使用可能な抗HBV作用薬は，大別して以下の２種類である．

１．IFN製剤

わが国では，1986年にβ型，1988年にα型のIFNが，HBe抗原陽性慢性活動性肝炎に対しての承認を獲得した．当初は投与期間が４週間であり，有効性は限定的であった．2002年から，24週間投与が可能となって現在に至っている．今後，徐放型IFNであるペグ・インターフェロン(PEG-IFN)の導入が期待されている．

２．核酸アナログ製剤

わが国では，2000年にラミブジン(LAM)が慢性肝炎に(2005年から肝硬変へも拡大)，2006年からエンテカビル(ETV)が肝炎・肝硬変症例に投与可能となった．また，アデホビル(ADV)は，2004年にLAM耐性例に対して追加併用が可能となり，さらに，2008年に単独投与の承認を得ている．

IFN製剤は，副作用，１回分の費用，投与可能期間などの点で核酸アナログ製剤に比べて負担が大きく，核酸アナログ製剤が導入された2000年以降は，核酸アナログ製剤投与例が多数を占める現状にある．

両者の作用機序には違いがあり，核酸アナログ製剤は，主にHBV逆転写の段階に作用し，増幅を抑制しているが，IFNは，この作用に加え免疫賦活作用を有しており，感染肝細胞自体を排除する効果を有する．この特徴を十分に理解し治療法を選択する必要がある．

IFNと核酸アナログ製剤の短期併用による抗HBV効果の評価

当科で行った，HBe抗原陽性例に対するIFNβの１日２分割投与(４週間)，LAM単剤投与，および，両者の併用(４週間．以後はLAMのみ継続)の検討について成績を示す．

対象は合計36例で，IFNβの１日２分割投与例をIFN２分割群，ラミブジン単剤投与例をLAM群，両者併用例を併用群と表すと，IFN２分割群18例，LAM群12例，併用群６例である．さらに，従来型のIFN１日１回４週間投与例85例をIFN従来群として比較した(表１)．

治療開始４週間でのHBV-DNA低下量は，IFNβ２分割群とLAM投与群で大きかった．また，HBV-DNAの減少は，開始後数日間の比較的急峻な低下と，それ以降の緩徐な低下に分けられる例が多く，これを，第１相，第２相と表して，減少

表1　HBe抗原陽性例の短期治療(4週間)検討対象

		IFN2分割群	LAM群	併用群	IFN従来群
症例数		18	12	6	85
男女比		12/6	10/2	3/3	56/29
平均年齢	(歳)	32.6±2.3	37.5±11.1	25.0±3.7	32.5±7.5
治療開始前		$P<0.05$ (IFN2分割群 vs LAM群)			
ALT値	(IU/l)	176.7±157.1	465.9±491.0	263.5±290.4	205±188.3
HBe抗原	(CI)	136.1±94.9	121.8±64.4	99.0±66.4	124.9±78.4
HBV-DNA量	(LGE/ml)	8.0±0.7	7.8±0.8	7.5±0.8	7.8±0.8
開始後4週間					
HBV-DNA低下量	(LGE/ml)	2.4±1.3	3.2±1.4	3.3±1.2	1.4±1.0
HBe抗原低下率	(%)	50.7±62.7	60.3±46.0	57.9±41.1	20.9±34.0

(平均±標準偏差)

表2　短期治療例における治療早期のHBV-DNA減少速度

	第1相	第2相
	(LGE/ml/日)	(LGE/ml/日)
IFN2分割群	0.23±0.12	0.08±0.05
LAM群	0.34±0.22	0.09±0.08
併用群	0.44±0.15	0.07±0.03

速度を算出した．併用群で，第1相での減少速度が大きい傾向を認めた(表2)．

これらの成績は，IFNと核酸アナログ製剤の併用により，抗ウイルス効果を増強させる可能性を示している．

当科におけるB型肝炎に対する併用療法

当科では，HBe抗原陽性慢性肝炎患者を対象として，IFN-βの24週間投与と核酸アナログ製剤16週間の同時投与を行ってきた．これは，現在，保険治療が可能なIFNの用法用量に核酸アナログ製剤を併用し，段階的に投薬を終了する形でプロトコールを作成している(図2)．

当科の同時併用治療の特徴は，

①2剤同時使用により，抗ウイルス効果および免疫賦活効果を増強させた．

②タイミングを考慮した上で，初期に強力な抑制効果を期待する．

③治療中止後の強いリバウンドによる肝不全の出現を回避する．

④核酸アナログ投与の短期化による耐性出現を抑制する．

などで，結果的に，若年層へも期間限定的な核酸アナログを用いた抗HBV治療を可能とさせている．

IFN(24週)+核酸アナログ製剤同時併用療法の成績

当科における，HBe抗原陽性慢性肝炎患者に対して，表1で示したプロトコールで，IFNβ24週間と核酸アナログ製剤16週間の同時併用治療を行った成績を示す．対象は49例で，IFNβ1日300万単位を，初期4週間は週に5～6回，以後20週間を週3回投与とし，核酸アナログ製剤は，16週間，連日内服して終了とした．投与した核

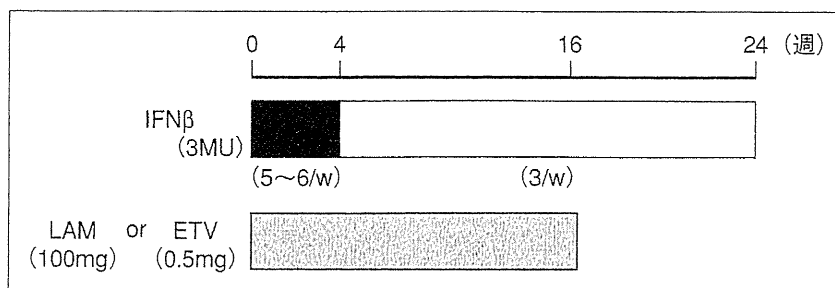

図2　当科のIFN+核酸アナログ併用方法

表3　核酸アナログ製剤およびIFN(24週間)併用のHBe抗原陽性症例

		NA単独群 (Lam or ETV)	併用群 (IFNβ＋Lam or ETV)
症例数		75	49
男/女		54/21	25/24
年齢	(歳)	40.9±13.4	38.7±12.8
開始時ALT値	(IU/l)	277.7±319.1	182.7±168.1
HBV-DNA量	(LC/ml)	7.4±1.2	7.4±1.0
Precore変異	：wild優位/その他	34/12	32/13
CP変異	：wild優位/その他	4/42	8/37
genotype	：B/C	1/33	1/35
F score	：0/1/2/3/4	5/10/11/10/7	2/10/18/9/3
A score	：0/1/2/3	0/16/20/1	1/11/15/4
観察期間	(月)	17.2±10.2	19.9±17.2

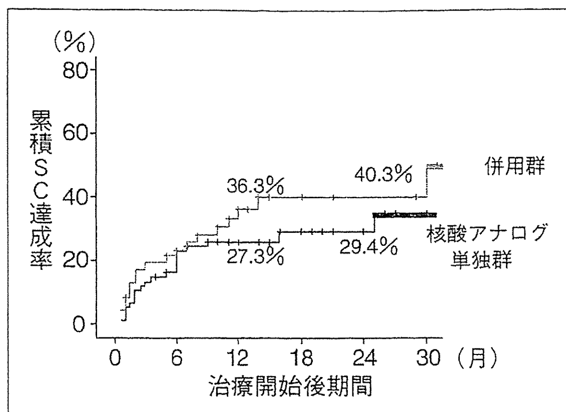

図3　治療法別の累積SC達成率

酸アナログは，LAMが23例，ETVが26例であった．対象として，同時期に核酸アナログ製剤を単独投与したHBe抗原陽性例75例(LAM 37例，ETV38例)の効果と比較した(表3)．両群の背景では，単独群の治療開始時ALT値が高値，併用群の女性比率が高率の傾向があった．単独投与群では，投与期間に制限は設けず，SC達成後，少なくとも1年以上の継続投与後に投与中止とし，それ以外は継続中である．

Kaplan-Meier法による累積SC達成率として2群を比較した場合，12か月後，24か月後のSC達成率は，核酸アナログ単剤群の27.3%，29.4%に対して，併用群では，36.3%，40.3%と高率の傾向を示したが，統計学的には有意な差には至らなかった(P=0.15)(図3)．

治療開始1年以内のSC達成例の割合は，単独投与の19例(25.3%)と比較して，併用群では19例(38.8%)と高率であった．治療開始3年以内ま

表4　治療開始後のHBe抗原/抗体の変化(開始後3年以内)

	SC達成例 1年以内	SC達成例 1〜3年後	HBe reversion (3年以内)
単独群(n=75)			
LAM37 / ETV38	19 (25.3%)	2 (2.3%)	1 (1.3%)
併用群(n=49)			
IFNβ＋(LAM23 / ETV26)	19 (38.8%)	3 (6.1%)	5 (10.2%)

表5　治療開始後の経過(開始後3年以内)

	再治療 (3年以内)	→SC	耐性株出現 LAM	耐性株出現 ETV
単独群(n=75)				
LAM37 / ETV38	2 (2.3%)	0 (0%)	16/37 (43.2%)	0/38 (0%)
併用群(n=49)				
IFNβ＋(LAM23 / ETV26)	14 (28.6%)	4 (8.2%)	1*/23 (4.3%)	0/26 (0%)

* 再治療にてLAM単独投与.

でのSC例は，単独群は21例(27.6%)，併用群では22例(44.9%)となり，併用群で高率の傾向を認めた(P=0.06)．一方，SC後3年以内にHBe抗原が再陽転する症例は，単独群では1例のみであるが，併用群では5例に認めた．しかし，この結果は，単独群と併用群とで，継続する治療期間が異なることに起因すると考えられる(表4)．併用群では，初回治療開始後3年以内に再治療を行う例が14例(28.6%)であり，その中からSCを達成する症例もあり，結果的には，3年間の経過の中では，一定のSC達成を維持していた(表5)．

一方，核酸アナログ製剤に対する耐性変異株

の出現については，LAMの単独投与群では高率に認め，37例中16例(43.2％)に耐性株を認めた．ETVに関しては，現時点では耐性変異の出現例は認めていない．

検討対象の背景を限定して，投与法別の有効性をKaplan-Meier法による累積SC達成率で評価すると，性別では男性，年齢では30歳以上の併用群で有効性が向上する傾向を認めた(図4, 5)．B型肝炎治療でHBe抗原の陰性化が得られやすい要因として，治療前ALT値，年齢，性別などがあげられるが，今回の併用治療は，難治が予想される条件において有効率を向上させる可能性を示している．

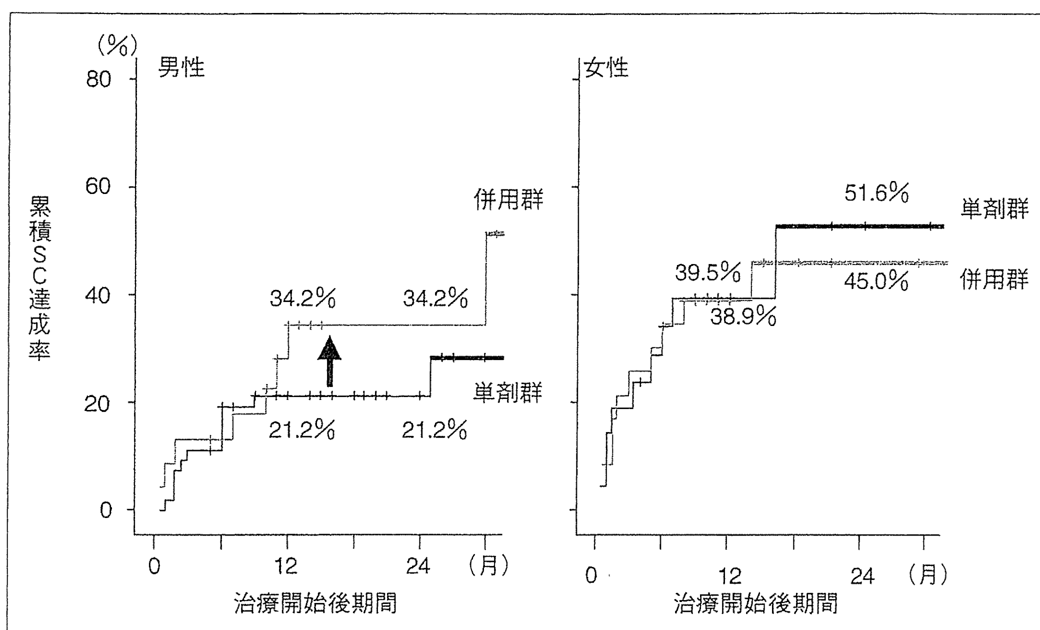

図4　性別および治療法別の累積SC達成率

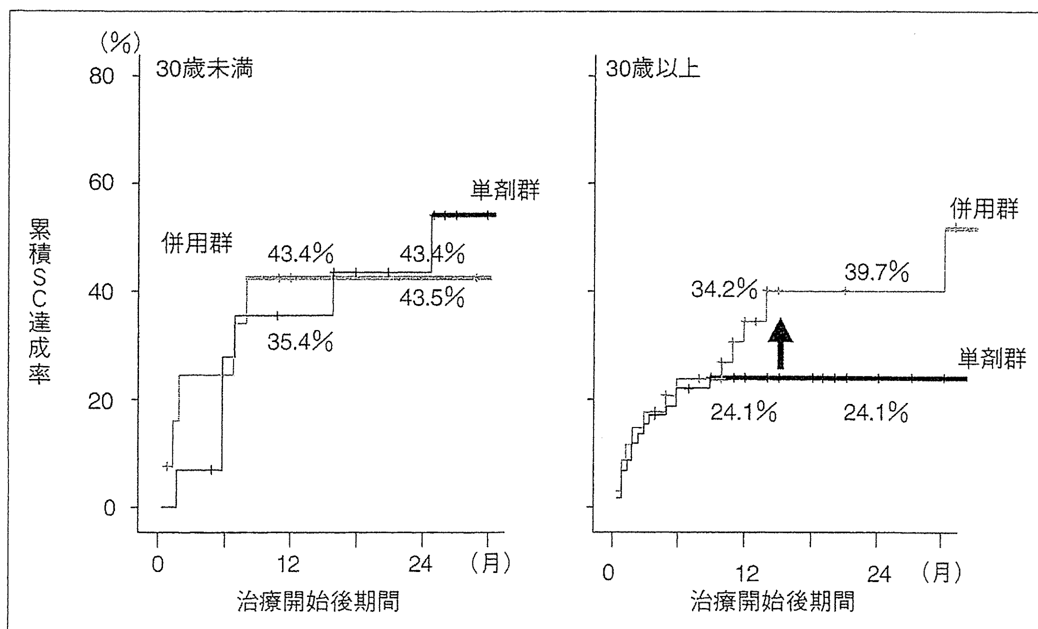

図5　年齢および治療法別の累積SC達成率

海外におけるIFN製剤＋核酸アナログ製剤併用の治療成績

従来型のIFN製剤と核酸アナログ製剤の単独・併用投与に関して，Schalmら[3]が無作為比較試験(RCT)の成績を報告している．230例のHBe抗原陽性者を，IFNα-2b(1,000万単位，週3回，16週間)，LAM(100mg，連日，52週間)，両者併用(8週間のLAM先行投与後，16週間の併用)，の3群で治療し，52週後のSC率は，それぞれ，19%，18%，29%で，特に，治療開始時ALT値が正常上限2倍以上の併用群で約40%と高率のSCを達成している．

2000年代前半の試験では，HBV genotype別の検討は行われておらず，また，併用治療とLAM単独投与での解析が多かったが[4)5)]，海外では，PEG-IFN製剤の導入後，B型肝炎治療ガイドラインも，IFN製剤はPEG-IFNαが中心となってきている[6)~8)]．

近年，PEG-IFNα製剤と核酸アナログ製剤の併用に関する比較的大規模なRCTの結果が報告されてきている．HBe抗原陽性例に対して，Lauら[9]は，LAM単独，PEG-IFNα-2a単独，両剤併用の3群814例による48週のRCTを行った．また，Janssenら[10]は，PEG-IFNα-2b単独群とLAM＋PEG-IFNα-2b併用群の2群307例で行った52週間のRCTを行っている．後者の試験は，70%以上が白人，genotypeはAまたはDの対象であった．前者は，85%強がアジア人を対象としているが，genotype別には，B型が約30%，C型が約60%と，日本国内での分布とやや異なる．いずれの試験においても，PEG-IFNα製剤と核酸アナログ製剤を同時併用した場合の最終観察時期のHBe抗原陰性化は，PEG-IFNα単独治療時とほぼ同等であり，併用治療の上乗せ効果は認めなかった．これらは多数例のRCTによる信頼性の高い結果であるが，わが国での治療対象と異なる要因もあり，今後，PEG-IFN導入後に国内での評価が必要である．

2つのRCTに共通しているのは，治療期間中のHBV-DNA量は，PEG-IFN単独投与群に比べ併用群で有意に減少するものの，終了後には併用群の再上昇幅が大きく，結果的に両群のHBV-DNAレベルが同等である点である．これは，HBe抗原陰性症例を対象としたRCTでも同様の傾向を示している[11)12)]．

Wooら[13]は，B型肝炎治療薬剤の効果を，ベイズ法にてメタ解析を行い，PEG-IFN＋LAM併用群のPEG-IFN単独投与群に対する有効性は，オッズ比として，HBV-DNA陰性化は5.75，ALT正常化は1.83，HBe抗原からのSCは0.99，HBe抗原陰性化は1.26，HBs抗原陰性化は1.40，組織学的改善は0.82と報告している．さらに，種々の治療選択肢の中で有効性が高いと評価される順位でも，PEG-IFN＋LAM併用療法は，HBV-DNA陰性化，HBe抗原陰性化，HBs抗原陰性化において，PEG-IFN単独よりも上位に位置づけられている．

併用療法の評価

前述のRCTにおける治療終了後の抗HBV効果は，PEG-IFN投与の有無が最も大きな規定要因となっており，長期効果に関するIFNの意義が示されている．すなわち，核酸アナログ製剤の主作用であるHBV増幅抑制効果よりも，IFNの免疫賦活による感染肝細胞排除の方が，長期的な有効性には強く作用し，海外のRCTでは，PEG-IFN単剤に対する併用療法の優位性は示されない結果となった．

一方で，治療中のHBV-DNA減少効果は，IFNと核酸アナログの併用が単剤投与に比較して明らかに強いことが示され，われわれの検討でも同様の結果が示されている．また，海外でのRCT検討対象はすべてPEG-IFN治療例であるが，われわれが行っている従来型IFN投与方法は，初期4週間の頻回投与(週5～6回)であり，PEG-IFNとは違う評価が必要と思われる．

B型肝炎治療の評価は，症例により大きく結果が異なる．特に，HBe抗原陽性者では治療開始時の背景要因の影響を受け，ALT値の変動を加味したタイミングの判断が最終結果にも影響する．今後は，このような併用時の増強効果が最終的有効性に寄与する要因を解析し，症例の選択を行っていく必要があると思われる．

まとめ

IFN製剤と核酸アナログ製剤の併用により，治

療期間中のHBV-DNA低下作用は明らかに増強されるが，HBe抗原陽性例のSC達成率に関しては，PEG-IFN単独投与に対する核酸アナログ製剤併用の上乗せ効果が明確にはなっていない．今後，わが国と海外の症例の比較，IFN製剤・投与法の比較，有効性に関連する要因などの解析を進める必要があると思われる．

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Sensitive Assay for Quantification of Hepatitis B Virus Mutants by Use of a Minor Groove Binder Probe and Peptide Nucleic Acids▽†

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Lamivudine is the first nucleoside analogue that was shown to have a potent effect on hepatitis B virus (HBV). However, the emergence of mutants resistant or cross-resistant to nucleoside/nucleotide analogues remains a serious problem. Several assays for the detection and quantification of antiviral-resistant mutants have been reported, but it has been difficult to measure the amounts of mutants accurately, especially when the target strain is a minor component of the mixed population. It has been shown that accurate measurement of a minor strain is difficult as long as a matching reaction with a single probe is included in the assay. We developed a new method for the quantification of lamivudine-resistant strains in a mixed-virus population by real-time PCR using minor groove binder probes and peptide nucleic acids, and we achieved a wide and measurable range, from 3 to 10 $\log_{10}$ copies/ml, and high sensitivity, with a discriminative limit of 0.01% of the predominant strain. The clinical significance of measuring substitutions not only of M204 but also of L180 residues of HBV polymerase was demonstrated by this method. This assay increases the versatility of a sensitive method for the quantification of a single-nucleotide mutation in a heterogeneous population.

Chronic liver diseases due to hepatitis B virus (HBV) infection are still serious problems worldwide, and many patients suffer from liver cirrhosis and hepatocellular carcinoma (2, 6, 21). Nucleoside/nucleotide analogues have been introduced in the treatment of chronic hepatitis B, and inhibition of disease progression has been achieved (20). However, since several analogues have been developed and have come into wide use, the risk of the emergence of resistant or cross-resistant mutations is increasing. This is a serious problem, since it may lead to the virological and biochemical breakthroughs that result in the loss of therapeutic effects (18).

Lamivudine (3TC) is the first nucleoside analogue that was demonstrated to have a potent effect on HBV (8, 17). The results of many studies on lamivudine-resistant mutants have been reported, and the impact of alteration from M204 to I204 or V204 (YMDD mutations) was initially investigated (20). Thereafter, the clinical significance of other regions of HBV polymerase was clarified. We reported the significance of dual mutations of the YMDD motif and the LLAQ motif (specifically the L180 residue) for the severity of breakthrough hepatitis (29). Recently, alteration of the L180 residue has been noted as one of the most crucial changes for the emergence of entecavir-resistant mutants.

It is important to detect mutants as early as possible in order to confirm genotypic or phenotypic resistance that may be followed by virological breakthrough and rebound. Several methods using PCR or hybridization for the qualitative detection of antiviral-resistant mutants have been reported.

Some assays have been proposed as sensitive methods for the quantitative measurement of mutant strains (33), and we have reported a quantification assay using type-specific minor groove binder (MGB) probes (40). However, we found that it was difficult to measure the amounts of mutants accurately, especially when the target strain was a minor component of the mixed population.

In this study, we developed a new sensitive assay for the quantification of hepatitis B virus mutants by introducing peptide nucleic acids (PNAs) into real-time PCR with MGB probes. PNAs are DNA mimics that bind to their complementary nucleic acid sequences with high specificity and have PCR-clamping effects (30, 31). We evaluated the efficacy of this assay and demonstrated the clinical significance of estimating the quantitative follow-up of dual mutants.

MATERIALS AND METHODS

Serum samples. HBs antigen-positive samples were obtained from chronic hepatitis B patients and were stored at −20°C. DNA was extracted and purified from the serum by a spin column method by using a QIAamp DNA minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The study was conducted with the approval of our institute's Ethics Committee, and written informed consent was obtained from all patients.

Construction of MGB probes. Minor groove binder (MGB) probes for the YMDD motif were designed to hybridize with HBV containing reverse transcriptase 204 (rt204) in domain C of the polymerase region in order to be complementary to the wild-type (rt204M) and mutant (rtM204I and rtM204V) strains. For the YIDD motif, two kinds of MGB probes were prepared because of the different kinds of codons for the same amino acid: YIDD1 for codon ATC and YIDD2 for codon ATT. The sequences of primers and MGB probes used

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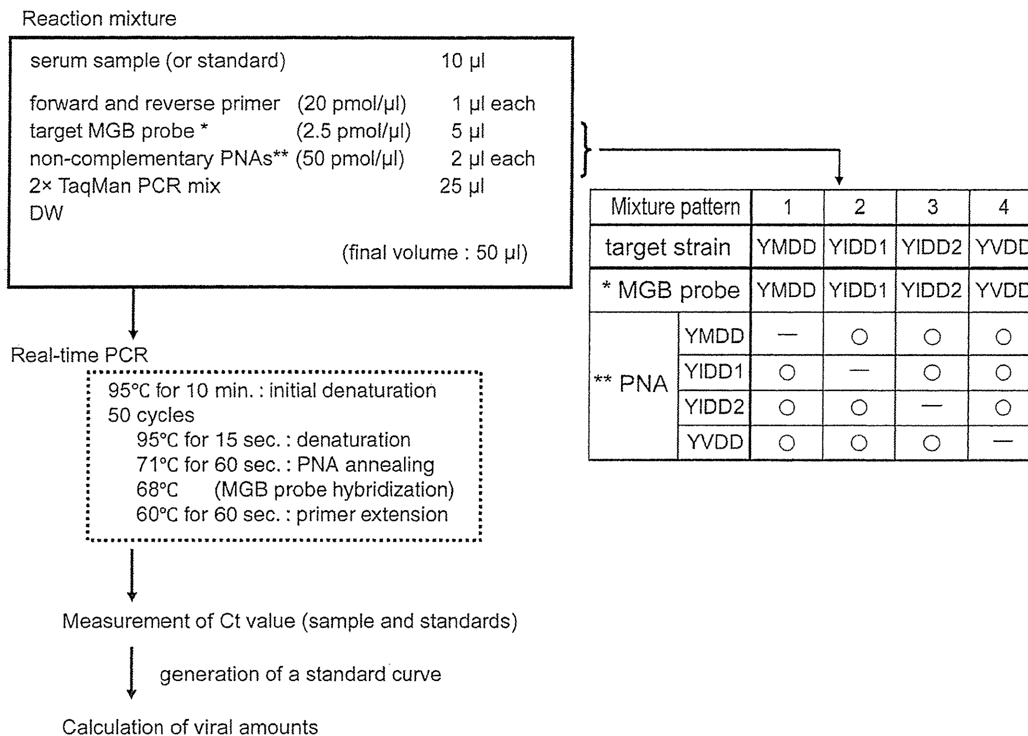

FIG. 1. Measurement of viral amounts by real-time PCR with the MGB probe and PNAs. The table shows patterns of combination of the MGB probe and noncomplementary PNAs.

were as follows: forward primer, 5′-GGGCTTTCCCCCACTGTT-3′; reverse primer, 5′-AAAGGGACTCAAGATGTTGTACAGACT-3′; YMDD probe, 5′-CTTTCAGTTATATGGATGATGTG-3′; YIDD1 probe, 5′-CTTTCAGTTAT ATCGATGATGTGG-3′; YIDD2 probe, 5′-CTTTCAGTTATATTGATGATG TGG-3′; YVDD probe, 5′-CTTTCAGTTATGTGGATGATGT-3′.

MGB probes for the LLAQ motif (rt179 to rt182) in domain B were also designed to be complementary to the wild type (rt180L) and a mutant (rtL180M). The sequences of primers and MGB probes used were as follows: forward primer, 5′-CCTATGGGAGTGGGCCTC-3′; reverse primer, 5′-AACA GTGGGGGAAAGCCCT-3′; LLAQ probe, 5′-CTCCTGGCTCAGTTTA-3′; LMAQ probe, 5′-TTCTCATGGCTCAGTTTACTA-3′. Those probes were purchased from Applied Biosystems Japan, Ltd. (Tokyo, Japan).

Construction of PNAs. PNAs were designed to match the sequence of each MGB probe exactly and were provided by Greiner Bio-One (Tokyo, Japan). The sequences of the PNAs were as follows: PNA-YMDD, 5′-GTTATATGGATG ATGTG-3′; PNA-YIDD1, 5′-GTTATATCGATGATGTGG-3′; PNA-YIDD2, 5′-GTTATATTGATGATGTGG-3′; PNA-YVDD, 5′-AGTTATGTGGATGA TGT-3′; PNA-LLAQ, 5′-TCCGTTTCTCCTGGCTC-3′; PNA-LLAQ, 5′-CCG TTTCTCATGGCTCA-3′.

Construction of HBV plasmids for the standard. HBV plasmids were prepared for the construction of a standard for quantification. PCR products that contained both YMDD and LLAQ motifs obtained from HBV-positive patients were cloned, and *Escherichia coli* XL-1 competent cells were transformed by each of the mutant-specific DNA fragments. Finally, plasmid DNA corresponding to each MGB probe was constructed.

Real-time PCR. Real-time PCR with the MGB probe and PNAs was performed using an ABI real-time PCR system, model 7300 (Applied Biosystems, Foster City, CA). A sample was measured with an MGB probe that is complementary to a target strain and with PNAs that are complementary to each strain except for the target strain (Fig. 1).

The reaction was carried out in a final volume of 50 μl in each well of a plate containing 10 μl of a serum sample, 400 nM forward and reverse primers, 250 nM MGB probe, 2 μM PNAs, and 1× TaqMan Universal PCR master mix (Applied Biosystems, Foster City, CA). Optimal concentrations of MGB probes and PNAs were determined by our own examinations as described below.

The conditions of the PCR were as follows: 10 min at 95°C for the initial denaturation, followed by 50 cycles at 95°C for 15 s for denaturation, 71°C for 1 min for PNA annealing, and 60°C for 1 min for primer extension.

A TaqMan probe has a reporter dye and a nonfluorescent quencher at the 5′ and 3′ ends, respectively. The reporter dye emission is quenched when the probe remains intact. When an MGB probe hybridized with a complementary strain, the fluorescence of a reporter dye became detectable (Fig. 2a).

When PCR was performed with a complementary PNA and an MGB probe noncomplementary to the target nucleotide, the extension and amplification were blocked by the preferentially bound PNA (Fig. 2b).

Real-time PCR was performed separately with each MGB probe and with noncomplementary PNAs for all sets of target strains. After the exclusion of background signals by subtracting the signals of early cycles of PCR, a threshold was set in the linearly increasing region of accruals of fluorescence signals. Then the cycle at which the signal reached the threshold (threshold cycle [C_T]) was measured. The amount of the target strain was calculated from the C_T number and a linear regression curve for standards of each strain (Fig. 3).

RESULTS

Standard curve for the measurement of a single strand using an MGB probe. HBV plasmids that had been quantitatively adjusted beforehand were used for 10-fold serial dilutions from 10 $\log_{10}$ copies/ml to 1 $\log_{10}$ copies/ml; from these, the standard curves were generated. A strong inverse correlation between the logarithmic concentration of samples and C_T values was obtained, and linearity was confirmed within a wide range from 3 to 10 $\log_{10}$ copies/ml.

Measurement of a target mutant in the mixed strains. The accuracy of quantification of a single HBV strain was determined in a mixture of multiple strains by real-time PCR with a type-specific MGB probe without PNA. HBV plasmids serially diluted 10-fold within a range of 10 $\log_{10}$ copies/ml to 4 $\log_{10}$

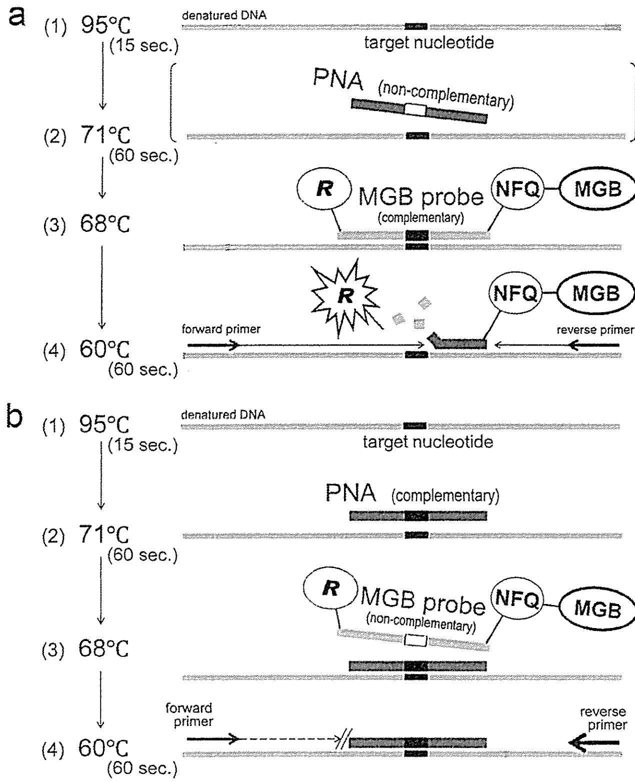

FIG. 2. (a) PCR with a complementary MGB probe. During the extension phase with primers, the DNA polymerase cleaves the reporter dye from the probe, and the dye emits its characteristic fluorescence. When a PNA added to the reaction mixture is noncomplementary to the target sequence, it does not affect amplification with a complementary MGB probe. NFQ, nonfluorescent quencher. (b) PCR with a complementary PNA. The mismatch of an MGB probe noncomplementary to the target sequence is blocked by the PNA, which is preferentially bound at a higher melting temperature than the MGB probe.

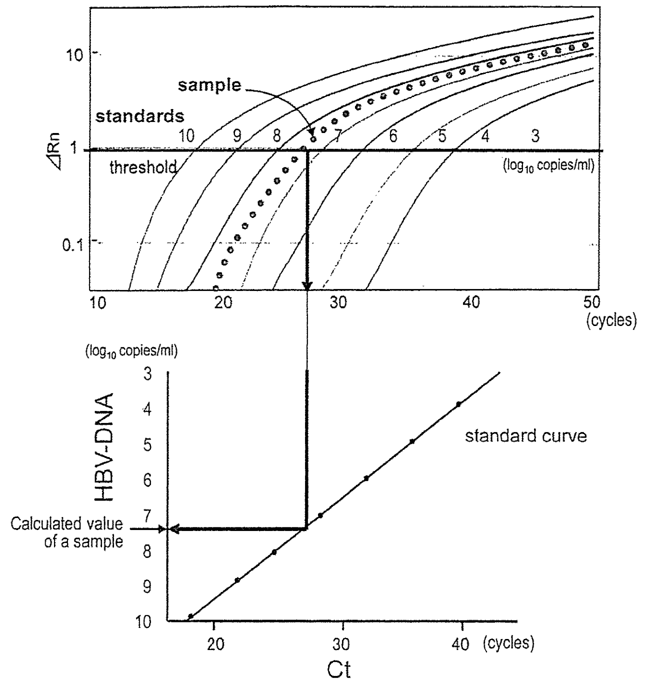

FIG. 3. Calculation of values from the results obtained by real-time PCR. The threshold was set in the linearly increasing region of accruals of fluorescence signals (ΔRn), and the threshold cycle (C_T) was measured. The value of the target strain was calculated from the C_T number and a standard curve for each strain. The measurement was performed separately with each MGB probe and noncomplementary PNAs for all sets of target strains. Solid lines, 10-fold serially diluted standards; dotted line, a sample of the target strain.

copies/ml were used for quantification under the condition of coexistence of 6 $\log_{10}$ copies/ml of another strain with the same position. The target strain was correctly measured when the concentration of the target strain predominated over that of another strain, and the measurement error was estimated to be less than twice the amount theoretically calculated. However, measurements were overestimated when the concentration of the target strain was lower than that of another strain (Fig. 4).

Clamping effects of PNA on PCR. PNAs were introduced into the assay system to reduce quantitative errors due to mismatching of MGB probes. The clamping effects of PNAs were investigated beforehand in order to determine the concentration at which the noncomplementary PNAs had a negative impact.

Six $\log_{10}$ copies/ml of HBV plasmids were quantified together with an MGB probe and a PNA that were complementary to the target strain. Delays in the C_T with different concentrations of PNA were measured (Fig. 5). The delay correlated with the PNA concentration, and a clamping effect of 1/100 to 1/1,000 was observed when 100 pmol of PNA was used. When real-time PCR was performed with a complementary MGB probe and with all sets of the PNAs that are complementary to the motifs in each strain except for the target strain, a delay in the C_T was observed when noncomplementary PNA was present at high concentrations. However, the delays were modest when the amount of PNA was less than 100 pmol.

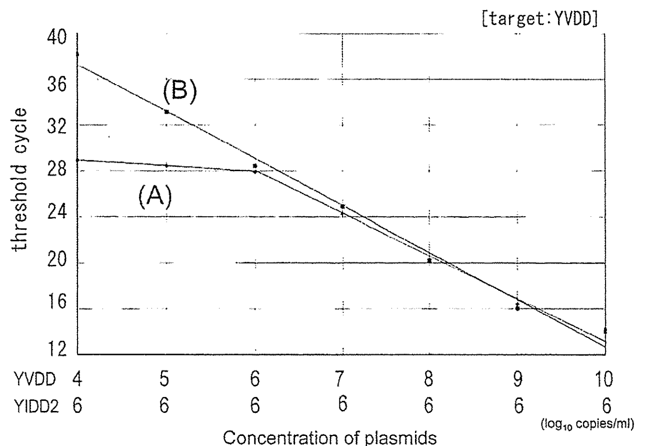

FIG. 4. Measurement of a target strain in mixed strains. Samples of a 10-fold serial dilution of the YVDD strain were measured under the condition of coexistence of 6 $\log_{10}$ copies/ml of the YIDD2 strain. Line A, MGB for YVDD; line B, MGB for YVDD with PNAs for YMDD, YIDD1, and YIDD2. Linearity was maintained even at the lower concentration range.

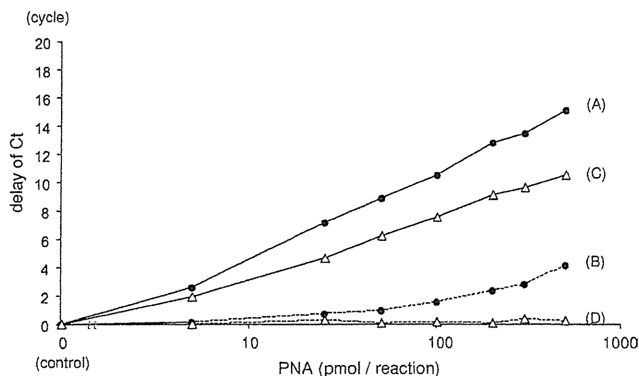

FIG. 5. Clamping effects of PNA on PCR. Delays in the C_T with different concentrations of complementary (solid lines) and noncomplementary (dashed lines) PNAs were measured with the MGB probe for the LLAQ strain (filled circles) and the LMAQ strain (open triangles). PNAs used were complementary (A) or noncomplementary (B) to the LLAQ strain and complementary (C) or noncomplementary (D) to the LMAQ strain. A delay in the C_T was observed even with noncomplementary PNAs when the concentration was high. The differences in delays between PNAs complementary and noncomplementary to the LLAQ strain are similar to those between PNAs complementary and noncomplementary to the LMAQ strain.

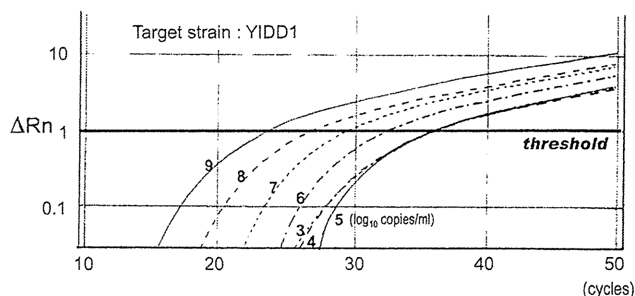

FIG. 6. Real-time PCR with the MGB probe and PNAs. Samples of a 10-fold serial dilution of the YIDD1 strain were measured under the condition of coexistence of 9 $\log_{10}$ copies/ml of YMDD strain with an MGB probe complementary to YIDD1 and PNAs complementary to an YMDD motif other than YIDD1 (YMDD, YIDD2, and YVDD). ΔRn, accruals of a fluorescence signal.

The concentration of PNA for the assay was determined from this result.

Measurement using both MGB probes and PNA. For confirmation of the proper method of determination by this assay, the amount of the HBV strain was measured in a mixture of two different strains of the same position using a new method of real-time PCR with the MGB probe and PNA. At first, 10-fold-diluted samples of target HBV plasmids from 9 $\log_{10}$ copies/ml to 3 $\log_{10}$ copies/ml were measured under the condition of coexistence of 3 $\log_{10}$ copies/ml of another strain. There were no significant differences in the values obtained for the target strain regardless of the presence of the other strain. Next, the amount of a minority strain was determined in the presence of a predominant strain. Tenfold serial dilutions of target HBV plasmids ranging from 9 $\log_{10}$ copies/ml to 3 $\log_{10}$ copies/ml were measured under the condition of coexistence of 9 $\log_{10}$ copies/ml of a different strain. A linear correlation between the C_T values and sample concentrations was observed in the range from 9 $\log_{10}$ copies/ml to 5 $\log_{10}$ copies/ml. However, a target strain at a concentration less than 5 $\log_{10}$ copies/ml could not be quantified accurately. Therefore, the detection limit of a nondominant strain was determined to be 0.01% of the predominant strain (Fig. 6).

A clinical case. The clinical and virological course of a case of breakthrough hepatitis after the emergence of lamivudine-resistant mutants is described below (Fig. 7a).

A 42-year-old male was administered 100 mg of lamivudine once a day (q.d.) in 1999. At first, HBV DNA levels were steadily reduced, and the serum alanine aminotransferase (ALT) level also decreased to the normal range after the start of treatment. About 16 months later, viral breakthrough and mild elevation of the ALT level were observed. Despite the fact that HBV DNA and HBe antigen returned to relatively high levels, the ALT level remained almost within the normal range. Thereafter, the ALT level suddenly increased, despite the fact that HBV DNA remained at the same level. When we measured mutants with resistance to lamivudine by a direct sequencing method, the changes in ALT and HBV DNA levels were thought to be related to the changes in mutant patterns over time. However, we could not predict the time or the severity of breakthrough hepatitis by this limited information, especially when HBV DNA loads did not change.

Precise examination of changes in HBV mutants was performed by real-time PCR with the MGB probe and PNA (Fig. 7b).

ALT levels were stable, whereas total levels of HBV DNA remained high. An LLAQ YIDD strain was dominant in the earlier phase. Next, an LLAQ strain became measurable, and when a YVDD strain emerged and overcame the YIDD strain levels, breakthrough hepatitis occurred. This case is a good example that demonstrates the clinical significance of measuring the amounts of HBV mutants with not only the YMDD motif but also the LLAQ motif by this new assay.

DISCUSSION

Inhibition of disease progression for patients with chronic HBV infection has been achieved by treatment with nucleoside/nucleotide analogues (20). Treatment with these analogues has reduced not only the incidence of hepatic decompensation but also the risk of occurrence of hepatocellular carcinoma (27). The same effect on recurrence has also been reported (4). However, the most serious issue is the emergence of viruses with resistance to nucleoside/nucleotide analogues, because this leads to virological and biochemical breakthroughs. Therapeutic effects were lost for patients who developed the resistant mutations (18).

From a resistance or a cross-resistance perspective, nucleoside/nucleotide analogues are classified into three groups based on their structural characteristics (35, 42): L-nucleoside analogues (lamivudine, telbivudine, clevudine, and emtricitabine), alkyl phosphonates (adefovir and tenofovir), and the D-cyclopentane group (entecavir). Resistance rates after 5 years of treatment have been reported to be 80% for lamivudine, 29% for adefovir, and 1.2% for entecavir. Although the rate of early-period resistance has been reduced as new drugs have been introduced, the long-term results have not yet been con-

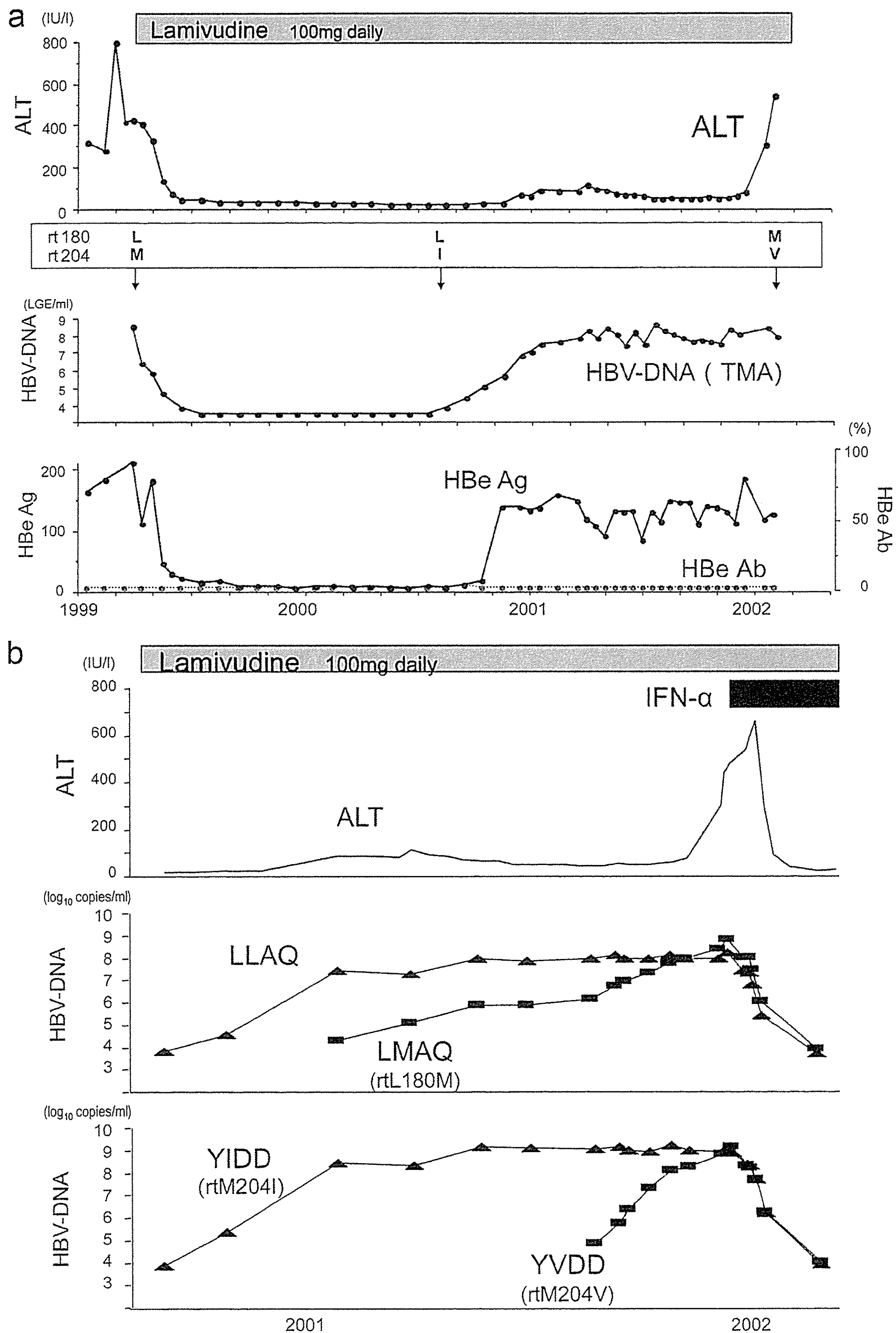

FIG. 7. (a) Course of a clinical case. HBV DNA was measured by a transcription-mediated amplification assay (logarithmic genome equivalent [LGE] per milliliter). L, M, I, and V of rt180 or rt204 represent the amino acids leucine, methionine, isoleucine, and valine, respectively. (b) Changes in viral amounts of the LLAQ and YMDD motifs. ALT levels were stable while the LLAQ/YIDD strain was dominant. When the LMAQ/YVDD strain became dominant over preexisting strains, breakthrough hepatitis occurred.

firmed. Moreover, the resistance rate becomes higher for patients with a lamivudine-resistant mutant, as high as 20% within 1 year for adefovir and 51% after 5 years for entecavir.

It is important to detect and quantify these mutant strains, and various methods have been developed for such measurements. The most common method for the detection of mutant viruses is direct sequencing after PCR amplification. However, the detection limit of a minor strain in the heterogeneous virus population is about 20%. Recently, a new method called ultradeep pyrosequencing has been developed (26, 36). This sequencing relies on the detection of DNA polymerase activity by measuring the pyrophosphate (PP_i) released by the addition of a deoxyribonucleoside monophosphate (dNMP) to the 3′ end of a primer. The detection limit decreased to 1% by this technique.

There are several methods for hybridization-based genotyping: the line probe assay (LiPA) (12, 22, 37), mixed hybridization-sequencing-PCR (minisequencing) (15), and the oligonucleotide tip assay (13). The sensitivity of the LiPA is 5 to 10%, and that of the minisequencing assay is 2 to 10%. The sensitivity of the high-density DNA chip reported by Tran et al. was 30 to 50%, but it was able to detect 245 mutations, 20 deletions, and 2 insertions at 151 positions (39). Hong et al. reported that mass spectrometry is another method for the detection of mutants. Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) had a detection limit of 100 copies/ml and a sensitivity of 1% (10).

Some modified real-time PCR methods have been developed for the quantification of mutant viruses: PCR with molecular beacons (32), PCR with a minor groove binder (MGB), PCR with a locked nucleic acid (LNA)-mediated probe (3, 19, 38), and amplification refractory mutation system PCR (ARMS-PCR) (33).

An MGB can fit snugly into the minor groove, which is the deep narrow space between the two phosphate-sugar backbones of a double-stranded DNA helix.

Afonina et al. reported that a DNA probe with a tripeptide {1,2-dihydro-(3H)-pyrrolo[3,2-e]indole-7-carboxylate} ($GDPI_3$), which is conjugated to the 5′ ends of short oligodeoxynucleotides (ODNs), formed unusually stable hybrids with cDNA and can be used as a PCR primer (1). This MGB probe has a higher melting temperature (T_m) and greater specificity than ordinary DNA probes, especially when a mismatch is in the MGB region of the duplex (7, 16). Zhao et al. reported a real-time PCR method using a TaqMan-MGB probe for the measurement of total amounts of HBV DNA (41), and we have reported the quantification of lamivudine-resistant mutants by the type-specific TaqMan MGB probe assay (40).

Several authors have reported a similar sensitivity of 10% for the detection of minor variants (9, 11, 25). Intraexperimental variability was reported to be 4.9% by Lole and Arankalle (24) and 1.0 to 2.2%, depending on the type of mutant, by our own evaluation (40).

However, our study revealed that the accuracy of quantification was reduced in the case of measurements of minor strains in a mixed population. Therefore, we introduced PNAs to be used in combination with the MGB probes.

PNAs are DNA mimics in which the deoxyribose phosphate backbone of DNA has been replaced by *N*-(2-aminoethyl)glycine linkages (30). They recognize and bind to their complementary nucleic acid sequences with high thermal stability and specificity (31). PNAs cannot function as primers for DNA polymerase and can be used to block a PCR amplification process in a sequence-specific manner. This PCR clamping allows for direct analysis of single-base mutations by PCR.

Kirishima et al. (14) reported a sensitive method for the detection of a lamivudine-resistant mutant by PNA-mediated PCR clamping with restriction fragment length polymorphism (RFLP) analysis, and Mori et al. performed semiquantitative measurements of mutants using PNA (28).

Under the real-time PCR conditions used in our assay, one reaction cycle started at 95°C for denaturation and finished at 60°C for extension. The T_m of PNA was designed to be 3°C higher than that of an MGB probe. Because of this difference in temperature, PNA is expected to clamp a noncomplementary strain first, and then an MGB probe will hybridize to the target strand. As a result, the assay was able to achieve a significant reduction in mismatches. However, an amplification delay was observed with an excessive amount of noncomplementary PNA. No matter how well an assay is constructed, mismatches cannot be completely avoided as long as the reaction includes a matching process. However, it is thought that the actual measurement can be performed correctly by setting the appropriate concentration of the reaction mixture. As for the limit of detection, we showed that a level of the minority strain equivalent to 0.01% of the predominant strain could be measured by our assay. However, more-robust analysis is required to determine the true analytical sensitivity of the assay by measuring a number of clinical samples.

This requires a precise knowledge of the sequence of the mutants prior to determination by this assay; as such, this assay cannot be construed as a general method for the study of viral quasispecies where mutants and emerging mutants will have an unknown sequence. However, it seems ideally suited for the approach of measuring a known mutant virus, such as HBV mutants resistant to nucleoside/nucleotide analogues.

Although the risk of breakthrough hepatitis is thought to correlate with the duration of infection with mutant viruses (23), not only the duration but also the change in the mutant strains is important for the occurrence of hepatitis. We reported that the patterns of the YMDD and LLAQ motifs often changed from YMDD and LLAQ to YIDD and LLAQ, YIDD and LMAQ, and YVDD and LMAQ, in that order, and that the degree of liver damage increased as the mutations accumulated (29). As demonstrated by the case presented in this report, breakthrough hepatitis seems to have a relationship with the specific types of mutants and the length of time from the emergence of a new mutant. The degree of increase in the viral load after the emergence of dual mutations was smaller than that after the emergence of the first mutation, despite the fact that the level of liver dysfunction was higher with the dual mutations. This result suggested that breakthrough hepatitis correlated closely with changes in viral characteristics. Das et al. (5) reported differences in inhibitory effects on HBV DNA polymerase between different lamivudine-resistant mutants. The inhibitory effects were measured by recombinant HBV DNA polymerase expressed in a baculovirus transfer vector. The fold changes in inhibition of the DNA polymerase were 8.0 for the M204I mutation, 15.2 for the L180M and M204I

mutations, and 25.2 for the L180M and M204V mutations, compared to the wild type. The results for our clinical case showed a tendency similar to that reported in the evaluations of Das et al. and similar to the results of a recent molecular modeling study. Sharon and Chu demonstrated that binding energy for mutant HBV polymerases was significantly different between dCTP and 3TC and that 3TC lost binding affinity with the mutants in comparison to the natural substrate dCTP (34). L180 residues did not interact directly with 3TC, but the M180 mutant caused loosening of the hydrophobic pocket formed by the residues A87, F88, P177, L180, and M204. Consequently, dual mutations (L180M plus M204I or L180M plus M204V) were more likely to show a steric clash between I204 or V204 and 3TC.

Despite the high risk of multidrug resistance evoked by lamivudine, it has been widely used, mainly due to its low cost. Substitution of any of the residues T184, S202, and M250, in addition to the M204V and L180M mutations, is necessary for the emergence of an entecavir-resistant mutant. Therefore, it is important to observe the change in the L180 substitution prior to M204V emergence, even for cases where a drug other than lamivudine has been administered.

In conclusion, we have developed a new method for the accurate quantification of lamivudine-resistant strains in a mixed virus population. This is the first report of the combined use of an MGB probe and PNAs for the quantification of viruses using real-time PCR. This methodology should prove immensely useful for better understanding of the dynamics of the emergence of HBV-resistant mutants. Moreover, this assay has the versatility to be applied as a sensitive method for the quantification of a single-nucleotide mutation in a heterogeneous population not only of viruses but also of other nucleic acids.

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Distribution of Hepatitis B Virus Genotypes among Patients with Chronic Infection in Japan Shifting toward an Increase of Genotype A[▽]

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Acute hepatitis B virus (HBV) infection has been increasing through promiscuous sexual contacts, and HBV genotype A (HBV/A) is frequent in patients with acute hepatitis B (AHB) in Japan. To compare the geographic distribution of HBV genotypes in patients with chronic hepatitis B (CHB) in Japan between 2005 and 2006 and between 2000 and 2001, with special attention to changes in the proportion of HBV/A, a cohort study was performed to survey changes in genotypes of CHB patients at 16 hospitals throughout Japan. Furthermore, we investigated the clinical characteristics of each genotype and examined the genomic characteristics of HBV/A isolates by molecular evolutionary analyses. Of the 1,271 patients, 3.5%, 14.1%, and 82.3% were infected with HBV/A, -B, and -C, respectively. In comparison with our previous survey during 2000 and 2001, HBV/A was twice as frequent (3.5% versus 1.7%; $P = 0.02$). The mean age was lower in the patients with HBV/A than in those with HBV/B or -C. Based on phylogenetic analyses of 11 full-length genomes and 29 pre-S2/S region sequences from patients, HBV/A isolates were imported from Europe and the United States, as well as the Philippines and India. They clustered with HBV/A from AHB patients and have spread throughout Japan. HBV/A has been increasing in CHB patients in Japan as a consequence of AHB spreading in the younger generation through promiscuous sexual contacts, aided by a tendency of HBV/A to induce chronic hepatitis. The spread of HBV/A infection in Japan should be prevented by universal vaccination programs.

Hepatitis B virus (HBV), a member of the *Hepadnaviridae*, is a circular, partially double-stranded DNA virus and is one of the major causes of chronic liver diseases, including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC).

The HBV genome is composed of approximately 3,200 nucleotides. HBV is classified into eight genotypes, designated A to H, based on an intergroup divergence of 8% or more in the complete nucleotide sequence (3, 23, 26, 37). They have distinct geographical distributions and are associated with differences in clinical and virological characteristics, such as severity of liver disease and response to antiviral therapies (7, 8, 12, 13, 22, 28). Furthermore, subgenotypes have been reported for HBV/A, -B, and -C and named A1 to -3 (17, 38), B1 to -6 (31, 32, 40), and C1 to -6 (20, 31, 45). Equally, other genotypes are classified into subgenotypes. There have been increasing lines of evidence to indicate influences of HBV subgenotypes on the outcome of liver disease and the response to antiviral therapies (1, 39, 44).

In 2001, we reported the geographic distribution of HBV genotypes in Japan (27). Of the 720 Japanese patients with chronic HBV infection (CHB), 12 (1.7%) harbored HBV/A, 88 (12.2%) HBV/B, 610 (84.7%) HBV/C, 3 (0.4%) HBV/D, and 7 (1.0%) mixed genotypes. HBV/C was detected in over 94%

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of patients on the Japanese mainland, while HBV/B was found in 64% of those in Okinawa, the southernmost islands, and 44% of those in the Tohoku area in the northern part of the mainland.

Recently, acute HBV infection (AHB) has been increasing in Japan, predominantly through promiscuous sexual contacts. In addition, it was reported that HBV/A was more frequent in patients with acute hepatitis than in those with chronic hepatitis (29, 41, 49). Recent studies suggest that the chances for progression to chronic disease may differ among patients acutely infected with HBV of distinct genotypes (21, 25); patients infected with HBV/A run an increased risk of becoming HBV carriers. Hence, it is of utmost concern whether chronic HBV/A infection is increasing in Japan.

In the present study, we compared the geographic distribution of HBV genotypes in Japan during 2005 and 2006 with 2000 and 2001, with special attention to changes in the proportion of HBV/A. Furthermore, we investigated the clinical characteristics of each genotype and examined the genomic characteristics of HBV/A isolates by molecular evolutionary analyses.

MATERIALS AND METHODS

Patients. From September 2005 to October 2006, sera were collected from 1,370 consecutive patients with CHB at 16 representative hospitals that were liver centers in their respective regions throughout Japan for the purpose of investigating the geographic distribution of HBV genotypes in Japan. All of the patients were diagnosed after they had been followed for at least 12 months. Patients diagnosed with AHB were excluded from the study; they had a sudden onset of clinical symptoms of hepatitis, along with high-titer antibody to HBV core antigen of the immunoglobulin M class in serum. Their sera were tested for alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (γ-GTP), and hepatitis B e antigen (HBeAg), as well as antibody to HBeAg (anti-HBe) (Dinabot, Tokyo, Japan). Four clinical diagnoses were established for them. The inactive carrier state was defined by the presence of HBV surface antigen (HBsAg) with normal ALT levels over 1 year (examined at least four times at 3-month intervals) and without evidence of portal hypertension. Chronic hepatitis was defined by elevated ALT levels (>1.5 times the upper limit of normal [35 IU/liter]) persisting over 6 months (with at least three bimonthly tests). Cirrhosis was diagnosed principally by ultrasonography (coarse liver architecture, nodular liver surface, blunt liver edges, and hypersplenism), platelet counts of <100,000/cm^3, or a combination thereof. Histological confirmation by fine-needle biopsy of the liver was performed as required. HCC was diagnosed by ultrasonography, computerized tomography, magnetic resonance imaging, angiography, tumor biopsy, or a combination thereof.

The study protocol conformed to the 1975 declaration of Helsinki and was approved by the ethics committees of the respective institutions. Every patient or his/her next of kin gave informed consent to the purpose of the study.

Genotypes and subgenotypes of HBV. The six HBV genotypes (A to F) were determined serologically by enzyme immunoassay (EIA) using commercial kits (HBV Genotype EIA; Institutes of Immunology Co., Ltd., Tokyo, Japan). The method depends on the combination of epitopes on pre-S2 region products detected by monoclonal antibodies that were specific for each of them (46, 47). Subgenotypes of HBV/A, designated A1 and A2, were determined by direct sequencing of the pre-S2/S gene, followed by a phylogenetic analysis.

Quantification of HBV DNA and sequencing. HBV DNA levels in sera were quantitated with a commercial kit (Amplicor HBV Monitor; Roche Diagnostics, Basel, Switzerland) with a detection range from 2.6 to 7.6 log copies/ml. Nucleic acids were extracted from 100 μl of serum using the Qiaamp DNA Blood Minikit (Qiagen GmbH, Hilden, Germany). Eleven complete HBV/A genomes and 29 pre-S2/S region sequences were amplified by PCR with appropriate primer sets, as described previously (40). The amplified HBV DNA fragments were directly sequenced using the ABI Prism Big Dye kit version 3.0 (Applied Biosystems, Foster City, CA) in an ABI 3100 automated DNA sequencer (Applied Biosystems). All sequences were analyzed in both forward and reverse directions. Complete and partial HBV genome sequences were aligned using GENETYX version 11.0 (Software Development Co., Ltd., Tokyo, Japan).

TABLE 1. Characteristics of 1,271 CHB patients

Parameter	Value
Characteristic	
Male gender [no. (%)]	766 (60.3)
Age (yr; mean ± SD)	51.4 ± 14.0
Diagnosis	
Inactive carrier state [no. (%)]	206 (16.2)
Chronic hepatitis [no. (%)]	786 (61.8)
Cirrhosis [no. (%)]	175 (13.8)
HCC [no. (%)]	104 (8.2)
Antiviral treatment [no. (%)]	577 (45.4)
Blood tests	
Platelets (10^4/mm^3)	21.4 ± 30.2
ALT (IU/liter)	59.8 ± 103.0
ALP (IU/liter)	270.4 ± 136.0
γ−GTP (IU/liter)	47.4 ± 66.1
HBV markers	
HBeAg [no. (%)]	399 (31.4)
HBV DNA (median [range] [log copies/ml])	4.2 (<2.6 to >7.6)

Molecular evolutionary analysis of HBV. Reference sequences were retrieved from the DDBJ/EMBL/GenBank databases with their accession numbers for identification. To investigate the relationship between HBV isolates from patients with chronic and acute hepatitis B in Japan, HBV/A isolates (AH1 to -10) were randomly retrieved from them and sequenced in our previous study (29). Nucleotide sequences of HBV DNA were aligned by the program CLUSTAL X, and genetic distance was estimated by the six-parameter method (10) in the Hepatitis Virus Database (36). Based on these values, phylogenetic trees were constructed by the neighbor-joining method (30) with the midpoint rooting option. To confirm the reliability of the phylogenetic trees, bootstrap resampling tests were performed 1,000 times.

Statistical analysis. Categorical variables were compared between groups by the χ^2 test or Fisher's exact test and noncategorical variables by the Mann-Whitney U test. A P value of less than 0.05 was considered significant.

Nucleotide sequence accession numbers. The DDBJ/EMBL/GenBank accession numbers of the complete genome sequences of HBV isolates JPN_CH1 to -11 are AB453979 to AB453989.

RESULTS

Distribution of HBV genotypes among patients with CHB. Of the 1,370 serum samples, the genotype could not be determined for 99 (7.2%) by EIA due to low HBsAg levels, leaving 1,271 for analysis in this study (Table 1). Of these, 206 (16.2%) were inactive carriers, 786 (61.8%) had chronic hepatitis, 175 (13.8%) cirrhosis, and 104 (8.2%) HCC. They had a mean age of 51.4 ± 14.0 years and included 766 (60.3%) men. They had a median HBV DNA level of 4.2 log copies/ml, and 399 (31.4%) of them were positive for HBeAg. Antiviral treatment had been given to 577 (45.4%) of them with interferon, lamivudine, adefovir pivoxil, or entecavir.

The genotypes were HBV/A in 44 (3.5%), HBV/B in 179 (14.1%), HBV/C in 1,046 (82.2%), and HBV/D in 2 (0.2%) (Table 2). In comparison with our previous report on the distribution of genotypes in Japan in 2001 (27), HBV/A was more frequent in this study (3.5% versus 1.7%; P = 0.02). Of the 16 hospitals in this study, 10 overlapped with those in our previous report from 2001. In these 10 hospitals, HBV/A was more frequent in the present than in the previous survey (3.6% versus 1.7%; P = 0.04).

The distribution of HBV genotypes in Japan differed by

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TABLE 2. Distribution of HBV Genotypes

Genotype	No. (%)	
	2005–2006 (n = 1,271)	2000–2001[a] (n = 720)
A	44 (3.5[b])	12 (1.7)
B	179 (14.1)	88 (12.2)
C	1,046 (82.3)	610 (84.7)
D	2 (0.2)	3 (0.4)
Mixed	0 (0.0)	7 (1.0)

[a] From Orito et al. (27).
[b] P = 0.02.

geographic location (Fig. 1). HBV/C was the most prevalent in the majority of areas. In the Tohoku area, the northern part of the Japanese mainland (Honshu), HBV/B was more prevalent than in the other areas of the Japanese mainland. In Okinawa, the southernmost islands of Japan, HBV/B was predominant. Of note, HBV/A was more frequent in the Kanto area (9.5%), the metropolitan area, and Okinawa (9.1%) than in the other areas.

Clinical differences among HBV/A, -B, and -C. Clinical backgrounds were compared among the patients infected with HBV/A, -B, and -C (Table 3). HBeAg was significantly less prevalent in the patients infected with HBV/B than in those infected with HBV/A or -C (P < 0.01 for each). When the positivity of HBeAg was stratified by age, HBeAg was markedly less common in patients infected with HBV/B than in those infected with HBV/A or -C who were older than 40 years of age (7/157 [4.5%] versus 4/19 [21.1%] [P < 0.05] or 215/755 [28.5%] [P < 0.01]) (Fig. 2). There were no significant differences in HBV DNA levels among patients infected with the three genotypes. As antiviral treatments might have influenced the severity of liver disease, clinical states were compared among patients infected with HBV/A, -B, and -C who did and did not receive it; antiviral treatments did not affect the above-mentioned trends represented in Table 3 in age, diagnosis, and HBeAg, as well as ALT and HBV DNA levels (data not shown).

Additionally, we compared the distributions of age and liver diseases in patients infected with HBV/A, -B, and -C. In patients infected with HBV/C, the prevalence of cirrhosis and HCC increased in those older than 50 years of age compared to younger patients (Fig. 3), whereas in the patients infected with HBV/B, cirrhosis and HCC were rare in elderly patients. The proportion of patients younger than 40 years of age was higher in those infected with HBV/A than in those infected with HBV/B or -C (25/44 [56.8%] versus 22/179 [12.3%] or 288/1,046 [27.5%]; P < 0.01 for each), while cirrhosis and HCC were also found in those older than 50 years of age infected with HBV/A.

Coinfection with human immunodeficiency virus type 1 (HIV-1) was found in 6 of the 44 (13.6%) patients infected with HBV/A compared to only 3 of the 1,046 (0.3%) patients infected with HBV/C (P < 0.0001); it occurred in none of the 179 patients infected with HBV/B.

Phylogenetic analyses. Among the 44 HBV/A isolates, the complete genome was sequenced successfully in 11 (JPN_CH1 to -11). Seven of them were classified as HBV/A2 and four as HBV/A1. A phylogenetic tree was constructed based on the complete genome sequences of these 11 isolates, along with those from two patients with AHB and those from 40 HBV/A isolates retrieved from the database (Fig. 4). Of the seven HBV/A2 isolates, the four from patients with CHB in this study formed a cluster with the Japanese isolates retrieved from the database and two from patients with AHB. Of the other three isolates, JPN_CH5 clustered with French and U.S. isolates, JPN_CH6 with German isolates, and JPN_CH7 with

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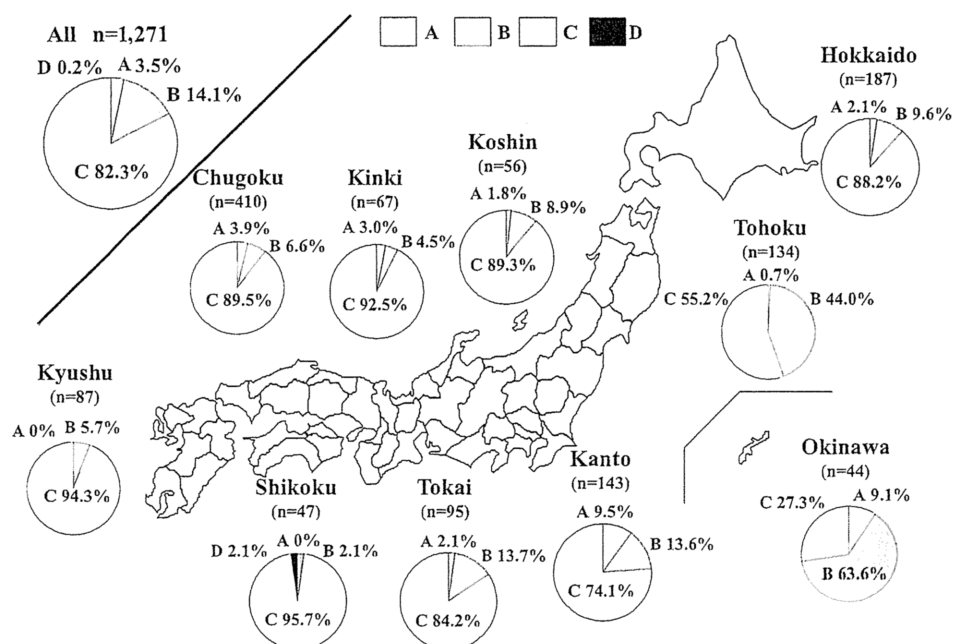

FIG. 1. Geographic distribution of HBV genotypes in patients with chronic HBV infection in Japan during 2005 and 2006.

TABLE 3. Clinical characteristics of individuals chronically infected with HBV of different genotypes

Parameter	Value for genotype:		
	A (n = 44)	B (n = 179)	C (n = 1,046)
Male gender [no. (%)]	32 (72.7)	112 (62.6)	621 (59.4)
Age (yr [mean ± SD])	41.3 ± 14.9[a]	55.8 ± 13.7[b]	48.8 ± 13.3
Diagnosis			
Inactive carrier state [no. (%)]	13 (29.5)[c]	63 (35.2)[b]	129 (12.3)
Chronic hepatitis [no. (%)]	26 (59)	103 (57.5)	656 (62.7)
Cirrhosis [no. (%)]	3 (6.8)	10 (5.6)[b]	162 (15.5)
HCC [no. (%)]	2 (4.5)	3 (1.7)[b]	99 (9.5)
Anti viral treatment [no. (%)]	13 (29.5)[d]	48 (26.8)[b]	516 (49.3)
Blood tests			
Platelet (10^4/mm^3)	23.3 ± 21.9	25.9 ± 35.9[e]	20.6 ± 29.5
ALT (IU/liter)	56.2 ± 83.8	42.2 ± 104.2[e]	63.0 ± 103.3
ALP (U/liter)	247.1 ± 123.0	255.5 ± 97.9	273.9 ± 141.9
γ-GTP (U/liter)	39.6 ± 34.6	49.3 ± 63.4	47.5 ± 67.6
HBV markers			
HBeAg [positive rate(%)]	15 (34.0)[f]	17 (9.5)[b]	367 (35.1)
HBV DNA (median [range]) (log copies/ml)	4.2 (<2.6–>7.6)	4.1 (<2.6–>7.6)	4.2 (<2.6–>7.6)

[a] $P < 0.01$, A versus B or C.
[b] $P < 0.01$, B versus C.
[c] $P < 0.01$, A versus C.
[d] $P < 0.05$, A versus C.
[e] $P < 0.05$, B versusC.
[f] $P < 0.01$, A versus B.

Spanish and Italian isolates. All four HBV/A1 isolates in this study formed a cluster with Philippine and Indian isolates.

In addition, the pre-S2/S region sequences of a total of 29 isolates were determined, including the 11 isolates whose complete genomes were sequenced. Of these, 21 (72%) were classified as HBV/A2 and the remaining 8 as HBV/A1. A phylogenetic tree was constructed based on the pre-S2/S region sequences from the 29 isolates, along with those from 10 patients with AHB infected with HBV/A and 47 HBV/A isolates retrieved from the database (Fig. 5). The 21 HBV/A2 isolates in the present study formed a cluster with Japanese, American, and European isolates retrieved from the database and those from patients with acute hepatitis. In addition, some of them were highly homologous with each other. Likewise, HBV/A1 isolates from eight patients with chronic hepatitis in this study were highly homologous with those from two patients with acute hepatitis and isolates from the Philippines and India. Based on the phylogenetic analyses, HBV/A isolates were imported from Europe and the United States, as well as the Philippines and India, and had infiltrated throughout Japan.

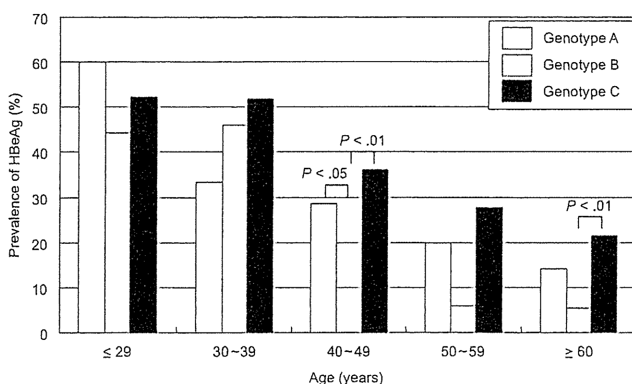

FIG. 2. Prevalence of HBeAg among patients infected with HBV of different genotypes stratified by the age.

DISCUSSION

Perinatal transmission from carrier mothers to their babies has been the principal route for establishing persistent HBV infection in Asian countries (19). In Japan, passive and active immunoprophylaxis with HBV immune globulin and vaccine has been mandated for babies born to HBeAg-positive carrier mothers since 1986; this was extended to HBeAg-negative carrier mothers in 1995. As a result, HBsAg has become rare in Japanese born after 1986; it was detected in only 0.2% of first-time blood donors younger than 19 years of age in 2000 (24). However, AHB has been increasing in Japan, predominantly through promiscuous sexual contacts.

In Japan, HBV/A is detected rarely among patients with CHB but is frequent in those with acute hepatitis (14, 25, 29, 41, 43). Yotsuyanagi et al. reported the distribution of genotypes in 145 Japanese patients with AHB and found HBV/A in 27 (19%), HBV/B in 8 (5%), and HBV/C in 109 (75%) (49). HBV/A is more frequent in metropolitan areas than other areas. The majority of patients with HBV/A infection in metropolitan areas have had extramarital sexual contacts with multiple irregular partners, through which they could have contracted infection. In support of this view, among men who have sex with men (MSM) who are coinfected with HBV and HIV-1 in Tokyo, most were infected with HBV/A (15, 35).

In Japan, AHB in adulthood becomes chronic in only ~1%

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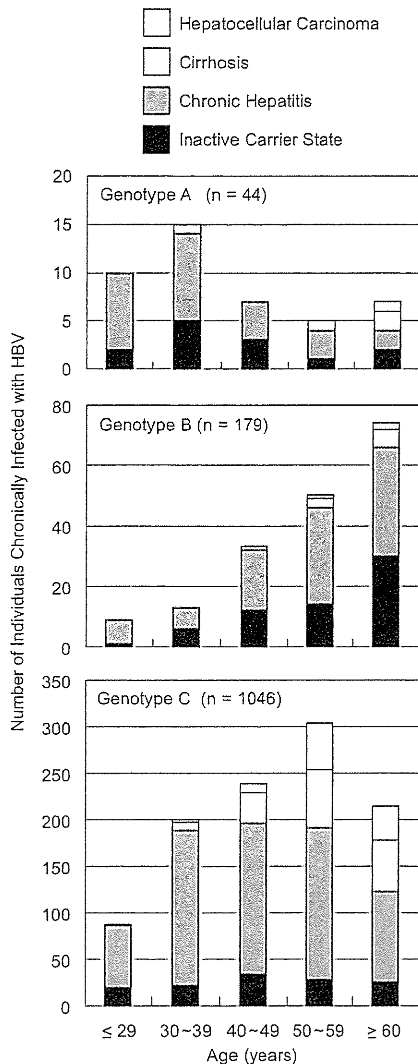

FIG. 3. Distribution of HCC, cirrhosis, chronic hepatitis, and inactive carrier state among the 1,271 patients infected with HBV of different genotypes stratified by the age.

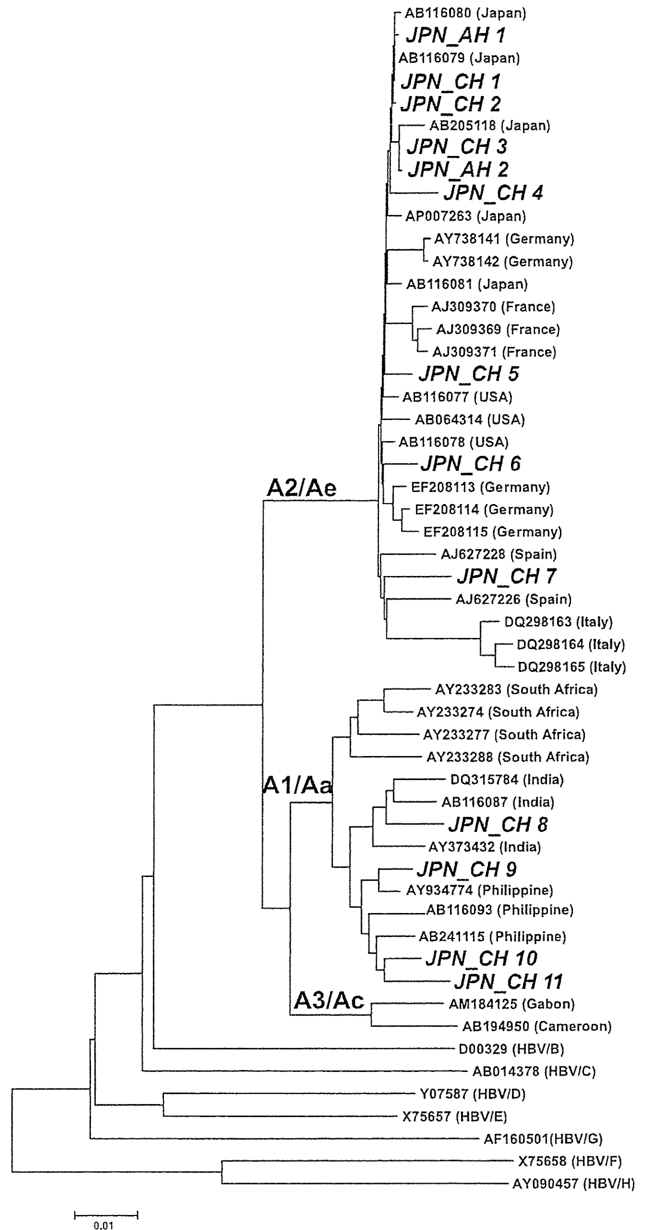

FIG. 4. Phylogenetic tree constructed based on the complete genome sequences of HBV/A isolates. Those from 11 patients with chronic infection in this study are shown in boldface italic (JPN_CH1 to -11), along with two isolates (JPN_AH1 and -2) from patients with acute hepatitis in Japan reported in our previous study (17). Representative isolates were retrieved from the DDBJ/EMBL/GenBank databases, including 21 HBV/Ae, 10 HBV/Aa, and 2 HBV/Ac isolates, along with 7 HBV isolates representative of the other seven genotypes. Isolates from the databases are identified by accession numbers, followed by the country of origin. The bar at the bottom spans 0.01 nucleotide substitutions per site.

of cases. This is much less than the progression to chronic disease (close to 10%) in Europe and the United States, where HBV/A prevails (34). Recent studies have suggested that the chances for persistence may differ among patients acutely infected with HBV of distinct genotypes (21, 25). In particular, acute infection with HBV/A may bring about an increased risk of progression to chronic disease. Therefore, an increase of acute infection with HBV/A would result in a surge of HBV/A among patients with CHB in Japan. In actuality, in comparison with our previous results during 2000 and 2001 (27), HBV/A was twice as frequent in this study (3.5% versus 1.7%; $P = 0.02$). HBV/A has been increasing in patients with CHB in the Kanto area, where HBV/A in patients with acute hepatitis is more frequent than in the other areas. In the islands of Okinawa, also, HBV/A was found to be prevalent in this study. Of the four patients infected with HBV/A there, two were coinfected with HIV-1. They were both MSM, and they were suspected to have been infected with HIV through sexual contacts on the Japanese mainland. It has been reported that HIV infection increases the probability that AHBs will become chronic (2, 11, 33, 48). Because they share routes of transmission and the risk for HIV-1 and HBV infections, approximately

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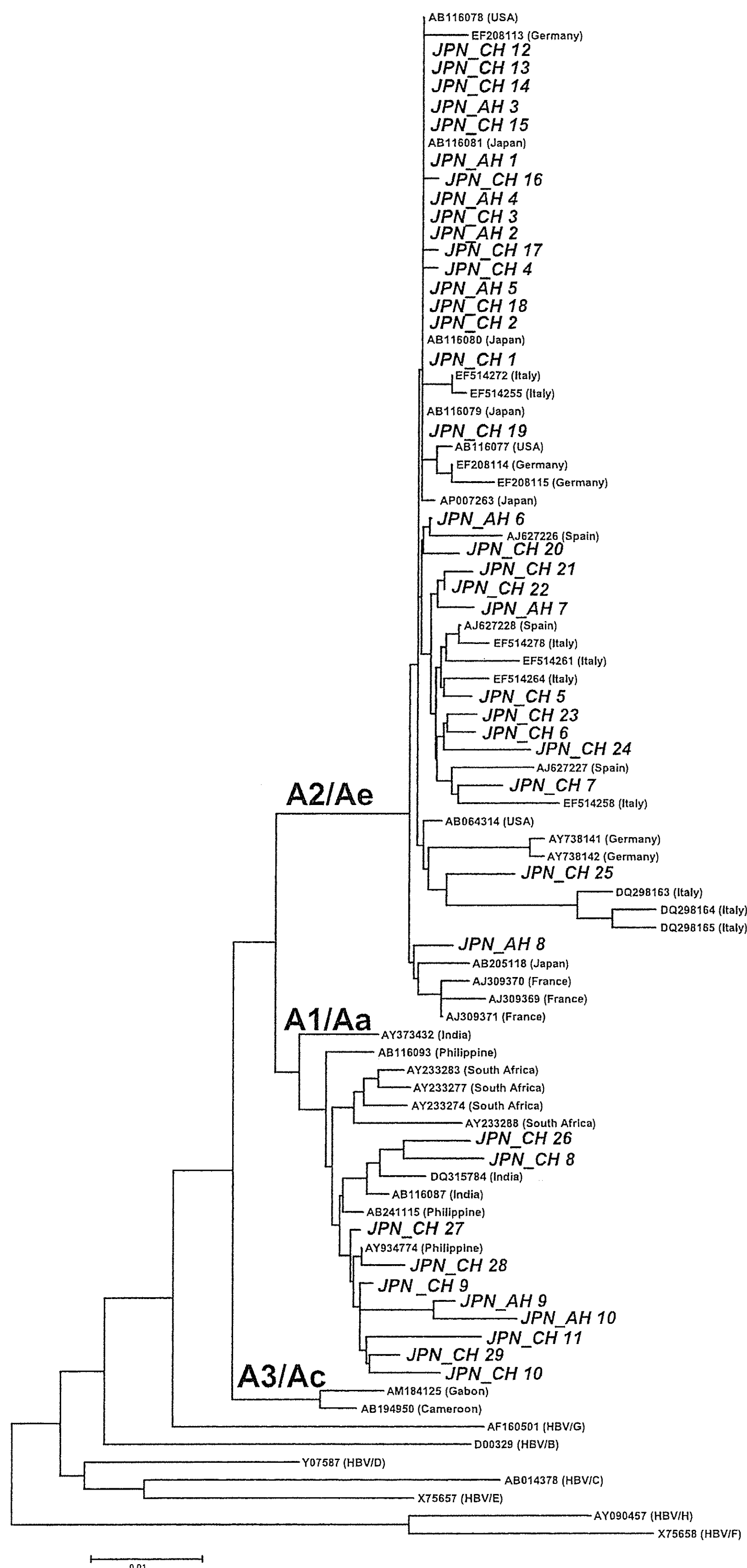

FIG. 5. Phylogenetic tree constructed based on pre-S2/S region sequences of HBV/A isolates. Those from 29 patients with chronic infection in this study are shown in boldface italic (JPN_CH1 to -29), along with 10 isolates (JPN_AH1 to -10) from patients with acute hepatitis in Japan reported in our previous study (17). Representative isolates were retrieved from the DDBJ/EMBL/GenBank databases, including 28 HBV/Ae, 10 HBV/Aa, and 2 HBV/Ac isolates and 7 HBV isolates representative of the other seven genotypes. Isolates from the databases are identified by accession numbers, followed by the country of origin. The bar at the bottom spans 0.01 nucleotide substitutions per site.

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