Predominance of Hepatitis B Virus YMDD Mutants Is Prognostic of Viral DNA Breakthrough

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Background & Aims: Hepatitis B virus (HBV) tyrosine, methionine, aspartate, aspartate (YMDD) mutants with or without additional compensatory mutations occur in chronically infected patients during lamivudine therapy and may be associated with accompanying viral breakthrough. The aim of this study was to determine whether a predominance of YMDD mutants could be a prognostic marker for occurrence of viral DNA breakthrough.

Methods: YMDD genotypes in 740 consecutive samples collected from 116 patients throughout lamivudine treatment were retrospectively analyzed using a matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS)-based genotyping assay, termed restriction fragment mass polymorphism (RFMP). RFMP exploits differences in molecular masses between wild-type and variant bases of rtM204V/I following PCR amplification of HBV DNA with a lower limit of detection being 100 copies/mL.

Results: The study demonstrated that YMDD mutants occur throughout the course of lamivudine therapy irrespective of occurrence of viral DNA breakthrough, indicating that a mere detection of YMDD mutants could not sufficiently predict the viral DNA breakthrough, although presence of YMDD mutants is associated with high incidence of viral DNA breakthrough (odds ratio, 7.8; P<.0012; relative risk 8.7%), and a 5-fold predominance of YMDD mutant to wild-type virus was significantly associated with viral DNA breakthrough (odds ratio, 604.5; P<.0001; relative risk 93.8%).

Conclusions: Close and periodical testing by RFMP assay should be useful to detect the predominance of YMDD mutants for monitoring drug resistance, enabling early intervention and prevention.

It is well known that long-term lamivudine therapy often is associated with the selection of resistant hepatitis B virus (HBV) mutants. Mutations in the highly conserved tyrosine, methionine, aspartate, and aspartate (YMDD) motif (rtM204V/I) are primarily responsible for resistance to lamivudine, and the rtL180M mutation, which usually appears in conjunction with rtM204V/I after long-term lamivudine therapy, augments resistance.1,2 Other compensatory mutations such as rtV173L, rtT128N, rtW153Q, or G1896A in the B subdomain of HBV polymerase, overlapping the surface reading frame, or precore region also have been found in conjunction with YMDD mutant viruses.3–6 YMDD mutations can be found as early as 2 weeks after initiation of lamivudine therapy, and, in a few cases, the mutations have been reported in patients without lamivudine therapy.7 These observations make it unclear how the presence of YMDD and other mutations predict phenotypic viral DNA breakthrough during lamivudine therapy.8,9

We introduced a novel genotyping assay based on matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS), termed restriction fragment mass polymorphism (RFMP), to detect variation in the sequences of YMDD mutants. RFMP utilizes molecular mass information of restriction enzyme-cleaved DNA fragments.10 This technology is very useful for the detection of viral quasispecies and subtle genetic variations present at viral load as low as 100 copies/mL because of the intrinsic sensitivity of MALDI-TOF MS. We have shown that quasispecies of wild-type and YMDD mutants during lamivudine treatment exist, even though HBV DNA remained below the detection limit of the Digene hybrid capture assay (Digene Diagnostics, Beltsville, MD) with a lower limit of 1.4 × 10^5 copies/mL, and have suggested that dominance of mutants over wild-type virus may precede HBV viral DNA breakthrough. Because it is still questioned how the presence of YMDD mutations is associated with viral DNA breakthrough, more defined studies have been needed to establish mutants that will serve as predictable markers for viral breakthrough. Consequently, we have

Abbreviations used in this paper: HBV, hepatitis B virus; MALDI-TOF MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; RFMP, restriction fragment mass polymorphism; YMDD, tyrosine, methionine, aspartate, aspartate.
undertaken a longitudinal study of 116 patients treated by lamivudine with a median follow-up of 21 months before discontinuation of lamivudine treatment. We analyzed their YMDD genotypes during the course of lamivudine therapy to assess whether the presence of YMDD mutants during the period of HBV DNA negativity determined by Digene assay is associated with viral DNA breakthrough, whether a predominance of activity determined by Digene assay is associated with YMDD mutants during the period of HBV DNA negativity, whether the dynamic status of YMDD mutants is a prerequisite for subsequent HBV viral DNA breakthrough, and whether the dynamic status of YMDD mutants are prognostic for development of viral resistance.

Materials and Methods

Patients and Sera

Included in the study were a total of 116 chronically infected hepatitis B patients who initially responded to lamivudine (GSK, Greenford, UK) at the Liver Clinic Center of Korea University Guro Hospital in Seoul, Korea, between March 1997 and November 2002. Each patient was administered orally with 100 mg of lamivudine daily. Sera were collected and analyzed over a 3- to 6-month interval. During the median follow-up of 21 months before lamivudine treatment was discontinued, HBV DNA breakthroughs were experienced by 26 patients, and no viral DNA breakthroughs occurred in 90 patients. None of the subjects was positive for either antihepatitis C virus antibodies or anti-human immunodeficiency virus antibodies. A needle biopsy of each patient was performed to confirm the presence of chronic hepatitis. Also, none of the patients received immunosuppressive or antiviral therapy at least 6 months before lamivudine therapy. Informed consent was obtained form each patient, and the experimental protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected by prior approval by the Korea University Guro Hospital human research committee. HBV DNA was measured by the Digene hybrid capture assay (Digene Diagnostics).

HBV DNA Extraction and PCR Amplification

HBV DNA was extracted from serum samples (200 µL) using QIAamp blood kit (Qiagen, Chatsworth, CA) according to the manufacturer’s instructions and dissolved in 20 µL distilled water. Two µL sample was used for the PCR reaction. For MALDI-TOF MS-based genotyping, PCR was performed in 18 µL reaction mixture containing 20 mmol/L Tris-acetate, 10 mmol/L magnesium acetate, 1 mmol/L diethiothreitol, and 1 unit of FdI. The reactions were incubated at 37°C for 2 hours and then at 45°C for 2 hours with BsaFI. The digests were desalted by vacuum filtration through a 384-well sample preparation plate containing 5 mg polymeric sorbent (Waters, Milford, MA) per well. Each cleavage reaction mixture was added to 70 µL of 1 mol/L triethylammonium acetate (pH 7.6), and loaded into a well containing 90 µL of 1 mol/L TEAA, pH 7.6. After rinsing 5 times with 85 µL of 0.1 mol/L TEAA, pH 7.0, the plate was reassembled on a vacuum manifold and eluted with 60 µL 60% aqueous isopropanol into a collection plate, which was placed on a heating block at 115°C for 90 minutes. The desalted reaction mixtures were resuspended with matrix solution containing 50 mg/mL 3-hydroxy picolinic acid (Sigma Chemical Co, St. Louis, MO), 0.05 mol/L ammonium citrate (Sigma), and 30% acetonitrile (Sigma) and spotted in 3 µL volumes on a polished anchor chip plate. Mass spectra were acquired on a linear MALDI-TOF MS (Biflex IV; Bruker Daltonics, Billerica, MA) workstation in a positive ion, delayed extraction mode. Typically, time-of-flight data from 20–50 individual laser pulses were recorded and averaged on a transient digitizer, after which the averaged spectra were automatically converted to mass by data processing software (Bruker DataAnalysis for time of flight 1.6 m; Bruker Daltonics). A lower limit of detection of the RFMP assay was found to be 100 copies of HBV genome per milliliter of serum as previously reported.12

Data Analysis

The capacity of the RFMP assay to show relative abundance among viral species enabled the effective monitoring of YMDD genotype dynamics in longitudinal samples.10 To estimate relative proportions of each virus genotype, ratios were calculated using the mean peak heights generated from
multiple experiments. Predominance is defined as a 5-fold or greater amount of a genotype virus than others.

Data were analyzed by the Mann–Whitney test for the continuous ordinal data, Cochran Mantel Haenszel test, Pearson’s χ² test, and Fisher exact test for the association between 2 qualitative variables using the statistical package SAS (version 8; SAS Institute Inc., Cary, NC). The multivariate analysis was done using logistic regression to adjust for possible confounders such as age and sex. P values of less than .05 were considered as statistically significant.

**Results**

RFMP Genotyping of YMDD Mutations in Patients With or Without HBV DNA Breakthrough

The RFMP assay is based on mass spectrometric analysis of small DNA fragments containing sites of variation (Figure 1). The first step requires PCR amplification using primers flanking the altered bases. The forward primer was designed to introduce a FokI site (an isoschizomer of BstF5I) in the amplified product by substituting the restriction recognition sequence GGATG for 1 nucleotide present within 8 bases away from mutated site. The backward primer was designed to make the resulting amplicon as short as possible; both primers’ Tm values matched with each other for better PCR yield. Both FokI and BstF5I are type IIIS restriction enzymes that cleave DNA outside the recognition sequence. The FokI enzyme cleaves DNA 9 bases 3’ to the recognition site on one strand and 13 bases from the recognition site on the other strand, leaving a 4-base overhang protruding 5’ end. BstF5I cleaves DNA 2 bases 3’ to the recognition site on one strand and immediately 3’ to the recognition site on the opposite strand, leaving a 2-base overhang. As summarized in Table 1, the 7mer fragments contain the polymorphic bases at the first bases of codon 204, and the 13mer fragments contain the 3 bases of codon 204 and an additional 2 bases from the HBV sequences. By analysis of artificial pools constructed by mixing appropriate amounts of wild-type and rt204I variant viruses in plasmids, we tested whether estimated peak ratios in mass spectrum reflect real relative abundance between the virus populations. Results showed that there is a linear relation of the estimated to the expected ratios in the range from 5% to 95% (Figure 2). Thus, the RFMP assay could detect mixtures of wild-type and variant viruses and determine their relative abundance quantitatively.

HBV DNA breakthrough was observed at a median of 16 months after initiation of lamivudine treatment in 25 of 116 patients, whereas 91 patients did not experience viral DNA breakthrough until the discontinuation of lamivudine treatment (median time of follow-up: 23 months). To investigate the relationship between viral DNA breakthrough and the presence of YMDD mutation, YMDD genotypes were compared between patients with or without DNA breakthrough. Eighty-nine serial sera from 25 patients collected at 3-month intervals during HBV DNA breakthrough and 374 serial sera from 91 patients who achieved sustained repression of viral replication on lamivudine treatment were examined.
for YMDD genotypes by RFMP. As shown in Table 2, YMDD mutants were found in 81 of 89 sera at the time of viral DNA breakthrough (91.0%). rt204I, rt204V, and mixed mutant genotypes of rt204I and rt204V were found in 46 (51.68%), 15 (16.85%), and 20 (22.47%) of the samples, respectively. However, the wild-type and a mixed genotype of YMDD wild-type and mutant viruses were found only in 8 sera (8.99%) during the stage of HBV viral DNA breakthrough. On the other hand, among 374 sera from the patient group without HBV DNA breakthrough, RFMP assay detected a mixed genotype of YMDD wild-type and mutant viruses in 253 (67.6%) and the sole wild-type virus in 120 (32.1%) sera, respectively (Table 2). The YMDD mutants mixed with wild-type virus were first seen within the average of the first 6-month period of treatment and continued to persist up to the end of the study without viral DNA breakthrough (data not shown). One serum sample (0.3%) harbored only rt204V mutant virus, showing that the incidence of pure YMDD mutants in those 374 sera was drastically lower than in sera taken during viral breakthrough (0.3% vs 91.0%, \( p < .0001 \)). These results demonstrate that patients with HBV DNA breakthrough had higher percentages of pure YMDD mutants without presence of wild-type virus compared with patients without HBV DNA breakthrough, consistent with previous findings.\(^{13}\) This suggests that YMDD mutants can be present throughout a course of lamivudine therapy regardless of the accompanying viral DNA breakthrough and that their presence is not a diagnostic marker of the viral DNA breakthrough.

**Association Between the Predominance of YMDD Mutants and Viral DNA Breakthrough**

In an attempt to study how the dynamic status of YMDD mutants prior to detectible viral rebound becomes associated with viral DNA breakthroughs, 429 serum samples with DNA levels below detection by the Digene Hybrid Capture assay (<1.4 \( \times \) 10^2 copies/mL) collected longitudinally from 116 patients during follow-up of a median 21 months were randomized, tested for YMDD genotype status, and compared with subsequent diagnosis of HBV DNA breakthrough within the 6 months of genotyping. The 429 sera could be grouped into 264 sera with detectable YMDD mutants and 165 sera without detectable YMDD mutants. Twenty-three of 264 sera with YMDD mutants (relative risk [RR] = 67.6%) and the sole wild-type virus in 120 (32.1%) sera, respectively (Table 2). The YMDD mutants mixed with wild-type virus were first seen within the average of the first 6-month period of treatment and continued to persist up to the end of the study without viral DNA breakthrough (data not shown). One serum sample (0.3%) harbored only rt204V mutant virus, showing that the incidence of pure YMDD mutants in those 374 sera was drastically lower than in sera taken during viral breakthrough (0.3% vs 91.0%, \( p < .0001 \)). These results demonstrate that patients with HBV DNA breakthrough had higher percentages of pure YMDD mutants without presence of wild-type virus compared with patients without HBV DNA breakthrough, consistent with previous findings.\(^{13}\) This suggests that YMDD mutants can be present throughout a course of lamivudine therapy regardless of the accompanying viral DNA breakthrough and that their presence is not a diagnostic marker of the viral DNA breakthrough.

**Figure 2.** The accuracy of measuring relative abundance in mixed genotypes by RFMP. Test was done for linearity between the mixing ratios and the corresponding 13mer peak ratios across artificial pools with different proportions of genotypes ranging from 5% to 95%. Expected percentages denote [rt204I/(rt204I+rt204V)] \( \times \) 100. The error bars represent the standard deviation. A diagonal line would be expected for complete concordance between measured and real values. \( R^2 = 0.992 \). Slope = 1.055. The slope was not significantly different from 1 (\( p = .810 \)).

**Table 1.** Expected and Observed Masses of Oligonucleotides Resulting From Restriction Enzyme Digestion of PCR Products Spanning YMDD Motif

<table>
<thead>
<tr>
<th>Codon 204</th>
<th>Amino acid</th>
<th>Expected fragments</th>
<th>7mer</th>
<th>13mer</th>
<th>Expected mass (daltons)</th>
<th>Observed mass (daltons)</th>
</tr>
</thead>
<tbody>
<tr>
<td>aTg</td>
<td>Met</td>
<td>AGTTATA</td>
<td>2199.4</td>
<td>3997.6</td>
<td>2199.6</td>
<td>3998.0</td>
</tr>
<tr>
<td>gTg</td>
<td>Val</td>
<td>AGTTAGG</td>
<td>2215.4</td>
<td>3982.6</td>
<td>2215.9</td>
<td>3982.9</td>
</tr>
<tr>
<td>aTt</td>
<td>Ile</td>
<td>AGTTATA</td>
<td>2199.4</td>
<td>4021.6</td>
<td>2199.6</td>
<td>4021.8</td>
</tr>
<tr>
<td>aTc</td>
<td>Ile</td>
<td>AGTTATA</td>
<td>2199.4</td>
<td>4037.6</td>
<td>2199.5</td>
<td>4037.6</td>
</tr>
<tr>
<td>aTa</td>
<td>Ile</td>
<td>AGTTATA</td>
<td>2199.4</td>
<td>4012.6</td>
<td>2199.6</td>
<td>4012.6</td>
</tr>
</tbody>
</table>

\( ^a \)The first and third bases of codon 204 in YMDD motif are noted by a small letter.
development of viral breakthrough in the presence of YMDD mutant (P = .0012) (Table 3). Although statistically significant, mere detection of YMDD mutants in a serum sample did not have sufficient statistical power to predict viral breakthrough, considering that a majority (91.3%) of mutant-harboring patients did not show occurrence of viral breakthrough. When the 429 sera were classified according to relative abundance of mutant against wild-type viruses expressed as fold excess, most (93.7%) of sera harboring 5-fold or greater excess of mutant over the wild-type virus showed viral breakthrough within their 6-month follow-up, whereas those with mutant detected less than 2.5-fold of wild-type virus rarely exhibited viral breakthrough, similar to sera without detectible mutant (Figure 3). Using a criterion of mutant predominance, defined as at least a 5-fold or greater amount of the mutant virus than wild-type virus (Figure 4), 16 sera were assigned as mutant predominance, whereas 413 sera did not satisfy the cut off that contained the sole wild-type or mutant virus present less than 5-fold excess over wild-type virus (Table 3). Of those 413 sera, 10 sera showed viral DNA breakthrough in the corresponding consecutive sera taken within their next 6 months (RR = 2.4%). In contrast, 15 of 16 sera with mutant predominance genotypes exhibited viral DNA breakthroughs in their consecutive sera (RR = 93.8%). Thus, YMDD mutant predominance indicated significantly tighter association with viral DNA breakthrough within 6 months (OR, 604.5, P = .0001) of the diagnosis and higher positive predictive value of 93.8% compared with mere detection of YMDD mutant virus.

A similar statistical correlation was observed when 429 sera were analyzed by patient basis (Table 3). Of a total 116 patients, 100 had a mixed genotype or sole wild-type viruses, and 16 patients had at least one test of mutant predominance in their consecutive samples during the course of lamivudine treatment. Among those 100 patients, 10 patients experienced viral DNA breakthrough (RR = 10%), and 15 of 16 patients with mutant predominance experienced viral DNA breakthrough (RR = 93.8%) during the course of lamivudine treatment (OR, 135.0; P = .001). By contrast, regression

### Table 2. Relationship Between YMDD Genotypes and Viral DNA Breakthrough

<table>
<thead>
<tr>
<th>Patient groups</th>
<th>No. of sera</th>
<th>Median follow-up in months (range)</th>
<th>Median HBV DNA in pg/mL (range)</th>
<th>Median ALT in IU/L (range)</th>
<th>Median AST in IU/L (range)</th>
<th>rt204</th>
<th>No. of genotypes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viral DNA breakthrough</td>
<td>89</td>
<td>16 (7–41)</td>
<td>286 (2.1–6000)</td>
<td>71 (13–1600)</td>
<td>59 (15–1260)</td>
<td>Val, Ile</td>
<td>20 (22.47)</td>
</tr>
<tr>
<td>(n = 25)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>15 (16.85)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>46 (51.68)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3 (3.37)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5 (5.62)</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 (0.3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>120 (32.1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>253 (67.6)</td>
</tr>
</tbody>
</table>

NOTE. For patient group with HBV breakthrough, test values were obtained at the time of viral breakthrough; for patient group without viral DNA breakthrough, test values are taken since viral DNA became negative upon lamivudine treatment; mix, a mixed genotype of wild type and mutant viruses.

### Table 3. Odds Ratios for the Development of Viral DNA Breakthrough by Relative Levels of YMDD Mutant Virus in Mixed Infection

<table>
<thead>
<tr>
<th>Mutant status</th>
<th>HBV DNA breakthrough</th>
<th>Relative risk of breakthrough (%)</th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sera (n = 429)</td>
<td>Presence of YMDD</td>
<td>Yes</td>
<td>23</td>
<td>241</td>
<td>8.7</td>
</tr>
<tr>
<td></td>
<td>mutants</td>
<td>No</td>
<td>2</td>
<td>163</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>Predominancea of</td>
<td>Yes</td>
<td>15</td>
<td>1</td>
<td>93.8</td>
</tr>
<tr>
<td></td>
<td>YMDD mutants</td>
<td>No</td>
<td>10</td>
<td>403</td>
<td>2.4</td>
</tr>
<tr>
<td>Patients (n = 116)</td>
<td>Presence of YMDD</td>
<td>Yes</td>
<td>23</td>
<td>47</td>
<td>32.9</td>
</tr>
<tr>
<td></td>
<td>mutants</td>
<td>No</td>
<td>2</td>
<td>44</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>Predominancea of</td>
<td>Yes</td>
<td>15</td>
<td>1</td>
<td>93.8</td>
</tr>
<tr>
<td></td>
<td>YMDD mutants</td>
<td>No</td>
<td>10</td>
<td>90</td>
<td>10</td>
</tr>
</tbody>
</table>

aPredominance of mutant represents approximately 5-fold or greater excess of mutant over the wild type virus.
by selection from a preexisting pool of quasispecies of HBV. However, an evident correlation has not been made between the presence of YMDD mutants, particularly detected at early stages of therapy and viral DNA breakthrough at late stages of lamivudine treatment, although many studies have reported detection of YMDD mutants a few months before viral breakthrough.21–23

Currently, YMDD genotyping is largely performed by sequence analysis and hybridization-based assays.24,25 Sequencing can provide information on the majority of species present in the viral populations but, generally, cannot detect species composing fewer than 25%–50% of a viral population.26 It is possible to sequence multiple clones to determine the genetic heterogeneity of a population. However, it is not practical for large cohort studies or clinical laboratories because it is hard to standardize and is time-consuming. Although generally reliable hybridization techniques suffer from labor intensiveness and complex hybridization steps, the limitations of hybridization-based methods arise largely from being qualitative, rather than quantitative. As shown in Figure 2, there is a good correlation between estimated peak heights and real proportions in mixed genotype pools, indicating that the RFMP assay enables better quantitative detection of mixed populations without need for population-based cloning and subsequent sequencing. By combining the merits of a unique assay chemistry and the mature nature of MALDI-TOF MS, the RFMP assay is able to screen for viral mutants in a robust high-throughput manner capable of analyzing 384 samples in 3 hours simultaneously, which is almost 10 times faster than existing methods. In terms of cost-effectiveness, we estimated the direct cost per test (reagents and labor) of the RFMP assay for YMDD genotyping to be about $30, including viral nucleic acid extraction, PCR, restriction digestion, and restriction fragment purification and matrix, which is much cheaper than the sequencing or hybridization assays that are approximately $50 to $100 per test. These costs do not include the capital equipment, which are slightly greater for the RFMP method. However, in many laboratories already using mass spectrometers for prenatal metabolite screening or for clinical genotyping, these costs could be avoided because the same equipment is executed for the RFMP assay.

To see whether viral DNA breakthrough and sustained repression of viral replication without HBV DNA breakthrough (viral DNA negative) could be differentiated by the dynamic status of YMDD mutants, genotypes of sera were determined at both states. As shown in Table 2, YMDD mutants without detectable wild-type virus were found almost exclusively in sera during HBV

Figure 3. Distribution of 429 HBV DNA-negative sera by relative abundance of YMDD mutant over wild-type viruses determined by RFMP analysis. Four hundred twenty-nine serum samples were collected longitudinally from 116 patients during a median 21 months of lamivudine therapy. The 429 samples were found to be HBV DNA negative when tested by the Digene Hybrid Capture assay (<1.4 × 10^5 copies/mL) and were tested for YMDD genotype by RFMP and classified according to relative abundance (fold excess) of mutant against wild-type viruses into 7 groups in ascending order; 165 sera of 0 (no mutant detected), 120 sera of less than 1, 103 sera of less than 2.5, 25 sera of less than 5, 10 sera of less than 7.5, 3 sera of less than 10, and 3 sera of 10 or over (including mutant only). Number in parentheses and solid part of a bar denote the cases of showing HBV DNA breakthrough within 6 months of genotyping.

Analysis of mere detection of mutants also revealed weaker linkage to viral breakthrough (OR, 10.7; P = .0003) and lower positive predictive value of 32.9%. Thus, correlation of patients with mutant predominance with subsequent breakthrough was much stronger than those with mere presence of YMDD mutant, inconsistent with in sera-based analysis.

**Discussion**

Lamivudine has revolutionized the treatment of chronic hepatitis B and opened options for the management of patients with decompensated cirrhosis or recurrent hepatitis B after liver transplantation.14,15 The suppressive effect, relatively low cost, and lack of significant adverse effects make lamivudine a frontline of treatment for chronic hepatitis B. However, long-term lamivudine therapy is associated with selection of HBV polymerase mutants that become resistant to lamivudine. Reports of lamivudine association with severe hepatitis and mortality have been increasing, which may be the result of a cytotoxic T-lymphocyte-mediated immune response directed against the YMDD mutant.16–20 That YMDD mutants exist even before lamivudine treatment suggests that emergence of YMDD mutants by lamivudine occurs...
viral DNA breakthroughs, whereas solely wild-type or mixed wild-type and YMDD mutant viruses were exclusively found in sera from patients without viral DNA breakthrough. This showed that YMDD mutant viruses can be present throughout a course of lamivudine therapy irrespective of accompanying viral DNA breakthrough and that the mere presence of YMDD mutants observed in a mixed genotype at a time point does not always foretell viral DNA breakthrough. It also conforms to the observation that drug-resistant mutants increase in viral titers only after the wild-type virus is eliminated from the hepatocyte population, presumably representing a lack of replicative space within the liver for infection of cells by lamivudine-resistant virus. Therefore, patients with YMDD mutants without the presence of wild-type virus should be monitored closely because they have a greater chance of HBV DNA breakthrough.

YMDD mutants have been detected 1 to 6 months before DNA breakthrough and can become dominant or the only species present. This suggests a transition period when viral population changes from wild-type to YMDD mutant viruses preceding DNA breakthrough. If we understood the dynamics among YMDD mutant and wild-type viruses during the transition period, we could foretell precisely whether HBV DNA breakthrough occurs. When a total of 429 sera showing undetectable serum HBV DNA as determined by Digene hybrid capture assay was analyzed for YMDD genotype status, we found a much stronger association of YMDD mutant predominance in serum, defined as at least a 5-fold higher amount of the mutant virus than wild-type virus with the following viral DNA breakthrough within 6 months and incomparable positive predictive values compared with mere mutant presence as shown in Table 3 (OR, 604.5 vs 7.81; positive predictive values, 0.938 vs 0.087, respectively). This indicates that detection of mutant virus as pure virus or at least a 5-fold higher amount of the mutant virus when mixed with wild-type virus can provide a prognostic marker for subsequent occurrence of HBV DNA breakthrough.

Ten of 25 sera (Table 3) showed viral DNA breakthrough in their following sera, although they had not met the criteria for mutant predominance. In the majority (90%), the wild-type virus disappeared at the time of HBV DNA breakthrough (data not shown). Recently, Pallier et al have observed such genotype transition to mutant predominance in the dynamic process of appearance of YMDD mutants during lamivudine therapy. The transition to mutant predominance took place in a short period of time of 2 to 6 months (median, 4 months) in our study. Consistently, Pallier et al reported a progressive switch from 100% wild-type to 100% YMDD

![Figure 4](image-url)
mutant over 2 to 4 months through a quasispecies analysis. This suggests that YMDD genotype transition across viral breakthrough might happen in the diverse span of time, and a 3-month interval for genotyping is too sparse to fully pinpoint such events. Factors such as the physiologic conditions surrounding mutant emergence and other compensatory mutations could affect the rate of transition. Compensatory mutations such as rtV173L, rtL180M, rtT128N, rtW153Q, or G1896A mutations in the B subdomain of HBV polymerase, overlapping surface reading frame, or precore region have been frequently found in conjunction with YMDD mutations in patients. These mutations give restored or enhanced replication fitness to YMDD variant viruses. It is worthwhile to test those mutations for subcategorizing kinetics of resistance emergence by composite genotypes of mutations related with drug sensitivity. Previously, Gaillard et al showed that the decrease in replication fitness of YMDD mutant HBV strains results from the lower affinities (increased Km values) of the YMDD mutant polymerase for the natural dNTP substrates and physiologic intracellular concentrations of dNTPs that are limiting for the replication of YMDD mutant HBV strains. Thus, host factors such as intracellular concentration of dNTPs could influence the transition rate to viral DNA breakthrough. More detailed study of factors governing the transition event will be valuable to understand ultimately the dynamics of HBV DNA breakthrough.

Since lamivudine was introduced for the treatment of chronic hepatitis B, the emergence of lamivudine-resistant mutant mutations have been suggested as a major drawback, despite its proven efficacy and safety profiles. Clinical experiences report severe hepatitis exacerbations and even hepatic decompensation on continuing lamivudine therapy after emergence of YMDD mutants and continued worsening of disease even after discontinuation of the therapy. Recent results also suggest that there is no benefit to continued lamivudine therapy after emergence of YMDD mutant. Particularly for patients at high risk for disease progression, it is crucial to identify early and precisely a prognosis for appearance of phenotype resistance when viral load in the patient is very low and/or when mutant viruses represent only a minor fraction of the total viral population, especially because alternative antiviral such as adefovir and entecavir, which are effective against lamivudine-resistant HBV, have become available. Beyond the observation that the presence of YMDD mutants is associated with high incidence of viral DNA breakthrough, our study showed that detection of predominant YMDD mutants rather than of mere mutant presence has a better prognostic value for occurrence of viral resistance. A close and periodic testing for detecting predominant YMDD mutants should enable early intervention and prevention of drug resistance as it develops.

References


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