Evaluation of methods for monitoring drug resistance in chronic hepatitis B patients during lamivudine therapy based on mass spectrometry and reverse hybridization

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A matrix-assisted laser desorption/ionization time-of-flight mass spectrometry-based genotyping assay, termed restriction fragment mass polymorphism (RFMP) has been recently developed for detecting hepatitis B virus (HBV) mutants. The assay is based on PCR amplification and mass measurement of oligonucleotides containing sites of mutations that confer resistance to lamivudine. We compared the efficacy and usefulness of the RFMP assay with a commercial assay using a reverse hybridization line probe technology, namely INNO-LiPA HBV DR (referred to henceforth as the LiPA assay), for the detection of lamivudine-resistant HBV mutants. A total of 60 patient samples were analysed for the presence of mutations at rtL180M and rtM204I/V of HBV polymerase by the LiPA and RFMP assays. The ability to detect mutations at rtM204I/V was compared with defined mixtures of wild-type and mutant HBV cloned in plasmids at relative concentrations ranging from 1–25%. Concordance between methods was found to be 95.0% (57/60) when only the presence of resistance mutations was considered, regardless of quasispecies. In three cases, additional minor populations of resistant viruses were identified by RFMP. Defined mixtures were consistently successfully identified at a 1% relative concentration of mutant versus wild-type viruses by the RFMP assay and 4% by the LiPA assay. The RFMP assay proved to be an accurate and reliable tool for detection of lamivudine-resistant mutations and was more sensitive than the LiPA assay in detecting mixtures of mutant and wild-type viruses. The improved sensitivity of the RPMP assay can help monitor drug resistance as it develops, enabling early intervention and prevention.

Introduction

Chronic infection with hepatitis B virus (HBV) is a leading cause of cirrhosis and hepatocellular carcinoma and, in addition to morbidity and mortality, creates significant economic and social burdens [1]. Worldwide, approximately 400 million people have chronic hepatitis B infection, of whom 15–40% can be expected to develop serious hepatic complications [2]. Although both the spread of HBV infection and the development of hepatocellular carcinoma can be effectively checked by vaccination, treatment with antivirals remains the only therapeutic option available to chronically infected patients [3].

The objective of treating chronic HBV infection is to halt progression of liver injury by suppressing viral replication and infection [4]. The availability of lamivudine [(–)-β-L-2′,3′-dideoxy-3′-thiacytidine; 3TC] has revolutionized the treatment of chronic hepatitis B and made new options available for the management of patients with decompensated cirrhosis or recurrent hepatitis B, post liver transplantation [5]. Unfortunately, long-term lamivudine therapy promotes the selection of HBV mutants with altered DNA polymerases resistant to therapy. The altered DNA polymerases frequently exhibit changes in a highly conserved tyrosine, methionine, aspartate and aspartate (YMDD) motif, and so are named YMDD mutants. The most commonly described mutations are the substitution of valine or isoleucine for methionine at residue 204 (rtL204I/V) with or without the rtL180M mutation [6–8].

The incidence of YMDD mutants is increasing at a rate of 15–20% per year and correlates with higher pretherapeutic viral load, histological activity scores and

alanine aminotransferase (ALT) levels before therapy [9]. Recent studies have shown that acute exacerbations occur frequently and, in patients with lamivudine-resistant mutants during long-term lamivudine treatment, hepatic decompensation may develop, which can be life-threatening [10,11]. An additional concern is that individuals infected by HBV YMDD mutants pre-transplant may have an increased risk of recurrent infection post-transplant, with reappearance of high levels of serum HBV [12,13]. Advances in antiviral therapy, such as lamivudine, have brought about an increasing need for sensitive and early detection of emerging drug-resistant mutants to monitor drug resistance as it develops and aid early intervention [14–19]. The identification of such mutations is of increasing importance, especially as alternative antivirals such as adefovir and entecavir, which are effective against lamivudine-resistant HBV, have become available [20,21].

We have recently developed a matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)-based genotyping assay, termed restriction fragment mass polymorphism (RFMP), which exploits differences between YMDD wild-type and variant sequences [22]. This assay represents an improvement over gel-based assays because it relies upon precise information about the molecular mass of the analyte, both DNA strands can be analysed in parallel and it can be fully automated. In this study, we established a RFMP assay for rtL180M in addition to rtM204I/V and compared the results of the RFMP assay with those of the INNO-LiPA HBV DR line probe assay (hereafter referred to as the LiPA assay). The concordance and ability to identify mixed wild-type and mutant viral populations of the two methods were assessed in this evaluation.

Materials and methods

Specimens

Sera were collected from 60 hepatitis B patients who were predicted to develop viral breakthrough, defined as rebound of viral load, during lamivudine treatment at the Liver Clinic of Yonsei University Medical Centre in Seoul, Republic of Korea, between December 2003 and April 2004. All of the serum samples were determined to have genotype C by the LiPA assay. None of the patients was positive for either anti-hepatitis C virus antibody or anti-HIV antibody. Experimental protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in *a priori* approval by Yonsei University Medical Centre human research committee. Hepatitis B surface antigen and hepatitis B e antigen/antibody were determined by enzyme immunoassay (Abbott Diagnostics, Chicago, IL, USA). HBV DNA was measured by the Digene hybrid capture assay (Digene Diagnostics, Gaithersburg, MD, USA) with a lower limit of 0.5 pg/ml. HBV DNA was extracted from 200 μl of serum using the High Pure PCR Template Kit (Roche Diagnostics Corp, Indianapolis, IN, USA) according to the manufacturer's instructions; 2 μl of the viral DNA was used for the PCR reaction.

INNO-LiPA HBV DR line probe assay

This LiPA assay uses genotype-specific oligonucleotide probes attached to nitrocellulose strips to detect sequence variants located at amino acid positions 180, 204 and 207 in HBV polymerase [23]. The biotin-labelled PCR product was hybridized to the probes on the strip under stringent conditions. After hybridization and washing, streptavidin-labelled alkaline phosphatase was added, followed by incubation with a chromogen, which results in the development of a purple-brown precipitate when there is a match between the probe and the biotinylated PCR product. The test was performed according to the manufacturer's instructions (Innogenetics NV, Ghent, Belgium). A proficiency panel was tested to demonstrate adequate experience with the technique. Results were blinded until all tests were completed and taken for further analysis only when identical results were obtained from two independent experiments.

RFMP genotyping

PCR was performed in 18 μl of reaction mixture containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.2 mM of each dNTP, 10 pmol of each primer and 0.4 units of Platinum® *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA). The amplification conditions included initial denaturation at 94°C for 2 min, 10 cycles of denaturation at 94°C for 15 s, annealing at 50°C for 15 s and extension at 72°C for 30 s, followed by 35 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 15 s and extension at 72°C for 30 s. The sequences of forward and reverse primers used in the PCR are described in Table 1. Restriction enzyme digestion of PCR products was performed by mixing the PCR reaction mixture with 10 μl of buffer containing 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM dithiothreitol and 1 unit of *Fok*I. The reaction mixture was incubated at 37°C for 2 h and further incubated at 45°C for 2 h with *Bst*F5I. The resulting digest was desalted by vacuum filtration through a 384-well sample preparation plate containing 5 mg of polymeric sorbent (Waters, Miliford, MA, USA) per well. Each well was equilibrated with 90 μl of 1 M triethylammonium acetate (TEAA, pH 7.6). Each cleavage reaction mixture was added to 70 μl of 1 M TEAA (pH 7.6) and loaded into

Table 1. Primers used in amplification for RFMP assays of rtM204V/I and rtL180M

Primer	Sequences (5′–3′)	Position	Polarity
rfmp204f	TTCCCCCACTGTTTGGCTggatgTCAGTTAT	712–738	Sense
rfmp204r	TACAGACTTGGCCCCCAATACCACATGA	771–744	Antisense
rfmp180f	ATTCCTATGGGAGTGGGCCTCAGTggatgCGTTTCTC	634–666	Sense
rfmp180r	ACGAACCACTGAACAAATGGCACTAGTAAACTG	705–673	Antisense

Primers were designed based on the consensus sequences extracted from the multiple alignment of HBV sequences retrieved from the Entrez Nucleotide database of the National Center for Biotechnology Information, Bethesda, MD, USA and detailed information is available upon request. A five-nucleotide sequence (ggatg) embedded in the forward primer to introduce a *Fok*I site in amplicon is indicated by lower case letters. A single base mismatch (underlined G) was introduced to erase the naturally occurring *Fok*I site (nucleotides 741–745). Nucleotide sequence positions were numbered according to Ono *et al.* [34].

a well. After rinsing five times with 85 µl of 0.1 M TEAA pH 7.0, the plate was reassembled on a vacuum manifold and eluted with 60 µl of 60% aqueous isopropanol into a collection plate, which was placed on a heating block at 115°C for 90 min. The desalted reaction mixtures were resuspended with matrix solution containing 50 mg/ml 3-hydroxy picolinic acid (Sigma, Saint Louis, MO, USA), 0.05 M ammonium citrate (Sigma) and 30% acetonitrile (Sigma), and were spotted in 3 µl volumes on a polished anchorchip plate. Mass spectra were acquired on a linear MALDI-TOF MS (Bruker Daltonics Biflex IV, Billerica, MA, USA) 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 TOF 1.6m).

Validation of genotyping by clonal sequencing

To amplify the HBV polymerase gene encoding the YMDD motif for cloning and sequencing, PCR was performed with a primer pair BF111 and BF109 as described by Chayama *et al.* [24]. The PCR products were cloned into the pCR-Script Amp cloning vector (Stratagene, La Jolla, CA, USA) and subsequently sequenced with vector-specific primers. Twenty clones were initially sequenced for each sample; additional clones were sequenced when the presence of a mixed population could not be demonstrated. Sequence analysis was performed by ABI PRISM 310 Genetic Analyzer (Applied Biosystems, New York, NY, USA).

Results

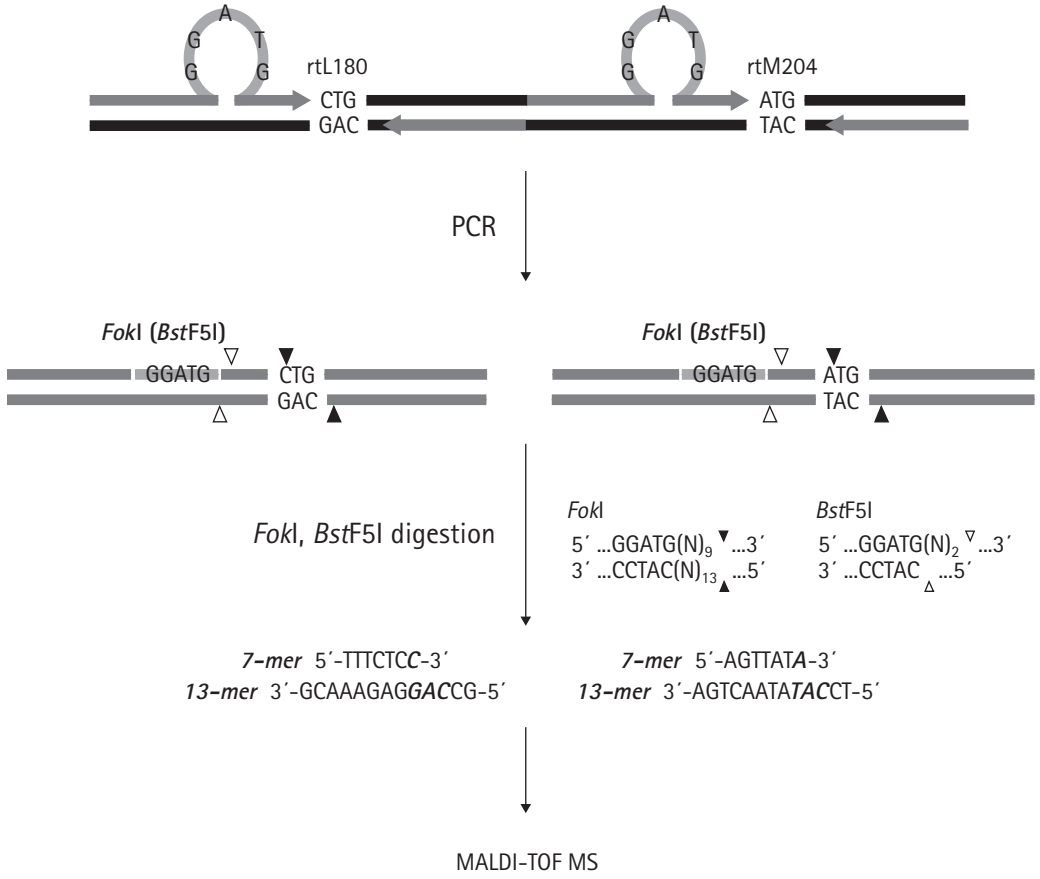
RFMP genotyping strategy

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 *Fok*I site (an isoschizomer of *Bst*F5I) in the amplified product by substituting the restriction recognition sequence GGATG for one nucleotide present within eight bases away from the mutated site. The reverse primer was designed to make the resulting amplicon as short as possible while both primers' T_m values matched with each other for better PCR yield. Both *Fok*I and *Bst*F5I are type IIS restriction enzymes that cleave DNA outside the recognition sequence. The *Fok*I enzyme cleaves DNA nine bases 3′ to the recognition site on one strand and 13 bases from the recognition site on the other strand, leaving a four-base overhanging, protruding 5′ end. *Bst*F5I cleaves DNA two bases 3′ to the recognition site on one strand and immediately 3′ to the recognition site on the opposite strand, leaving a two-base overhang. As summarized in Table 2, the 7-mer fragments contain the polymorphic bases at the first bases of codon 180 or 204, and the 13-mer fragments contain the three bases of codon 180 and 204 and two additional bases from the HBV sequences. These fragments were then analysed by mass spectrometry to identify the mutations at codon 180 and 204.

Concordance between RFMP and LiPA assays

Both genotyping methods were able to genotype all the samples enrolled (60/60). Identical results were found in 56 of the 60 samples (93.3%). In four samples, there were discrepancies observed between the results of the two assays, as presented in Figure 2. In all of these cases, RFMP indicated the presence of additional virus species (Table 3). In three cases (5.0%), RFMP revealed the presence of both mutant and wild-type viruses, while LiPA detected only wild-type virus. LiPA detected the mutant virus in specimens taken from these patients at a later time point, indicating that the mutant virus was already present in the previous sample and that RFMP detected it before LiPA did. In another sample, RFMP exhibited mixed rtM204I and rtM204V mutants along with wild-type while LiPA identified only the rtM204I mutant co-existing with wild-type virus. Existence of the additional viral species shown in RFMP was further verified by the clonal analysis using direct sequencing of multiple clones representing PCR

Figure 1. RFMP strategy



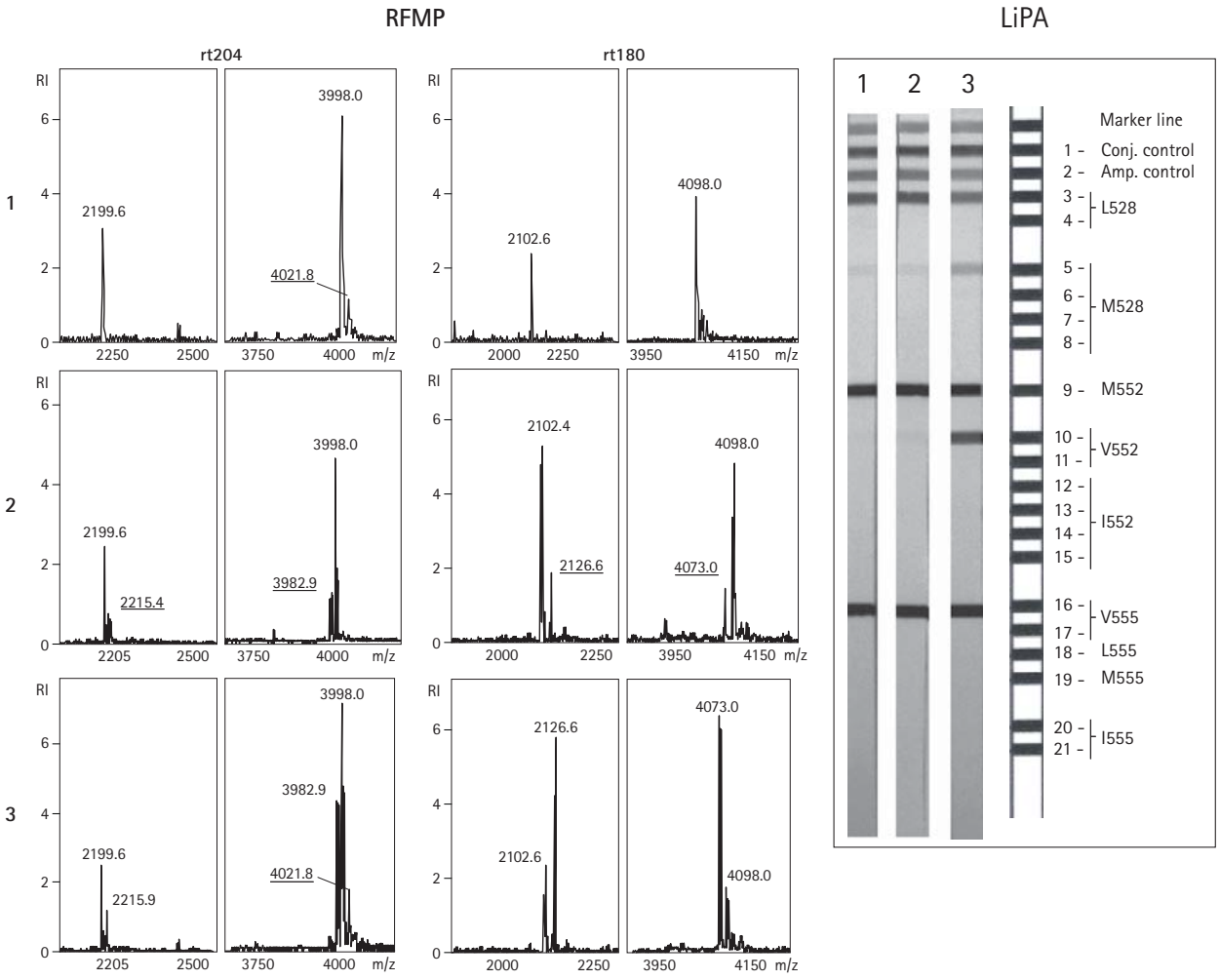
PCR was performed with primers containing a type IIS restriction endonuclease recognition sequence (GGATG; *Fok*I) embedded nine bases ahead of rtL180 and rtM204. The enzymatic digestion of the products released a pair of 7-mer and 13-mer fragments representing nucleotide sequences within the codons shown in bold italic, and then masses of the resulting oligonucleotide fragments were analysed by the mass spectrometer. Cleavage sites of *Fok*I and *Bst*F5I, an isoschizomer for *Fok*I, are indicated by black and white triangles, respectively, and restriction endonuclease recognition sites and primers are indicated by shaded bars and shaded arrows, respectively. One-base gaps (C at position 658 and T at position 720) replaced by the artificial sequences are denoted by blank spaces.

Table 2. Expected and observed masses of oligonucleotides resulting from restriction enzyme digestion of PCR products

		Expected fragments (5′–3′)*		Expected mass, Da		Observed mass, Da	
Sequence	Amino acid	7-mer	13-mer	7-mer	13-mer	7-mer	13-mer
Codon 204							
aTg	Met	AGTTATa	TCcAtATAACTGA	2199.4	3997.6	2199.6	3998.0
gTg	Val	AGTTATg	TCcAcATAACTGA	2215.4	3982.6	2215.9	3982.9
aTt	Ile	AGTTATa	TCaAtATAACTGA	2199.4	4021.6	2199.6	4021.8
aTc	Ile	AGTTATa	TCgAtATAACTGA	2199.4	4037.6	2199.6	4037.6
aTa	Ile	AGTTATa	TCtAtATAACTGA	2199.4	4012.6	2199.6	4012.6
Codon 180							
cTg	Leu	TTTCTCc	GCcAgGAGAAACG	2102.4	4097.6	2102.6	4098.0
cTt	Leu	TTTCTCc	GCaAgGAGAAACG	2102.4	4121.6	2102.6	4122.0
tTg	Leu	TTTCTCt	GCcAaGAGAAACG	2117.4	4081.6	2117.8	4081.8
aTg	Met	TTTCTCa	GCcAtGAGAAACG	2126.4	4072.6	2126.6	4073.0

*The first and third bases of codons 204 and 180 are shown in lower case letters.

Figure 2. Samples showing discordant results between RFMP and LiPA



Serum was taken from hepatitis B patients who showed rebound of viral load during lamivudine therapy and was subject to testing for lamivudine-resistant mutations by RFMP and LiPA assays. RFMP detected lamivudine-resistant mutations in all samples, whereas LiPA only showed the mutations in one sample (strip 3). Mass peaks indicating the presence of additional mutant virus in the RFMP results are underlined. The old nomenclature, L528M and M552V/I, was used in the reference LiPA strip instead of rtL180M and rt204V/I. amp., amplification; conj., conjugate; m/z, mass-to-charge ratio; RI, relative peak intensity.

products spanning sequences encoding the YMDD motif. Overall, RFMP had 95.0% agreement with LiPA when only the detection of mutant virus was considered, regardless of viral species.

Ability to detect mixed infection

To compare the ability to distinguish wild-type and mutant DNA in mixed populations, the samples composed of defined ratios of wild-type and mutant sequences (nucleotide 411–886) in plasmids were analysed by both the RFMP and LiPA methods. The plasmids containing the nucleotide sequence of the rtM204I or rtM204V mutants were diluted in known negative HBV DNA serum to make concentrations corresponding to 10^7 and 10^4 HBV genomic equivalents/ml, respectively. The dilution series were made at both DNA loads in a background of wild-type HBV DNA in the following ratios: 25, 10, 4, 1 and 0%. As shown in Figure 3, RFMP could detect 1% mutant virus DNA, in agreement with previous reports [22]. Results were identical at both viral DNA concentrations irrespective of mutant types. In contrast, the LiPA assay could not detect the mutant DNA mixed with a 25-fold greater amount of wild-type DNA species, even though the sample contained 10^7 copies of mutant plasmids. Thus, RFMP is more sensitive than LiPA in identifying a minor population of mutant virus DNA in a background of wild-type virus.

Table 3. Comparison of the results obtained by RFMP and LiPA analyses for 60 clinical specimens

	RFMP			LiPA			
Agreement	Codon 180	Codon 204	Mutation	Codon 180	Codon 204	Mutation	Samples, *n* (%)
Concordant	L	I	mt	L	I	mt	7 (11.7)
	M	I	mt	M	I	mt	2 (3.3)
	L,M	I	mt	L,M	I	mt	2 (3.3)
	L	I,M	mt+wt	L	I,M	mt+wt	2 (3.3)
	M,L	I,M	mt+wt	M,L	I,M	mt+wt	3 (5.0)
	M	V	mt	M	V	mt	7 (11.7)
	M,L	V,M	mt+wt	M,L	V,M	mt+wt	2 (3.3)
	M	I,V	mt	M	I,V	mt	2 (3.3)
	L,M	I,V	mt	L,M	I,V	mt	8 (13.4)
	M	I,V,M	mt+wt	M	I,V,M	mt+wt	2 (3.3)
	L,M	I,V,M	mt+wt	L,M	I,V,M	mt+wt	5 (8.3)
	L	M	wt	L	M	wt	14 (23.3)
Disconcordant	L	M,I	wt+mt	L	M	wt	2 (3.3)
	L,M	M,V	wt+mt	L	M	wt	1 (1.7)
	M,L	M,V,I	wt+mt	M,L	M,V	wt+mt	1 (1.7)
Total							60 (100)

Differing results are underlined. wt, wild-type; mt, mutant type.

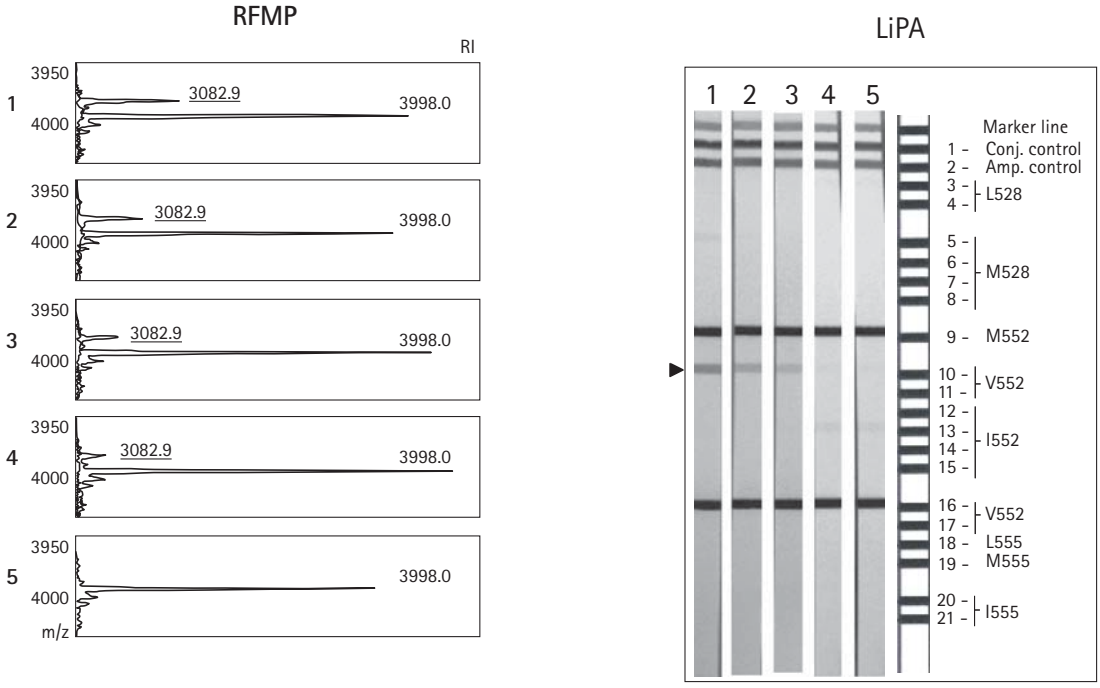
Discussion

Lamivudine is currently considered a primary therapeutic option for patients affected with chronic hepatitis B, irrespective of hepatitis e antigen status. Although the initial response to the therapy is favourable in the majority of patients, long-term response rates are challenged by the selection and build-up of HBV with mutations conferring drug resistance. Particularly for patients at high risk for disease progression, it is important to detect these mutants early and precisely during the emergence of viral 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.

The performance of the RFMP assay for detecting mutations associated with lamivudine resistance was compared with that of LiPA for 60 chronically HBV-infected patients who had shown viral DNA rebound in serum during lamivudine treatment. The results demonstrated a high level of concordance between the two methods. Discordant results observed in four samples could be attributed to the presence of additional virus species detected only by RFMP. This is probably due to the presence of minor viral subspecies that were missed by LiPA, which usually only allows the detection of minor virus mutants if they constitute about 10% of the viral population [25]. The hypothesis was confirmed by clonal analysis of the four samples, which revealed a mixture of virus populations with mutant genotype, and the analysis of a defined dilution series of wild-type and mutant DNA, which indicated a lower detection limit of 1% for RFMP. These results were of particular interest because they indicated that the RFMP assay can detect a mixed wild-type and mutant virus population earlier than LiPA during therapy, and can monitor a disappearing virus population for a longer time period after cessation of lamivudine treatment. Thus, the greater sensitivity of RFMP should be useful for detecting lamivudine-resistant mutants and for monitoring drug resistance as it develops, enabling early intervention and prevention.

At the onset of viral breakthrough, lamivudine-resistant mutations were identified in 46 (76.7%) of the 60 patients (Table 3). In agreement with previous reports [14], rtM204I is commonly found in lamivudine-resistant subjects, being present either alone or together with the rtL180M mutation. The significance of virological breakthrough due to HBV strains without mutations in the YMDD motif remains unclear. Such breakthroughs were observed in 14 of our 60 patients (23.3%). Recently, Papatheodordis *et al.* reported that YMDD variants were absent in two out of 32 patients with virological breakthrough; these individuals remained with fluctuating low viraemia and in biochemical remission during the following few months [26]. It has been reported that approximately 5–20% of patients with HBV breakthrough showed no detectable YMDD mutants; a possible explanation for this observation has been attributed to partial or non-responders to lamivudine, treatment non-compliance or the emergence of other mutations (such as rtA181T)

Figure 3. Comparison of RFMP and LiPA assays for detecting minor level of mutant virus with a defined mixture of rtM204 and rtV204 DNA



RFMP and LiPA results from mixed populations of wild-type rtM204 and mutant plasmids are shown. The wild-type plasmids, equivalent to 10^4 copies/ml of HBV, were mixed with the mutant plasmid to make 25, 10, 4, 1 and 0% of mutant versus wild-type DNA mixtures (strips or spectra numbers 1–5 in order). Only the mass spectra of the 13-mer fragments are shown. Mass peaks and hybridization bands corresponding to rtV204 are indicated by values with an underline and by the arrow, respectively. amp., amplification; conj., conjugate; m/z, mass-to-charge ratio; RI, relative peak intensity.

with clearance of original YMDD mutations during prolonged lamivudine therapy [14,27,28]. In addition, those assays may have been done at relatively early stages of viral breakthrough when mutant viruses represent an insufficient fraction of the total viral population to be detectable, although this is not highly probable considering the sensitivity of the assays adopted. It would be worthwhile investigating genotypes at viral breakthrough on lamivudine therapy, since patients with YMDD variants may not represent cases with typical resistance to lamivudine. Further study of the stratification of viral resistance based on viral genotypes should explain the significance of this phenomenon and provide prognostic markers to determine variance in the clinical outcome of antiviral therapy.

Previous reports suggest that genotypic succession of mutations of the HBV polymerase is associated with early-stage viral acquisition of resistance and occurrence of long-term resistance to lamivudine [29,30]. Moreover, compensatory mutations such as rtL180M, rtT128N, rtW153Q or G1896A, found 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 and give restored or enhanced replication fitness to YMDD variant viruses [13,30–32]. The RFMP genotyping assay is easily adaptable for the detection of other mutations or polymorphisms and can process 384 samples in one assay set-up. Thus, it could be very useful to study possible routes of genotypic change to a more stable and high-level resistance, which might take place dynamically with the selection and accumulation of mutants during long-term lamivudine treatment. When mutations associated with resistance to other antiviral agents are identified, the RFMP assay can easily be applied to detect these new mutations.

Hybridization-based assays such as LiPA, as well as RFMP, appear to be accurate and capable of contributing to the understanding of the occurrence of HBV mutants, but can only be used for the identification of known mutants. A crucial problem is the detection of emerging novel mutants. Recently, Niesters *et al.* and Bozdayi *et al.* reported detection of novel YMDD variants (rtM204S) during lamivudine treatment [15,33]. Since the RFMP assay uses the principle of identifying the genotype by reading the

mass composed of three bases in codon 204, it could detect unidentified variations as well as rtM204I and rtM204V. Though it has the drawback of not differentiating between two genotypes with the first base in common and with the second and third base having a different order (like ATG and AGT), we expect this problem can be resolved by repeating the strategy on the reverse strand, leading to the determination of the identity of the third base and, subsequently, the middle base.

The RFMP assay is able to screen for known viral mutants and to identify and characterize previously unidentified mutants in a sensitive, specific and robust high-throughput manner by combining the merits of its unique assay chemistry and the mature nature of MALDI-TOF MS, which is capable of running 384 samples simultaneously with high speed automatic data acquisition. In terms of cost-effectiveness, the RFMP assay is comparable with the LiPA assay, at approximately US $100 per test for reagents and labour. This cost does not cover the outlay for capital equipment, which is substantially greater for the RFMP method. However, in many specialty or reference laboratories already using mass spectrometers for prenatal metabolite screening or for single nucleotide polymorphism genotyping, the capital cost would be reduced because this equipment can also be used for the RFMP assay.

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