

Identification of Lamivudine-resistant Hepatitis B Virus by Oligonucleotide Microarray

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Abstract

Hepatitis B virus (HBV) is a partially double-stranded DNA virus that infects the human liver, causing cirrhosis and hepatocellular carcinoma. The nucleoside analogue lamivudine is an effective antiviral agent for HBV; however, drug-resistant HBV strains may emerge during prolonged lamivudine treatment. Lamivudine-resistant strains exhibit specific mutations, especially in the B and C domains of the HBV polymerase gene. The ability to detect such mutations is of increasing importance with increased availability of alternative antiviral agents such as penciclovir or famciclovir, which show promise as a treatment for chronic HBV infections. We have developed a rapid and accurate HBV drug-resistant oligonucleotide chip for the detection of specific mutations in the HBV polymerase gene. Synthesized oligonucleotide probes of 25-32 bases in length with modifications at the 5' end were spotted in duplicate and covalently linked onto a glass slide. The oligonucleotide probes were hybridized with fluorescence-labeled complementary HBV DNA target molecules amplified from 40 samples that were confirmed to have single mutations by sequencing. This study shows that the results of the HBV drug-resistance oligonucleotide chip were identical to those of sequencing and reverse hybridization. The HBV drug-resistance oligonucleotide chip is very simple to use and provides an alternative method for the detection of HBV drug-resistance mutants.

Keywords: Hepatitis B virus, HBV, Lamivudine, Drug resistance, Microarray, Oligonucleotide DNA chip

Introduction

Hepatitis B virus (HBV) is one of the major causes of liver disease worldwide; chronic infection may lead to cirrhosis and hepatocellular carcinoma. It is therefore of great interest to have potent antiviral therapies against chronic HBV (CHB) available^{1,2}. The development of nucleotide analogs that inhibit HBV reverse transcriptase activity, including lamivudine, famciclovir, and adfovir, has provided an alternative to interferon therapy for CHB³. The HBV reverse transcriptase polymerase contains 5 conserved regions: A, B, C, D and E. Region B is known to be associated with attachment to the RNA template that is undergoing reverse transcription to DNA, while Region C comprises the catalytic regions of the transcriptase. Regions B and C represent the most common areas of emergence of resistant viral variants during lamivudine therapy⁴.

Lamivudine, (-)- β -L-2',3'-dideoxy-3'-thiacytidine (3TC), is a cytosine analogue with potent antiviral activity against HBV⁵. The main mechanism of action of lamivudine is the effective inhibition of HBV polymerase activity and the cessation of HBV replication, resulting in reduced inflammatory activity in CHB patients⁶. Lamivudine is rapidly absorbed with good bioavailability, and is excreted in urine. It is generally considered a safe drug with rare and usually minor side effects^{7,8}. Nevertheless, during prolonged lamivudine therapy, random mutations in the HBV genome may arise resulting in the emergence of lamivudine-resistant viral mutants^{9,10}. Point mutations related with lamivudine resistance occur within the P open leading frame (ORF) of the HBV genome and affect domains B and C of the HBV reverse transcriptase (RT). This, in turn, leads to various amino acid substitutions (Figure 1)¹¹. Amino acid substitutions at position 528 and 552 of HBV DNA polymerase have been associated with lamivudine resistance, while an amino acid transition at position 555 has been associated with resistance to famciclovir¹². The first resistant mutations emerging under lamivudine therapy were detected within the YMDD motif (tyrosine-methionine-aspartate-aspartate), which is within the major catalytic region C of HBV polymerase and is well conserved in all reverse transcriptases. In particular, the most common nucleotide substitution in-

duces exchange of methionine (M) at position 552 with valine (V) or isoleucine (I) (M552V or M552I, respectively)¹³⁻¹⁶. The second most common resis-

Table 1. Probes used on the HBV drug resistance oligonucleotide chip for the detection of drug resistant HBV.

No.	Name	Sequence
(1)	28W1	5'-C6-aminolink-T10-ccgtttctcctggetcagt
(2)	28W2	5'-C6-aminolink-T10-ccgtttctcctggcgacagt
(3)	28M3	5'-C6-aminolink-T10-ccgtttctcatggcgacagt
(4)	28M4	5'-C6-aminolink-T10-ccgtttctcatggcgacagt
(5)	52W1	5'-C6-aminolink-T10-ggctttcagttatggatcatgtgga
(6)	52W2	5'-C6-aminolink-T10-ggctttcagttatggatcatgtggt
(7)	52W3	5'-C6-aminolink-T10-gctttcagtttaggggatgatgtgg
(8)	52M4	5'-C6-aminolink-T10-gctttcagtttaggggatgatgtgg
(9)	52M5	5'-C6-aminolink-T10-gctttcagtttaggggatgatgtgg
(10)	52M6	5'-C6-aminolink-T10-ctttcagtttaggggatgatgtgg
(11)	52M7	5'-C6-aminolink-T10-ggctttcagtttagccatgatgtggtattg
(12)	52M8	5'-C6-aminolink-T10-ggctttcagtttagatgatctggtattg
(13)	52M9	5'-C6-aminolink-T10-gctttcagtttagatgatctggtattg
(14)	52M10	5'-C6-aminolink-T10-ggctttcagtttagatgatctggtattg
(15)	52M11	5'-C6-aminolink-T10-gctttcagtttagatgatctggtattg
(16)	52M12	5'-C6-aminolink-T10-gctttcagtttagatgatctggtattg
(17)	Positive control	5'-C6-aminolink-T10-tcgtagggccttccccactgtctg
(18)	Negative control	5'-C6-aminolink-T10-tcgtagggcctgtacacgcgtctctg

Abbreviations: c, cytosine; g, guanine; t, thymine; a, adenine

tance mutation is the substitution of leucine (L) with M at position 528 of region B (L528M)¹⁷. During lamivudine therapy, it is important to detect the emergence of drug-resistant HBV as early as possible, thus assays for the detection of drug-resistant HBV must provide rapid and accurate diagnosis. Several techniques are available for the detection of variants, and each has advantages and disadvantages¹⁸. Nucleotide sequence analysis is a useful technique for mutation detection in nucleotide sequences and has the advantage that it is able to directly demonstrate the emergence of lamivudine-resistant HBV mutations. However, it has the disadvantage that it lacks sensitivity, is labor-intensive and cannot detect mixtures of variant and wild type virus^{19,20}. Specific mutation analysis techniques, such as PCR-restriction fragment length polymorphism analysis (RFLP) and reverse hybridization (INNO-LiPA HBV-DR), overcome some of the limitations of DNA sequencing and are more sensitive. However, these techniques are time-consuming and laborious²¹.

Oligonucleotide microarray technology has been reported as a useful tool for the easy, rapid and accurate detection of single nucleotide polymorphisms or point mutations for molecular diagnostics²²⁻²⁶.

In this study, an accurate oligonucleotide chip-bas-

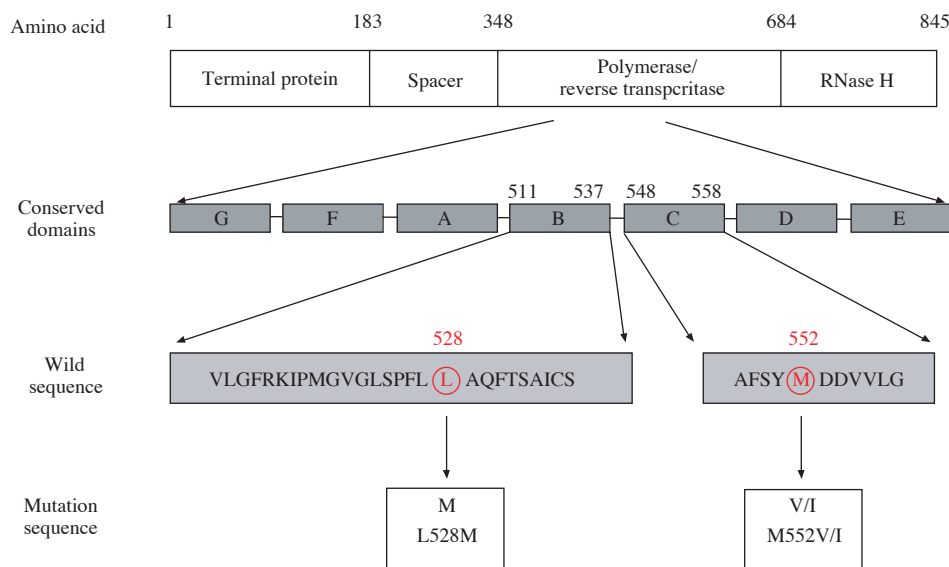


Figure 1. Schematic presentation of amino acid transitions (L528M and M552V/I) in the HBV DNA polymerase conferring resistance to lamivudine. Such transitions arise from point mutations within the B or C conserved domains of the reverse transcriptase region of the P gene. The most common mutations are the substitution of methionine (M) with valine (V) or isoleucine (I) at position 552 of domain C (M552V or M-552I, respectively) and/or substitution of leucine (L) with M at position 528 of domain B (L528M).

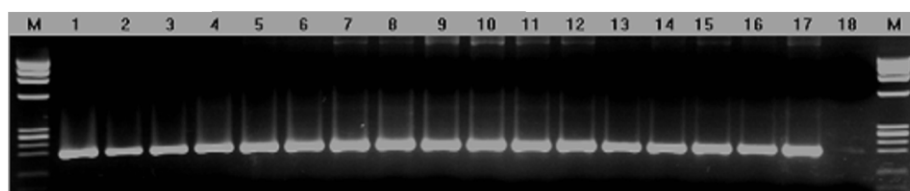


Figure 2. Amplified HBV DNA fragments separated by electrophoresis on a 2% agarose gel. The size of the specific HBV DNA band is 190 bp (arrow). M indicates phiX 174/HaeIII marker.

ed diagnostic assay was developed to detect lamivudine-resistant HBV mutations at codons 528 and 552. Experiments were also conducted to test whether it is possible to identify mutant strains in DNA extracted from patient serum using this technique. In addition,

the accuracy of oligonucleotide microarray results compared to those obtained by line probe assay (INNO-LiPA) and nucleotide sequence analysis were confirmed.

Results

Test of Probe Specificity

For analysis of amino acid 528 of the HBV polymerase gene, clones were constructed using plasmids containing either wild type strain (L528L) DNA with a CTG sequence at codon 528, or a point mutant (L528M) with an ATG sequence at codon 528. For analysis of amino acid 552, clones were constructed from wild type strain (M552M) with an ATG sequence at codon 552, or mutants (M552V, M552I, and M552I) with GTG, ATT and ATC respectively at codon 552 (Figure 1). These clones were used to determine the specificity of each probe in detecting wild type or mutant HBV. Plasmids were amplified by HBV DR nested PCR, giving a 190 bp PCR product that was confirmed by electrophoresis with 2% agarose gels (Figure 2). The amplified DNA was hybridized to the specific probes on oligonucleotide chips

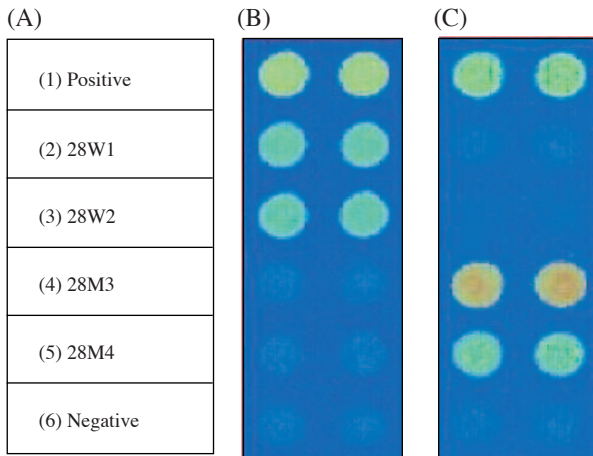


Figure 3. Probe specificity at codon 528. (A) The spot presentation of 528 probes. (B) Case for wild type such as CTG sequence. (C) Case for mutant type such as ATG sequence.

(1) Positive	(1) Positive	(1) Positive	(1) Positive
(7) 52W1	(10) 52M4	(13) 52M7	(16) 52M10
(8) 52W2	(11) 52M5	(14) 52M8	(17) 52M11
(9) 52W3	(12) 52M6	(15) 52M9	(18) 52M12

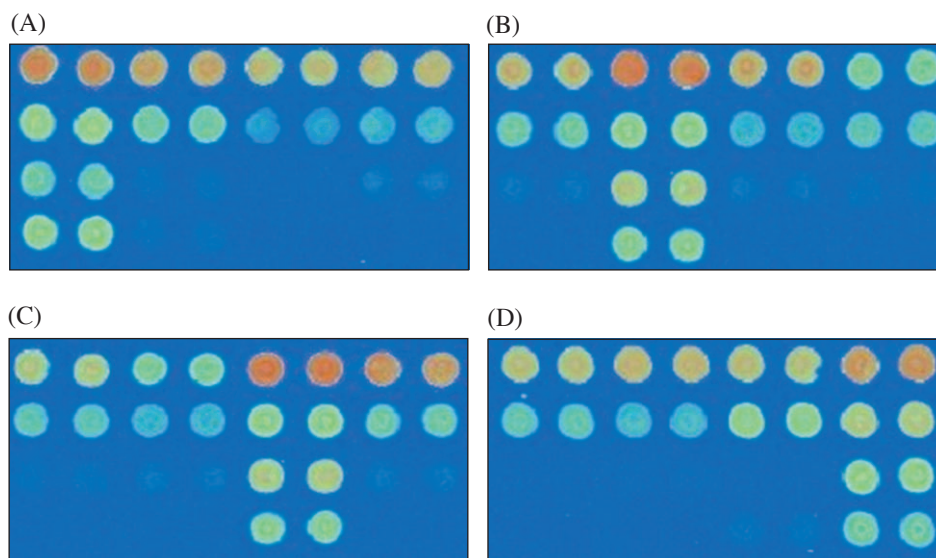


Figure 4. Probe specificity at codon 552. (A) Case for wild type such as ATG sequence. (B) Case for mutant types such as GTG sequence, (C) ATT sequence, (D) ATC sequence.

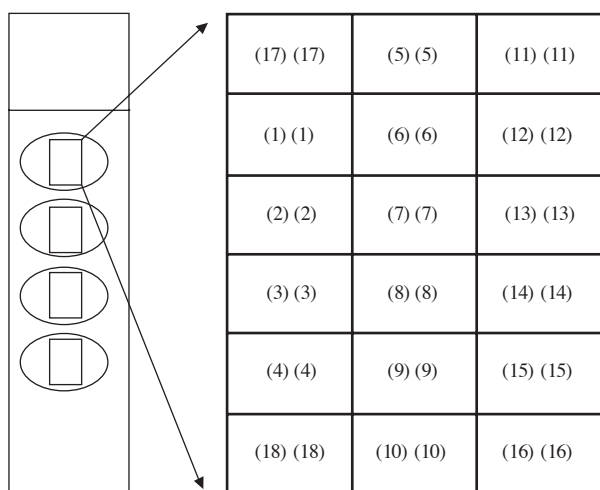


Figure 5. Schematic presentation showing the oligonucleotide probe layout for the sequences shown in Table 1. The oligonucleotide probes were printed onto slides as duplicate arrays. The positive control probe is a part of a highly conserved region in HBV DNA polymerase. The negative control probe has 7 random nucleotide substitutions compared with the positive control probe. PC, positive control probe; NC, negative control probe. The circled numbers indicate probe numbers.

under described conditions in method and the results were obtained as images and as fluorescent signal intensities of each spot.

First, to test the specific probes for codon 528, namely 28W1, 28W2, 28M3 and 28M4, the probes were printed onto a glass slide as shown in Figure 3 (A). The amplified products from template plasmid were hybridized with the probes onto the microarray. In the case of L528L wild types, fluorescent spots were detected in the 28W1 and 28W2 probes, as shown in Figure 3(B). L528M mutants containing ATG sequences appeared as fluorescent spots in the 28M3 or 28M4 probes, confirming that PCR products from L528M mutants only reacted with the specific probes (Figure 3(C)).

Second, the specificities of the probes designed for position 552 (52W1, 52W2, 52W3, 52M4, 52M5, 52M6, 52M7, 52M8, 52M9, 52M10, 52M11, and 52M12) were confirmed. When the spots resulting from the wild type 52W1, 52W2 and 52W3 probes had high fluorescent signal intensity, the other probes did not give a fluorescent signal (Figure 4(A)). It was concluded that the designed probes for the HBV DR oligonucleotide chip are very specific, because non-specific reaction or cross-reactivity between wild type probes and mutant type probes could not be detected (Figure 4(B), (C), (D)).

PC	52W1	52M4
28W1	52W2	52M5
28W2	52W3	52M6
28M3	52M1	52M7
28M4	52M2	52M8
NC	52M3	52M9

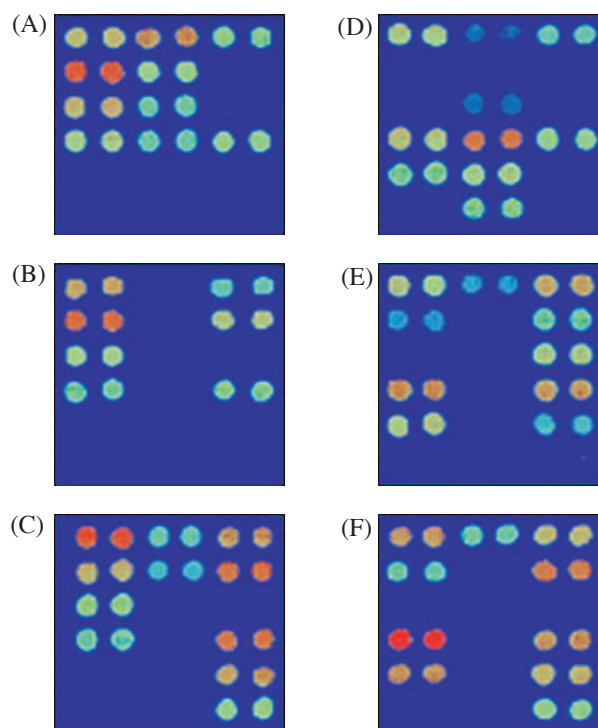


Figure 6. HBV DR oligonucleotide chip analysis of HBV clinical samples. (A) Wild type at both codons 528 and 552; (B) and (C) wild type at codon 528 and mutant type at codon 552; (D), (E), and (F) mutant type at both codons 528 and 552.

Distinction of Wild Type or Mutant Type HBV Using an Oligonucleotide Chip

To evaluate the efficiency of the HBV DR oligonucleotide chip, 42 clinical samples were tested and the results of the microarray assay were used to determine whether the samples were drug resistant. HBV DNA was extracted and amplified from patient serum according to the above described protocol, and the amplified target was hybridized with spotted probes on a HBV DR oligonucleotide chip.

The clinical samples were clearly divided into 3 classes: wild type at codons 528 and 552 (Figure 6 (A)); wild type at codon 528 and mutant type at cod-

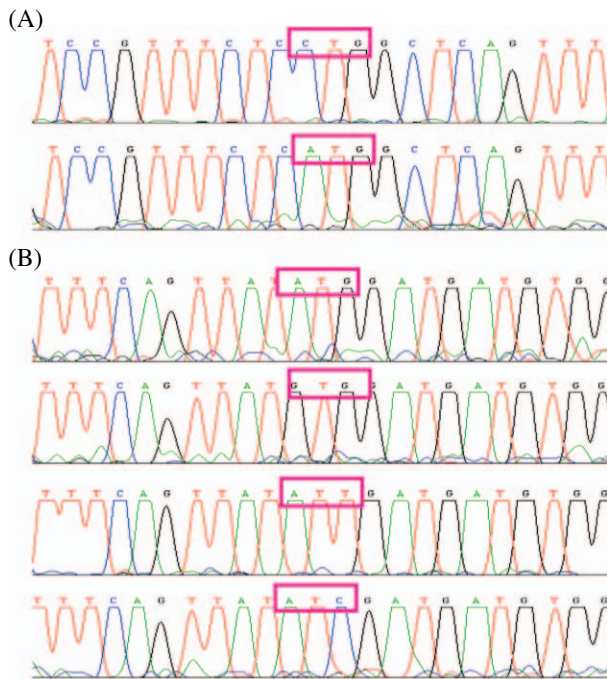


Figure 7. Nucleotide sequence analysis of part of the DNA polymerase gene from HBV clinical samples. The nucleotide sequences of region 528 (A) and region 552 (B) are shown inside the frame box.

on 552 (Figure 6(B), (C)); and mutant type at codons 528 and 552 (Figure 6(D), (E), (F)).

Finally, it was confirmed that the assay performed correctly with positive and negative control probes. Thus, the positive control probe reacted with all of the samples, while the negative control probe did not react with any of the samples (Figure 6).

Detection of HBV Mutation for Drug Resistance by INNO-LiPA and Sequencing

To confirm the results obtained by the HBV DR oligonucleotide chip assay, two molecular assays, sequence analysis and INNO-LiPA analysis were performed to detect lamivudine-resistant HBV. The results of sequence analysis at codons 528 and 552 are shown in Figure 7(A) and (B), respectively. Each sample had the expected triplet CTG or ATG in position 528, and ATG, GTG, ATT, or ATC in position 552. In addition, it was confirmed that lamivudine resistance mutations were found in clinical samples using the INNO-LiPA assay (Figure 8). Finally, for each HBV clinical sample, results of sequence analysis, INNO-LiPA analysis and HBV DR oligonucleotide chip analysis were compared (Table 2). There was complete concordance between the results of the three techniques for all clinical samples tested.

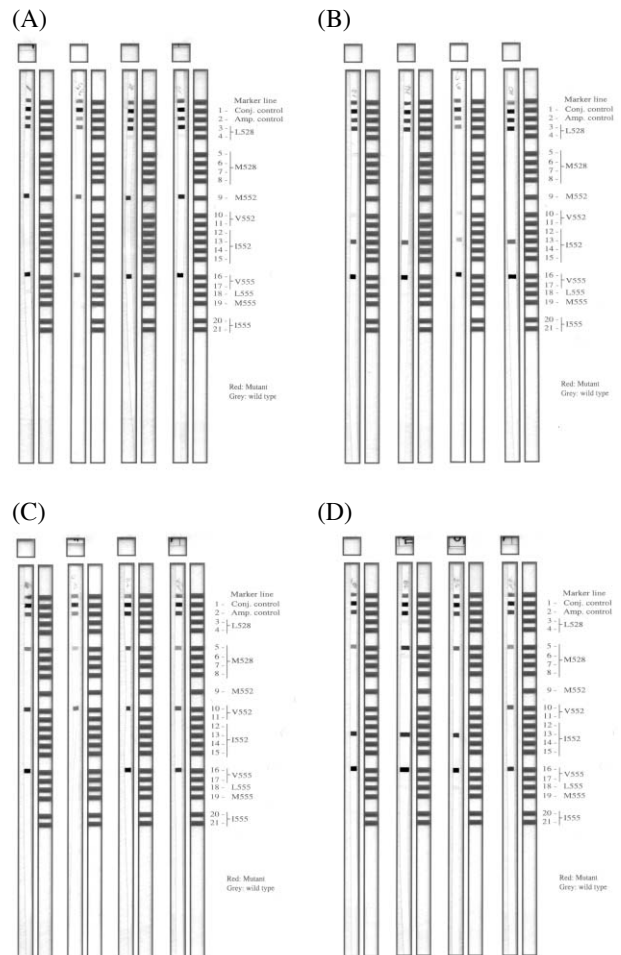


Figure 8. INNO-LiPA HBV DR blots. (A) Wild type at both codons 528 and 552; (B) wild type at codon 528 and mutant type at codon 552; (C) mutant type at codon 528 and wild type at codon 552; (D) mutant type at both codons 528 and 552.

In the HBV DR oligonucleotide chip analysis reported herein, point mutations associated with drug resistance were detected in 32 patients, and wild type sequences were detected in 8 patients. These results agree with those of direct sequence analysis and INNO-LiPA (Table 2).

Discussion and Conclusion

HBV resistance to lamivudine is a frequent and significant therapeutic problem in patients in prolonged lamivudine therapy. Emergence of mutant HBV has been associated with reduced susceptibility to lamivudine²⁷. To obtain accurate information on the emergence of these mutants, novel reliable assays are re-

Table 2. Comparison of the results of sequence analysis, INNO-LiPA assay and HBV DR oligonucleotide chip assay for clinical samples.

Sample ID	Sequence analysis		INNO-LiPA assay		HBV DR Oligonucleotide chip	
	Codon 528	Codon 552	Codon 528	Codon 552	Codon 528	Codon 552
HBV-001	ATG	GTG	Met	Val	ATG	GTG
HBV-002	ATG	ATT	Met	Ile	ATG	ATT
HBV-003	ATG	ATT	Met	Ile	ATG	ATT
HBV-004	CTG	ATT	Leu	Ile	CTG	ATT
HBV-005	CTG	ATG	Leu	Met	CTG	ATG
HBV-006	ATG	ATC	Met	Ile	ATG	ATC
HBV-007	ATG	ATT	Met	Ile	ATG	ATT
HBV-008	ATG	ATT	Met	Ile	ATG	ATT
HBV-009	ATG	ATT	Met	Ile	ATG	ATT
HBV-010	CTG	ATT	Leu	Ile	CTG	ATT
HBV-011	ATG	ATC	Met	Ile	ATG	ATC
HBV-012	ATG	GTG	Met	Val	ATG	GTG
HBV-013	CTG	ATT	Leu	Ile	CTG	ATT
HBV-014	ATG	ATT	Met	Ile	ATG	ATT
HBV-015	ATG	GTG	Met	Val	ATG	GTG
HBV-016	ATG	GTG	Met	Val	ATG	GTG
HBV-017	CTG	ATG	Leu	Met	CTG	ATG
HBV-018	CTG	ATG	Leu	Met	CTG	ATG
HBV-019	ATG	ATT	Met	Ile	ATG	ATT
HBV-020	CTG	ATG	Leu	Met	CTG	ATG
HBV-021	CTG	ATC	Leu	Ile	CTG	ATC
HBV-022	CTG	ATG	Leu	Met	CTG	ATG
HBV-023	ATG	ATT	Met	Ile	ATG	ATT
HBV-024	CTG	ATG	Leu	Met	CTG	ATG
HBV-025	ATG	ATC	Met	Ile	ATG	ATC
HBV-026	ATG	ATT	Met	Ile	ATG	ATT
HBV-027	CTG	ATG	Leu	Met	CTG	ATG
HBV-028	ATG	GTG	Met	Val	ATG	GTG
HBV-029	CTG	ATT	Leu	Ile	CTG	ATT
HBV-030	ATG	GTG	Met	Val	ATG	GTG
HBV-031	ATG	GTG	Met	Val	ATG	GTG
HBV-032	CTG	ATT	Leu	Ile	CTG	ATT
HBV-033	ATG	ATT	Met	Ile	ATG	ATT
HBV-034	CTG	ATG	Leu	Met	CTG	ATG
HBV-035	ATG	GTG	Met	Val	ATG	GTG
HBV-036	ATG	ATT	Met	Ile	ATG	ATT
HBV-037	ATG	GTG	Met	Val	ATG	GTG
HBV-038	CTG	ATG	Leu	Met	CTG	ATG
HBV-039	CTG	ATC	Leu	Ile	CTG	ATC
HBV-040	ATG	GTG	Met	Val	ATG	GTG

Abbreviations: C, cytosine; G, guanine; T, thymine; A, adenine; Met, Methionine; Leu, Leucine; Val, Valine; Ile, Isoleucine.

quired which enable detection of small amounts of mutant HBV in the presence of wild type HBV. In general, viral nucleotide sequences are very diverse, but sequences around the YMDD motif in the HBV genome are highly conserved^{28,29}. Therefore, this region was used in the development of an oligonucleotide chip assay for the detection of resistance to lamivudine.

Using this assay, mutations in codons 528 and 552 of HBV DNA polymerase were specifically detected.

Using specific primer pairs to amplify the B and C domains of HBV polymerase, HBV DNA extracted from patient serum was amplified, and the nested PCR products were used as templates for hybridization with probe on an oligonucleotide chip. Positive samples were amplified sufficiently through the nested PCR process, and the 190 bp products could be detected on agarose gels.

The chip included 4 probes for region 528 and 12 probes for region 552. Both 28W1 and 28W2 probes

reacted only with samples that contained wild type nucleotide sequence (CTG) in region 528. The mutant sequence contained the ATG nucleotide sequence in region 528 and reacted with both 28M3 and 28M4 probes. Using the HBV DR oligonucleotide chip, clear results were obtained demonstrating that the HBV sequence was a mutant at position 528, thus indicating resistance to lamivudine. In addition, the specificity of 52W1, 52W2, 52W3, 52M4, 52M5, 52M6, 52M7, 52M8, 52M9, 52M10, 52M11, and 52M12 probes were tested, and accurately detected mutant 552 sequences. For example, when the test sample had a copy of M552M, a fluorescent signal was detected in 52W1, 52W2, and 52W3 probes on the microarray. It was also demonstrated that samples with a copy of M552V or M552I only reacted with 52M4, 52M5 and 52M6 probes, or 52M7, 52M8, 52M9, 52M10, 52M11, and 52M12 probes, respectively. By means of these results, the specificity of the probes printed onto the HBV DR oligonucleotide chip was confirmed.

Moreover, direct sequence analysis and reverse hybridization (INNO-LiPA assay) were used as additional techniques for mutation detection, and the results from the HBV DR oligonucleotide chip were compared with these specific mutation detection methods. In this way it was confirmed that the HBV DR oligonucleotide chip results were in agreement with the results from direct sequence analysis and INNO-LiPA assay.

In conclusion, the newly developed HBV DR oligonucleotide chip technique is less time-consuming, is easier to use in a routine setting, and is a reliable assay for the detection of HBV variants resistant to lamivudine. This assay, therefore, may be an effective method for monitoring patients with prolonged lamivudine therapy or for screening patients for lamivudine resistance before starting lamivudine therapy.

Materials and Methods

Extraction of HBV DNA

HBV DNA was extracted from 200 μ L of patient serum with a QIAamp DNA blood Mini kit (Qiagen Inc., Valencia, Calif.) according to the protocol described by the manufacturer. DNA was stored at -20°C prior to use in nested polymerase chain reaction (nested PCR).

Target DNA Preparation

The HBV DNA polymerase gene was amplified by nested PCR using the outer forward primer (5'-AAA ATT CCT ATG GGA GTG GG-3'), the outer reverse

primer (5'-CCA (A/G)AG ACA AAA (G/T)AA AAT TGG TAA-3'), the inner forward primer (5'-ACCTG TATTCCCATCCCATC-3'), and the inner reverse primer (5'-Biotin-CAAAGACAA AAGAAAATTGG -3'). PCR conditions were as follows: initial denaturation at 94°C for 5 min, 40 amplification cycles with denaturation at 94°C for 1 min, primer annealing at 45°C for 1 min 30 sec, extension at 72°C for 1 min, followed by final extension at 72°C for 5 min. The second PCR was performed under the same conditions used for the first PCR.

Design of Target-specific Probes, Positive Control Probe, and Negative Control Probe

A total of 18 target-specific probes, whose sequences were based on the HBV polymerase gene sequence, were designed to detect lamivudine-resistant HBV (Table 1). The primers and probes used in this study were manufactured by Metabion (Germany).

Oligonucleotide Microarray Design and Fabrication

The probes were diluted to 50 pmol/L with spotting buffer (3X SSC and 3 M betaine). Probes were printed in duplex spots as shown in Figure 5, and were adhered to the silylated glass slide (Cel Associates, Texas, U.S.A.) using the microarrayer (QArray mini; Genetix Ltd, UK). The oligonucleotide chips were stored in a desiccator until use.

Hybridization, Signal Detection, and Analysis

5 μ L of nested PCR product was mixed with 90 μ L of prewarmed hybridization buffer I (3X SSC and 0.25% SDS) and 5 μ L of hybridization buffer II (Cy3-conjugated streptavidin diluted to 0.02X in 1X PBS and 15 mM NaN_3 buffer), and the oligonucleotide chip was incubated at 68°C for 60 min. After hybridization, the slide was washed once with distilled water for 2 min then dried. Oligonucleotide chip images were obtained with a scanner (GenPix 4000B; Axon Instruments, Inc., Union City, Calif.) with a 532 nm laser for excitation of Cy3.

Line Probe HBV Drug Resistance (INNO-LiPA HBV DR) Assay and Sequence Analysis

The INNO-LiPA HBV DR assay (Innogenetics, Gent, Belgium) was performed as recommended by Innogenetics.

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