Oligonucleotide Chip for Detection of Lamivudine-Resistant Hepatitis B Virus

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Hepatitis B virus (HBV) is one of the major causes of liver disease worldwide. It is important to conduct antiviral therapy against chronic hepatitis B to minimize the amount of liver damage. Lamivudine has been known to be an effective antiviral agent for the treatment of HBV infection. However, the emergence of viral mutants resistant to lamivudine is the main concern during the treatment of HBV-infected patients. Therefore, the detection of lamivudine-resistant mutants is of clinical importance. We have developed an oligonucleotide chip for the detection of lamivudine-resistant HBV which is rapid and accurate. The oligonucleotide chip consists of quality control probes, negative control probes, and specific oligonucleotide probes for the detection of lamivudine-resistant HBV. The specific probes consist of five probes for the detection of wild-type rtL180, rtM204, and rtV207 sequences and seven probes for the detection of HBV mutations. We tested 123 serum samples from patients with chronic HBV infection who had received lamivudine therapy. Eighty samples contained mutants with YMDD mutations. Among these, 17 contained rtM204V (YIDD), 24 contained rtM204I3 (YIDD3), 3 contained rtM204I2 (YIDD2), and 36 contained mixed types. We compared the results obtained with our oligonucleotide chip with those obtained by PCR-restriction fragment length polymorphism (RFLP) analysis and sequencing. The rate of concordance between the assay with the oligonucleotide chip and PCR-RFLP analysis for detection of the YMDD motif was 96.7%. The rate of concordance between the results obtained with the oligonucleotide chip for the detection of rtL180 and rtV207 and the results obtained by sequencing was 100%. Thus, the oligonucleotide chip is a reliable and useful tool for the detection of antiviral-resistant HBV.

Hepatitis B virus (HBV) is one of the major causes of liver disease worldwide, and chronic hepatitis B (CHB) can progress to cirrhosis and hepatocellular carcinoma. It is important to conduct antiviral therapy against CHB to minimize the amount of liver damage (15). The development of nucleotide analogs which inhibit HBV reverse transcriptase activity, such as lamivudine, famciclovir, and others, has provided an alternative to which overcome some of the limitations of DNA sequencing, and it is easy to perform tests for a multitude of mutations and polymorphisms simultaneously. Other molecular genetic techniques, such as PCR-restriction fragment length polymorphism (RFLP) analysis and peptide nucleic acid-mediated PCR clamping, which overcome some of the limitations of DNA sequencing, are available (8), but they are also time-consuming and laborious.

Oligonucleotide chips have been reported to be useful tools for molecular diagnostics. They are widely used for genotyping and the detection of single-nucleotide polymorphisms and mutations (3, 4, 6, 14). The oligonucleotide chip-based method is less time-consuming and is very sensitive for the detection of point mutations, and it is easy to perform tests for a multitude of mutations and polymorphisms simultaneously.

In the study described here, we established a rapid and accurate method for the detection of lamivudine-resistant mutations in HBV on the basis of assays with the oligonucleotide chip. In addition, the oligonucleotide chip included negative control (NC) probes as well as quality control (QC) probes for evaluating the quality of oligonucleotide chip fabrication. Fi-
tion mixture contained 1 μl of Taq DNA polymerase (Qiagen Inc.) and 1 μl of the QC probe to amplify the HBV polymerase gene. Briefly, the 25-mer biotin-CAAAAGAAAATTGGTAAC and primer bio-HBR2 [5'-TTTCGC-3'] were designed so that the base sequence of one point mutation was located in the 5'-end of each control probe. The 3'-end of a QC probe was labeled with a fluorescent dye (6-carboxytetramethylrhodamine [TAMRA]).

### MATERIALS AND METHODS

**Patients and extraction of HBV DNA.** Sera from 123 patients with CHB who had received lamivudine therapy were tested in this study. The mean duration of lamivudine therapy for the patients infected with isolates with phenotypic resistance was 16.7 months (standard deviation, 6.7 months). The viral titers in the sera from 123 patients with CHB who had received lamivudine therapy for the patients infected with isolates with phenotypic resistance were quantified by a plaque assay under the same conditions used for the other serologic tests.

**Codon Probe no. Probe name Sequence Tm (°C) G+C content (%)**

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<th>Codon</th>
<th>Probe no.</th>
<th>Probe name</th>
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* Abbreviations: M, mutant type; N, negative control probes; FD, TAMRA fluorescent dye; Tm, melting temperature.

**Design of target-specific probes, NC probes, and QC probes.** A total of 12 target-specific probes, whose sequences were based on the HBV polymerase gene sequence, were designed to detect lamivudine-resistant HBV. The probes were designed so that the base sequence of one point mutation was located in the center of a 15- or 17-mer probe (Table 1).

**Control probes (NC and QC probes) were used in this study.** We designed the control probes by the use of substitutions, insertions, and deletions with one- or two-base mismatches compared to the sequences of the target-specific probes (Table 1). In addition, the QC probes comprised poly(T) and were a 30-mer or a 35-mer (total length). The 5'-end of each control probe was aminated. The 3'-end of each control probe was aminated. The 3'-end of each control probe was aminated. The 3'-end of each control probe was aminated. The 3'-end of each control probe was aminated.

**Hybridization and scanning.** Biotin-labeled PCR products were denatured for 5 min at 95°C and then chilled on ice for 5 min. Two microliters of single-stranded biotin-labeled target DNA was mixed with 8 μl of hybridization buffer containing 1× SSC, 3 M formamide, salmon sperm DNA, bovine serum albumin, dH₂O, and cyanine 5 (Cy5)-streptavidin (Amersham Pharmacia Biotech, UK Limited, Little Chalfont, Buckinghamshire, England). The oligonucleotide chip was incubated for 30 min at 40°C. After hybridization, the slides were washed once with 50% formamide, 2× SSC and then once for 5 min with 0.2× SSC. The oligonucleotide chip images were obtained with a nonconfocal fluorescent scanner (GenePix 4000A; Axon Instruments, Inc., Union City, Calif.) with green and red lasers (532 nm for excitation of TAMRA and 635 nm for excitation of Cy5). As shown in Fig. 3, the fluorescent images obtained with the QC probe were obtained at 532 nm, and the images obtained with the specific probes were obtained at 635 nm.

**Mutant construction by site-directed mutagenesis.** To confirm the specificity of the probe, four clones of the wild type and seven clones of mutant types were made by site-directed mutagenesis. The primers used for mutagenesis are presented in Table 2. Site-directed mutagenesis comprised three PCR steps. For the mutagenesis of clone MC204I, the first PCR step was performed with primers BF111 and BR109. Primers BF111 and BR109 were described earlier (2). The second PCR step was performed with two tubes. One tube contained primer BF111 and primer rtV204-Rmg, and the other tube contained primer rtV204-Fmg and primer BR109. Primers rtV204-Fmg and rtV204-Rmg were designed to introduce one point mutation at the center of a 25-mer primer in each reference sequence, and the sequences of both sides were perfectly matched. Then, the products of the second PCR were purified by electrophoresis in a 2% agarose gel.

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and extracted with a QIAquick gel extraction kit (Qiagen Inc.). Finally, the third PCR with primers BF111 and BR109 in one tube amplified two DNA samples extracted from gels. One microliter of each DNA sample was added to the third PCR mixture. The third PCR-amplified DNA fragments were extracted with a gel extraction kit and cloned in vector pGEM-T (Promega, Madison, Wis.). We analyzed the clone to confirm the mutagenesis of its sequences. All types of clones were made by the same method used for clone MC204V.

**PC!R-RFLP analysis and sequencing.** We confirmed the results obtained with the oligonucleotide chip by comparing them with those obtained by PCR-RFLP analysis and PCR-direct sequencing. The PCR-RFLP assay was described earlier (2). For the sequencing of the partial sequence of an HBV DNA polymerase gene, 290-bp PCR products were generated by nested PCR. The nested PCR was performed with outer primers (primers BF105 and BR112) and inner primers (primers BF111 and BR109). Sequencing was performed two times with primers BF111 (forward) and BR109 (reverse). The sequence was determined with an ABI Prism BigDye Terminator Cycle Sequencing Ready reaction kit (PE Applied Biosystems, Inc., Foster City, Calif.) in an ABI Prism 3700 genetic analyzer (PE Applied Biosystems, Inc.).

**RESULTS**

Test of probe specificity by mutagenesis. The clones obtained by mutagenesis (Table 2) were used to confirm the specificities of the probes specific for codon positions rt180, rt204, and rt207. As shown in Fig. 1, clones MC204M (Fig. 1A), MC204V (Fig. 1B), MC204I1 (Fig. 1C), MC204I2 (Fig. 1D), and MC204I3 (Fig. 1E) hybridized to the specific probes at the expected locations (rtM204, rtM204V, rtM204I1, rtM204I2, and rtM204I3, respectively). Also, the probes specific for codons rt180 and rt207 were detected by using other specific probes (data not shown).

**Design of control probes and specific probes.** First, a QC probe was used to evaluate the quality of the oligonucleotide chip that was fabricated and the immobilized probe. The probes chosen for each codon are shown in Table 1. Second, NC probes were used to measure the background by nonspecific binding or cross hybridization and to determine a cutoff value. In Table 1, N1 and N2 indicate NC probes specific for codons 180 and the rtM204V mutation (YVDD) at codon 204, respectively. They were designed by using one-base substitutions specific for codon 207, and it was designed by using a one-base insertion at the position immediately adjacent to the target probe-specific sequences. N4 indicates an NC probe specific for codon 207, and it was designed by using two base substitutions specific for the mismatched two-base sequence of a target probe sequence. Probes for the detection of lamivudine-resistant HBV were designed so that the base
sequence with one point mutation was located in the center of a 15- or a 17-mer mutation-specific probe. Finally, positive control probes were prepared by mixing all of the probes. The positive control probes were used to check the amplified DNA according to the PCR and hybridization conditions.

**Detection of mixed types with NC probes.** We used NC probes to simultaneously detect mixtures of the wild type and one or more mutants in the serum samples. A schematic showing the layouts of the oligonucleotide probes is shown in Fig. 2. Figure 3 shows the signal intensities for the individual hybridization spots and positive spots on the basis of the signal intensities for the NC spots. Figure 3A1 and A2 shows the results of the analysis for mixed infections with clones MC204M and MC204V and spots positive for rtL180, rtM1204, rtM204V, rtM2204, and rtV207. The results for clones MC204M and MC204V showed that the signal intensities for each target-specific probe were 856 (probe rtM1204 [probe 5]) and 256 (probe rtM204V [probe 7]) and that the signal intensity for the NC probe was 10 (probe N2 [probe 6]). Therefore, by comparing the signal intensities of the positive spots with those of the spots obtained with an NC probe, we could confirm that clones MC204M and MC204V had mixed types of mutations. For codon 180, the positive spot could not be detected by scan imaging but could be detected by comparison of the signals obtained with specific probes and that obtained with an NC probe; the signal intensities were 88 (probe rtL180 [probe 2]), 5 (probe rtL180M [probe 4]), and 14 (probe N1 [probe 3]). As shown in Fig. 3B1 and B2, the spots showed positive results for clones rtL180, rtL180M, rtM204, rtM204V, rtM204I2, and rtV207. The results for the clones with mixed mutations, clones MC204M, MC204V, and MC204I2, showed signal intensities of 52 (probe rtL180 [probe 5]), 58 (probe rtM204V [probe 7]), and 206 (probe rtM204I2 [probe 11]) with the specific probes and 2 (probe N2 [probe 6]) and 38 (probe N3 [probe 9]) with the NC probes. Therefore, by comparing the signal intensity of a positive spot with that of an NC probe, we could confirm that clones MC204M, MC204V, and MC204I2 had mixed types of mutations.

**Sensitivity and limit of detection of minority populations of mixed-type HBV with the oligonucleotide chip.** To evaluate the sensitivity of the assay with the oligonucleotide chip, serial dilutions (10^2 to 10^6 copies of HBV/ml) of serum samples were prepared with sera with known viral titers, as determined by the Cobas AMPLICOR HBV Monitor test. The sensitivity of the oligonucleotide chip assay was 10^3 copies of HBV DNA/ml of serum (Fig. 4A). We evaluated the specificities of the probes and the detection limit in the mixture with the wild type and mutant types by using plasmids constructed by site-directed mutagenesis. The plasmids were mixed with the wild type (rtM204) and the mutant types (rtM204V or rtM204I3) at various ratios (Fig. 4B1 and B2). As shown in Fig. 4B1 and B2, we could detect the rtM204V and rtM204I3 mutants whenever the mutant was mixed with a 10-fold higher or a 10-fold lower amount of the wild type (rtM204). Namely, in tests for the detection of a minority population, the oligonucleotide chip assay could easily detect minority viral populations present at only 10% of the total viral load.

**Comparison of oligonucleotide chip assay, PCR-RFLP analysis, and sequencing.** The assay with the oligonucleotide chip
could specifically detect mutations in the B domain (codon 180) and the C domain (codon 204 and codon 207) of HBV DNA polymerase. We tested 123 clinical samples using an oligonucleotide chip. A schematic showing the layouts of the oligonucleotide probes is shown in Fig. 2. Figure 5A shows the specific signals obtained with two identical positive control probes (probe P [probe 1]) and the signals for the wild type detected with four other probes (probes rtL180 [probe 2], probe rtM1204 [probe 5], probe rtM204 [probe 8], and probe rtV207 [probe 13]). Figure 5B shows the specific signals obtained with probes specific for codon 180 (probes rtL180 [probe 2] and rtL180M [probe 4]), codon 204 (probes rtM1204 [probe 5], probe rtM204V [probe 7], and probe rtM2204 [probe 8]), and codon 207 (probe rtV207 [probe 13]) and the signals obtained for samples infected with mixed types, the wild type,
and a mutant with mutations (rtM204 and rtM204V) at codon 204. Furthermore, Fig. 5C shows the specific signals for the detection of a mixture of two mutants (rtM204V and rtM204I3) with mutations at codon 204 obtained with all six probes (specific probes rtL180 [probe 2], rtM204V [probe 7], rtM204I3 [probe 12], and rtV207 [probe 13] and two identical positive probes [probe P [probe 1]). Figure 5D and E shows the mutants detected according to the expression of the rtM204I2 signal and the rtM204I3 and the rtV207I1 signals, respectively. Figure 5F shows a high-quality image of an oligonucleotide chip. This image shows the optimum conditions by the size, shape, and amount for each spot, as confirmed with a QC probe before and after hybridization.

To confirm the results obtained by the oligonucleotide chip assay, the three methods used to detect lamivudine-resistant HBV were compared. Figure 6 shows one example of the results of PCR-RFLP analysis and sequencing for confirmation of the results obtained by the oligonucleotide chip assay. Figure 6A shows the results of detection of mixed types with an oligonucleotide chip. For each codon, mixed types consisting of rtM204V and rtM204I3, rtL180M, and rtV207 were detected at codons 204, 180, and 207, respectively. The results of PCR-RFLP analysis for a mixed type with rtM204V and rtM204I3 are shown in Fig. 6B. The results for each sample with rtM204V and rtM204I3 could be further analyzed by nested PCR, enzyme digestion, and gel electrophoresis. Figure 6C shows the results of sequencing for a mixed type with rtM204V and rtM204I3. The sequencing assay could detect a mixed type only when the type was present at a high level.

The results of the three methods for a total of 123 samples were compared and are summarized in Table 3. The oligonucleotide chip assay provided the same information as PCR-RFLP analysis for 96.7% (119 of 123) of the samples and the same information as sequencing for 78% (96 of 123) of the samples for codon 204. The concordance of the results of the three assays was observed for 78% (96 of 123) of the samples.

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<th>Types</th>
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Table 3. Comparison of results obtained by oligonucleotide chip assay, PCR-RFLP analysis, and sequencing

a A total of 123 samples were tested. The rates of concordance between the assays were as follows: 96.7% (119 of 123 samples) for oligonucleotide chip assay versus PCR-RFLP analysis for codon 204 and 100% for oligonucleotide chip assay versus sequencing for codons 180 and 207.

b PCR-RFLP analysis could not detect Y1DD2 at codon 204 because the PCR-RFLP assay of Chayama et al. (2) can detect only rtM204V (YVDD) and rtM204I3 (YIDD3).

c Oligonucleotide chips could not detect rtM204V in one sample.

d The sequencing assay could detect a mixed type but could detect that type only if it was present in large amounts.
For three samples with discordant results, the oligonucleotide chip assay showed the presence of mixed types consisting of the wild type (rtM204) and a mutant (rtM204I2) and two mutants (rtM204V and rtM204I2), but PCR-RFLP analysis and sequencing analyses could not detect rtM204I2 and rtM204V, respectively. For three samples with discordant results, the oligonucleotide chip assay provided additional information compared to that provided by PCR-RFLP analysis and sequencing. For one sample with discordant results, the oligonucleotide chip assay could not detect rtM204V, but PCR-RFLP and sequencing analyses did detect rtM204V. For the remaining 23 samples, the results of the oligonucleotide chip assay coincided with those of PCR-RFLP analysis. However, the oligonucleotide chip assay results did not coincide with those of sequencing, because the sequencing assay could detect a mixed type but could detect that type only when it was present at high levels. The results of the three methods corresponded for rtM204I3. In a comparison of the results of the oligonucleotide chip assay and sequencing, the results for some samples with mixed types were significantly different. For 36 samples with mixed types (34 samples with rtM204V and rtM204I3 and 2 samples with rtM204 M, rtM204V, and rtM204I3), the results of the oligonucleotide chip assay coincided with those of PCR-RFLP analysis. However, by the sequencing assay, 13 samples were found to have a mixture of types rtM204V and rtM204I3, 6 samples were found to have rtM204I3 mutants, and 15 samples were found to have rtM204V mutants. For two samples with mixed types (rtM204, rtM204V, and rtM204I3), the oligonucleotide chip and PCR-RFLP assay results coincided, but the results of the sequencing assay indicated the presence of the wild type, rtM204.

The results obtained with the oligonucleotide chip for codon 180 and codon 207 were compared with the sequencing results for 113 samples. Both methods recorded the same information for 100% of the samples.

**DISCUSSION**

HBV isolates with mutations in the YMDD motif of the polymerase gene have been detected in patients who have received long-term lamivudine therapy (1, 5, 8, 10, 12). Mutants with either the YVDD or the YIDD mutation were detected in 14 to 47% of patients after 1 year of lamivudine therapy. Moreover, the mutants with YMDD mutations were detected as early as a few weeks (12 weeks) after the start of lamivudine therapy in Korea (12). Rapid and accurate detection of lamivudine-resistant HBV provides us with important information about therapies (13, 17). To detect mutants and to identify the mutation types, many methods, such as the direct sequencing of PCR products, PCR-RFLP analysis, clonal analysis, and the line probe assay, have been used (18). The PCR-based method can detect only one specific marker per reaction (4). Hybridization tools, such as microplates or dot blots with membrane-bound probes, require a large amount of the target product, and the results are often difficult to interpret. In addition, these methods are time-consuming and laborious. However, nucleic acid hybridization is highly specific and can be controlled by the use of various reaction conditions.

In this study, we used three different types of technologies, an oligonucleotide chip, PCR-RFLP analysis, and PCR-direct sequencing, for the detection of lamivudine-resistant HBV in patient sera. Moreover, we developed control systems to evaluate the quality of oligonucleotide chip fabrication, differentiate positive and negative spots, and determine the presence of mixtures of viral types. Two types of control probes provided a useful standard for the monitoring of chip fabrication and measurement of the background activities on the oligonucleotide chips. The probes specific for the detection of lamivudine resistance comprised more than one probe for each codon because of natural variations and variations in base pairs for the same amino acid. This oligonucleotide chip contained three kinds of probes: a QC probe, NC probes, and target-specific probes. The QC probe was used to assess the surface quality, integrity, and homogeneity of each probe spot. The NC probes were used to generate cutoff values. Namely, all spots with signals with intensities greater than those of the NC probes were recognized as positive. Spots with signal intensities with specific probes that were equal to or less than those for the NC probes were recognized as negative. Finally, target-specific probes that detected YVDD mutations and three kinds of YIDD mutations at codon 204, two of the wild-type nucleotides at codon 180, and two types of mutations at codon 207 were contained in this oligonucleotide chip.

The sensitivity of the oligonucleotide chip was 10-5 copies/ml, and it detected minor populations present at levels as low as 10% of the total viral load. The oligonucleotide chip assay used in this study showed a sensitivity for the detection of minority populations of mixed types higher than that of PCR-RFLP analysis and sequencing.

We tested 123 patient serum samples. For codon 204, the rate of concordance between the results of the oligonucleotide chip assay and those of PCR-RFLP analysis was 96.7% (119 of 123 samples) and the rate of concordance between the oligonucleotide chip assay and sequencing was 78% (96 of 123 samples). The analysis of the samples with discordant results showed that the PCR-RFLP and sequencing methods could detect minority populations in samples with mixed viral populations but that the oligonucleotide chip assay was able to provide a more accurate means of typing for more samples than the other two methods. However, one sample with YVDD was not detected by the oligonucleotide chip assay. The PCR-RFLP analysis described by Chayama et al. (2) can detect only YVDD and YIDD3 at codon 204, and the sequencing assay can detect only major types. The oligonucleotide chip and PCR-RFLP analyses were able to detect mixed types. However, the oligonucleotide chip assay is more efficient than the PCR-RFLP method due to the absence of the post-PCR process. Also, the results of the oligonucleotide chip assay are simple to interpret. For the rtM204V and rtM204I3 mixed types, the results of the oligonucleotide chip and PCR-RFLP analyses coincided, but the results of the sequencing assay coincided only for the rtM204 wild type. Twenty-three of 36 samples with mixed types were found to contain the wild type or a mutant. In the case of mixed infections, sequencing often detected the most prevalent viral type.

The incidence of YVDD (rtM204V; 16.3%) was similar to that of YIDD3 (rtM204I3; 19.5%). L180M and M204V were detected, and contrary to a previous study (16), rtL180M-rtM204I2 and rtL180M-rtM204I3 were detected quite often. In this study, the distribution of mutations detected showed that
rtM204V and rtM204I are commonly found. There were many samples with the mixed type of rtM204V and rtM204I and three samples with rtM204I.

In conclusion, the oligonucleotide chip assay is a rapid, sensitive, and reliable diagnostic tool for the detection of lamivudine-resistant mutant types.

ACKNOWLEDGMENT

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REFERENCES