Oligonucleotide chip, real-time PCR and sequencing for genotyping of hepatitis B virus

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AIM: To compare the oligonucleotide chip, real-time PCR and sequencing for genotyping of hepatitis B virus in Chinese patients with chronic hepatitis B.

METHODS: Mixture of samples with different genotypes and clinical serum samples from 126 chronic hepatitis B patients were tested for hepatitis B virus genotypes by oligonucleotide chip, real-time PCR and sequencing of PCR products, respectively. Clinical performances, time required and costs of the three assays were evaluated.

RESULTS: Oligonucleotide chips and real-time PCR detected 1% and 0.1% genotypes, respectively, in mixed samples. Of the 126 clinical samples from patients with chronic hepatitis B, genotype B was detected in 41 (33%), 41 (33%) and 45 (36%) samples, and genotype C in 76 (60%), 76 (60%) and 81 (64%) samples, by oligonucleotide chip, real-time PCR and sequencing, respectively. Oligonucleotide chip and real-time PCR detected mixed genotypes B and C in 9 samples. Real-time PCR was the rapidest and cheapest among the three assays.

CONCLUSION: Oligonucleotide chip and real-time PCR are able to detect mixed genotypes, while sequencing only detects the dominant genotype in clinical samples.

INTRODUCTION

It is estimated that 350 million individuals are chronically infected with hepatitis B virus (HBV) and that more than 1 million die from cirrhosis and hepatocellular carcinoma (HCC) each year[1-3]. HBV has been classified into eight genotypes (A-H) based on the sequence divergence of > 8% in the entire genome, which consists of about 3200 base pairs[4-7]. Different HBV genotypes have distinct geographical distributions[8]. Genotype A is found mainly in Northwest Europe, the United States, India, and sub-Saharan Africa. Genotypes B and C prevail in East Asia. Genotype D is common in the Mediterranean countries. Genotype E is only found in Africa, while genotype F is found mainly in Central and South America. The distribution of HBV genotypes G and H still needs to be determined. In China, the most common HBV genotypes are B (41%) and C (53%), while genotypes A and D are also found in some provinces[9]. Several studies have revealed that genotype C is associated significantly with the severity of HBV infection, liver cirrhosis, and HCC compared with genotype B[10-13].

HBV genotypes may be associated with differences in response to antiviral therapy. Some studies indicate that HBV genotypes respond differently to interferon in patients with chronic hepatitis B. Genotype B responds better to interferon than genotype C[13,14]. Several technologies have been developed for genotyping of HBV[15-17], but the number of studies actually comparing these assays is limited.

Nucleotide sequencing of PCR products is routinely used to detect lamivudine resistance. However, this method is expensive and laborious. We have developed an oligonucleotide chip assay for determination of HBV genotypes[17]. In the present study, we compared sequencing of PCR products, oligonucleotide chip and a commercial real-time PCR kit (Fosun Diagnostics, Shanghai, China) for detection of YMDD mutants in mixed samples and clinical samples from patients with chronic hepatitis B. Clinical performances, time required, and costs were also analyzed to enable a comparison among these assays for their economic effectiveness.

MATERIALS AND METHODS

Patients, samples and extraction of HBV DNA

Serum samples were collected from 126 patients with chronic HBV infection. All the patients being positive for hepatitis B virus surface antigen (HBsAg) and HBV-DNA were from Changzhou, China. HBV DNA was extracted...
from serum samples using the QIAamp blood kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer’s instructions. HBV DNA levels were measured on PE5700 real-time PCR system (Applied Biosystems, Foster, CA, USA) with quantitative real-time PCR reagents (Fosun Diagnostics, Shanghai, China) approved by the State Food and Drug Administration of China for in vitro diagnostic use.

**Oligonucleotide chip test**

Oligonucleotide chip test was carried out as described previously. In brief, extracted HBV DNA was amplified by nested PCR with primers specific for the pre-S region of HBV. Cy5-labeled unpurified PCR products were mixed with DIG Easy Hyb (Roche Molecular Biochemicals, Canada). The mixture was denatured and added onto the chips and covered with a glass cover. Oligonucleotide chips were incubated at 40°C for 30 min in a moist incubator for hybridization and washed. Images were obtained with a fluorescent scanner (GenePix 4000B, Axon Instruments, Inc., CA, USA).

**Real-time PCR**

Real-time PCR for determination of HBV genotypes was performed according to the manufacturer’s instructions. In brief, parallel reactions were used to detect HBV genotypes B and C. The reactions differed in the TaqMan probes. The amplification was performed on PE5700 PCR system (Applied Biosystems, Foster, CA, USA) by incubating the reaction mixture (50 μL) at 50°C for 2 min, followed by 40 cycles of PCR amplification at 93°C for 30 s and at 60°C for 90 s. The Ct value is defined as the number of fractional cycles in which the fluorescence emitted by the reaction mixture exceeds a preset threshold and marks the beginning of an exponential growth of the fluorescence signal. The results were analyzed with ABI prism 5700 software (Applied Biosystems, Foster City, CA). The reaction system was provided by Fosun Diagnostics (Fosun Diagnostics, Shanghai, China).

**Sequencing of PCR products**

HBV DNA sequencing was performed as previously described. Extracted DNA was amplified by using primers specific for the pre-S1/pre-S2 region of HBV. PCR products were purified with QIAquick PCR purification kits (Qiagen, Chatsworth, California, USA) and eluted from the column with 80 μL of distilled deionized water. The quality and concentration of DNA were determined by absorbance measurements at 260 and 280 nm and gel electrophoresis. All sequencing reactions were performed on ABI 3100 DNA sequencer (Applied Biosystems, Foster, California, USA).

**Mixing experiments**

Mixing experiments were used to evaluate the abilities of oligonucleotide chip, real-time PCR, and sequencing to accurately detect minor HBV genotypes. Two previously tested samples of genotypes B and C were used to prepare the mixture. Both samples were diluted with HBV negative serum to a final concentration of 2 × 10⁶ copies/mL and mixed. HBV genotype B in the mixture was 50%, 10%, 1%, and 0.1%, respectively. The mixture was analyzed with the three methods.

**Serial dilution experiments**

A sample with 5.20 × 10⁴ copies/mL of HBV DNA in genotype C was diluted to 5.20 × 10³, 5.20 × 10², 5.20 × 10¹, and 5.20 × 10⁰ copies/mL, and tested with the three methods.

**Time study**

Time study was carried out as previously described. Three skilled technologists performed these assays. The time required for each assay was measured by direct observation by the technologists, with a work unit of 16 samples.

**Cost**

Costs for each assay were estimated based on the prices of reagents, including consumables and DNA extraction reagents, in China. The costs of instruments and labors were not included.

### RESULTS

**Detection of mixed samples**

The mixture of samples containing genotypes B and C at different ratios was detected by oligonucleotide chip, real-time PCR, and sequencing, respectively. Oligonucleotide chip detected genotypes B and C in the mixture containing 50%, 10%, and 1% of genotype B, but only genotype C in the mixture containing 0.1% of genotype B. Real-time PCR detected genotypes B and C in the mixture containing 10%, 1%, 0.1% of genotype B. Sequencing detected genotypes B and C only in the mixture containing 50% of genotype B, but only genotype C in the mixture containing 10%, 1%, 0.1% of genotype B (Table 1).

**Detection of diluted samples**

To determine the sensitivity of oligonucleotide chip, real-time PCR, and sequencing, a sample of genotype C was diluted and tested with the three methods. Oligonucleotide chip was able to detect all the dilutions, while real-time PCR and sequencing were able to detect dilutions containing 5.20 × 10³, 5.20 × 10², 5.20 × 10¹, and 5.20 × 10⁰ copies/mL, but not able to detect the dilution containing 20 × 10⁰ copies/mL.

### Table 1 Oligonucleotide chip, real-time PCR, and sequencing in detection of genotypes B and C in mixed samples

| Oligonucleotide chip | Real-time PCR | Sequencing | Percentage |
|---------------------|---------------|------------|------------|
| B and C             | B and C       | B and C    | 50         |
| B and C             | B and C       | C          | 10         |
| B and C             | B and C       | C          | 1          |
| C                   | B and C       | C          | 0.1        |

**Oligonucleotide chip, real-time PCR, and sequencing in detecting genotypes in clinical samples**

A total of 126 clinical serum samples from patients with chronic hepatitis B were tested. The results obtained by oligonucleotide chip, real-time PCR, and sequencing
were compared (Table 2). Completely concordant results were obtained in 117 (93%) samples by the three assays. Genotype B or C was detected by sequencing and genotypes B and C were detected by oligonucleotide chip and real-time PCR in the mixture of 9 samples. Genotype B, genotype C, and both of them were detected by oligonucleotide chip and real-time PCR in the mixture of 41 (33%), 76 (60%) and 9 (7%) samples, respectively. No other genotypes were observed in this study.

**Time required**
The time required for each assay depended on the sample size and the throughput of instruments used for detection. In this study, we measured the time required for each assay with a 16-sample work unit because the highest throughput of ABI 3100 sequencer was 16 samples per run. The total assay time for oligonucleotide chip, real-time PCR, and sequencing was 7, 6, and 3 h, respectively.

**Costs**
The cost for each assay was calculated based on the prices of reagents and consumables used. The cost per test for oligonucleotide chip, real-time PCR, and sequencing was 120, 70, and 100 Chinese Yuan (CNY), respectively (The costs of instruments and labors were not included).

**DISCUSSION**
HBV has been classified into eight genotypes of A-H. These genotypes are different in biological properties and show heterogeneity in their global distribution, prevalence of mutations, clinical outcome, and response to antiviral treatment[19,20]. To date, many assays have been used for genotyping of HBV, such as multiplex PCR with genotype-specific primers[20-22], PCR followed by restriction fragment length polymorphism (RFLP) analysis[15,23], serological assay[24,25], reverse hybridization[16], real-time PCR with melting curve analysis[26,27], and oligonucleotide chips[17,28-30].

However, the sensitivity, specificity, cost, and time required are different in these methods. In this study, we compared oligonucleotide chip, real-time PCR, and sequencing, in determination of HBV genotypes in patients with chronic hepatitis B. The results obtained by them were concordant in 93% of the samples. Oligonucleotide chip and real-time PCR were able to detect mixed genotypes in 9 samples, while sequencing only detected the dominant genotype.

Oligonucleotide chip has been used to detect hepatitis B virus resistance mutations and identify genotypes[31]. We have developed an oligonucleotide chip assay based on the reverse hybridization principle-specific oligonucleotide probes to detect the eight genotypes of HBV[17]. In the present study, oligonucleotide chip assay determined HBV genotypes in samples with HBV DNA load of 5.20 × 10^6 copies/mL, showing the highest sensitivity among the three assays. This may be due to the very high sensitivity of nested-PCR used before chip hybridization. However, in the mixture experiments, oligonucleotide chip could detect 1% of the genotypes while real-time PCR could detect 0.1% of genotypes in samples with 2 × 10^5 copies/mL DNA. This may be attributed to the competition of the dominant genotype in PCR amplification and hybridization. Further study is needed to explore the truth behind this conflict.

Commercial assay for genotyping of HBV is based on TaqMan technology, with genotype-specific probes for HBV genotypes A and D. This assay is the most rapid and cost-effective one among the three assays. However, oligonucleotide chip and sequencing could detect the three HBV genotypes, while real-time PCR in this study could only detect the two common genotypes in China. When real-time PCR is used to determine the eight HBV genotypes, eight reactions are needed and the cost is rather high.

Of the 126 clinical samples from patients with chronic hepatitis B, genotype B was detected in 41 (33%), 41 (33%) and 45 (36%) samples, and genotype C in 76 (60%), 76 (60%) and 81 (64%) samples, by oligonucleotide chips, real-time PCR, and sequencing, respectively. Oligonucleotide chip and real-time PCR detected mixed genotypes B and C in 9 samples, which is consistent with the reported data[9]. Compared to our previous study, however, the prevalence of genotype C was lower, possibly due to the different patient samples used in the two studies. Although genotypes A and D have been observed in China[9], they were not detectable in our study, showing that these genotypes are very scarce in Changzhou, China.

In conclusion, oligonucleotide chip and real-time PCR are able to detect mixed genotypes in clinical samples. Of the three assays, real-time PCR is the most rapid and cost-effective technique in detecting genotypes B and C.

### Table 2 Comparison of oligonucleotide chip, real-time PCR, and sequencing for detection of the clinical samples (n (%))

| Genotypes | Oligonucleotide chip | Real-time PCR | Sequencing |
|-----------|----------------------|---------------|------------|
| B         | 41 (33)              | 41 (33)       | 45 (36)    |
| C         | 76 (60)              | 76 (60)       | 81 (64)    |
| Mixture of B and C | 9 (7)             | 9 (7)         | 0          |

### COMMENTS

#### Background
Hepatitis B virus (HBV) genotypes may be associated with the severity of HBV infection and response to antiviral therapy. Sensitive and cost-effective methods for genotyping of HBV are needed in the management of HBV infection.

#### Research frontier
Several methods, such as sequencing, PCR-RFLP, oligonucleotide chip, real-time PCR, and line probe assay, have been used in genotyping of HBV. Geographical distribution, characteristics, and response to antiviral therapy of different HBV genotypes are the focus of research interest.

#### Related publications
Detection of hepatitis B virus genotypes using oligonucleotide chip among HBV carriers in Eastern China by Tang et al published in World J Gastroenterol 2007; 13: 1973-1977.

#### Innovations and breakthroughs
This article compared the oligonucleotide chip, real-time PCR and sequencing for genotyping of HBV in patients with chronic hepatitis B virus infection. Clinical performances, time required, and costs of the three assays were analyzed.

#### Applications
Both oligonucleotide chip and real-time PCR can be used in genotyping of HBV in patients with HBV infection.
**Terminology**

Oligonucleotide chip is an assay tool, by which short DNA oligonucleotides specific to different regions of the genomes spotted onto the chip can be detected.

**Peer review**

This article compared the clinical performances, time required, and costs of oligonucleotide chip, real-time PCR, and sequencing for genotyping of hepatitis B virus in Chinese patients with chronic hepatitis B. The methods they used in the study can be applied in clinical studies.

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