Microarrays for the Detection of HBV and HDV

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The increasing pace of development in molecular biology during the last decade has had a direct effect on mass testing and diagnostic applications, including blood screening. We report the model Microarray that has been developed for Hepatitis B virus (HBV) and Hepatitis D virus (HDV) detection. The specific primer pairs of PCR were designed using the Primer Premier 5.00 program according to the conserved regions of HBV and HDV. PCR fragments were purified and cloned into pMD18-T vectors. The recombinant plasmids were extracted from positive clones and the target gene fragments were sequenced. The DNA microarray was prepared by robotically spotting PCR products onto the surface of glass slides. Sequences were aligned, and the results obtained showed that the products of PCR amplification were the required specific gene fragments of HBV, and HDV. Samples were labeled by Restriction Display PCR (RD-PCR). Gene chip hybridizing signals showed that the specificity and sensitivity required for HBV and HDV detection were satisfied. Using PCR amplified products to construct gene chips for the simultaneous clinical diagnosis of HBV and HDV resulted in a quick, simple, and effective method. We conclude that the DNA microarray assay system might be useful as a diagnostic technique in the clinical laboratory. Further applications of RD-PCR for the sample labeling could speed up microarray multi-virus detection.

Keywords: Diagnosis, Gene probe, HBV, HCV, Microarray, PCR

Introduction

Viral hepatitis is the most common cause of acute and chronic hepatitis. The term viral hepatitis generally refers to infections

resulting from one of the hepatotropic viruses. Chronic infection by the hepatitis virus affects over 500 million people worldwide (Lok *et al.*, 2001), is associated with significant morbidity and mortality, and is considered a major public health problem in most areas of the world, which raises issues of its diagnosis, treatment, and prevention (Jay *et al.*, 1997). Hepatitis B and D viruses are parent rally transmitted, and occur both in the acute and chronic forms. When they persist in a chronic carrier state, they serve as a reservoir for infection and give rise to chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). In particular, the laboratory diagnosis of viral hepatitis is important to effectively plan immediate patient management, to determine treatment choices, and to limit the transmission of infection.

In the past decade, improvements in molecular biology-based techniques have yielded highly valuable tools for use in this setting. DNA microarray technology can allow the quantitative and simultaneous analysis of hundreds to thousands of genes (Schena *et al.*, 1995; Schena *et al.*, 1996; Shalon *et al.*, 1996), and allows the high-throughput screening of multiple pathogens in a large numbers of samples. The DNA chip represents a new category of this developing technology, and is characterized by expedient, large scale, highly automatic, and sensitive detection of biological information regarding DNA and RNA.

In this article, we describe a microarray technology developed for the simultaneous detection of HBV and HDV. The specific PCR primers used were designed using the Primer Premier 5.00 program according to the conserved regions of HBV and HDV. The PCR fragments were purified and cloned into pMD18-T vectors. Recombinant plasmids were extracted from positive clones and the target gene fragments were sequenced. The DNA microarrays were then prepared by robotically spotting PCR products onto the surfaces of glass slides.

Materials and Methods

Probe template The full-length plasmid of HDV pDL553 was

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presented by Dr S. Gudima of the Fox Chase Cancer Center (USA), and the full-length HBV plasmid was constructed in our laboratory.

Chemicals and reagents Premix Taq, dNTP, *Eco*RI, *Sau*3AI, pMD18-T vector, and T4 DNA Ligase were obtained from Takara Co. (Shiga, Japan). Plasmid Miniprep kits were purchased from Shen Neng Bo Cai Co. (China), PCR primers of HBV and HDV and the primers in pMD18-T vector were synthesized by BIOASIA Co. (China). The universal primer cy5-GTTTGGCTGGTGTGGATC was purchased from Gibco Co. (Gaithersburg, USA), CMT-GAPS coated glass slides were from Corning Co. (Corning, USA).

Bacterial strains The *E. coli* strain XL-1 used in these experiments was maintained in our laboratory.

Oligonucleotide primers design Oligonucleotide primers were designed using the Primer Premier 5.00 program and GenBank Blastn sequence alignments. Selected PCR primers met the following criteria: (i) primers hybridized to highly conserved sequence elements, (ii) each amplicon spanned approximately 250-700 bp, (iii) primer melting points generally fell between 50 and 54°C. We designed 10 and 4 primer pairs respectively according to the conserved region of HBV and HDV. The sequences of the synthetic primers used in the study are shown in Table 1.

PCR The plasmids of HBV and HDV (1 µl), respectively, were added to a PCR mixture of total volume 50 µl containing 25 µl 2 × premix, and 2 µl (each) of the oligonucleotide primers (0.25 µM).

HBV The mixture was denatured at 94°C for 5 min and then subjected to 30 thermal cycles (94°C for 55 s, 55°C for 55 s, 72°C for 55 s), followed by a final 5 min incubation at 72°C.

HDV The mixture was denatured at 94°C for 5 min and then subjected to 30 thermal cycles (94°C for 45 s, 50°C for 45 s, 72°C for 60 s), followed by a final 5 min incubation at 72°C. The mixture was then chilled and the products were sized by 1.5% agarose gel electrophoresis. The PCR products were purified, using a 3S PCR product purification kit V20 (Bocai Corp., Shanghai, China) and stored at -20°C.

Identification of the PCR products After purification using the PCR product purification kit, the PCR products were inserted into pMD18-T vector. The ligation mixture which contained 4 µl PCR products, 1 µl pMD18-T vector (50 ng/µl), and 5 µl loading buffer solution was incubated for 4 hours at 16°C, and then transferred into 100 µl XL-1 *E. coli* competent cells, which had been previously treated with solutions containing Ca^{2+} ions (0.1 M). To transform *E. coli*, the mixture of DNAs formed in the ligation reaction was combined with a suspension of competent cells for 30 min, then heat-shocked at 42°C for 1~2 min. The cells were then incubated in a growth medium and finally spread on an agar plate and incubated until single colonies of bacteria grew. Clones containing target fragments were then selected and identified with pMD18-T vector primer (primer A 5'-CTAAAACGACGGCC AGT-3', primer B 5'-CAGGA AACAGCTATGAC-3'). The sequence of target fragments were then analyzed using an ABI PRISM 377 DNA sequencer, and GenBank Blastn sequence alignments were performed.

Fig. 1. Arrangement of all spots on genechip. ● HBV probes(10 fragments of genes) ● Negative control ● Empty controls ● HBV Positive controls ● HDV Positive controls ● HDV probes (4 fragments of genes).

Microarray printing and processing The final concentration of each purified PCR product was adjusted to 0.3 mg/ml with DMSO and water (50% v/v). The DNA microarray was prepared by spotting the PCR products on special glass slides using a Cartesian 5,500 MicroArrayer. The DNAs of the target gene were then cross-linked with ultraviolet irradiation, baked at 80°C for 2 h, and maintained in closed containers until application. The arrangement of spots on the gene chip is shown in Fig. 1.

Samples labeling The plasmids of HBV and HDV were digested at 37°C with the restriction endonuclease *Sau*3AI. T4 DNA ligase was used to link gene fragments with universal adapters (SIP: 5'pGATCmCAC ACC AGC CAA ACC CA, SIR: 5'GGT TTG GCT GGT GTG). After 4 h of ligation at 16°C, 25 µl 2 × premix, 2 µl of the linkage products, 2 µl of the Cy5 labeled universal primer matched with the adaptor, and 21 µl water were added to a total volume of 50 µl. Reactions were performed in a GeneAmp PCR 9700 system with an initial denaturation of 10 min at 94°C, and then subjected to 30 thermal cycles (94°C for 30 s, 60°C for 30 s, 72°C for 60 s), followed by a final 5 min incubation at 72°C. Finally, the reaction was stopped by cooling to 4°C. After this labeling reaction, the sample DNA fragments of hundreds base pairs were labeled with fluorescent Cy5. The labeled PCR products were then further purified using a 3S PCR product purification kit V20 (Bocai Corp.). 5 µl of the 30 µl purified products was used for hybridization with the microarray probes.

Prehybridization Microarray slides were placed in a pre-hybridization solution (5 × SSC, 0.2% SDS, 25% formamide and 1% BSA) at 42°C for 1 h, rinsed at room temperature in Milli Q water for 5 times, dehydrated in isopropanol, and air dried.

Hybridization and data analysis 5 µl Cy5 labeled samples were mixed with 1 µl Cot-1 DNA (20 µg/µl, Life Technologies) and 6 µl 2 × hybridization buffer (50% formamide, 10 × SSC, 0.2% SDS), and denatured at 95°C for 5 mins, centrifuged at 14,000 rpm for

Table 1. Sequences of the synthetic primers used for preparing the HBV and HDV probes

Virus	No.	Primers (5' → 3')	Location (nt)	Product size (bp)
HBV	1	P1: ACTCGTGGTGGACTTCTCTC P2: GAACCACTGAACAAATGGCA	251-270 701-720	470 bp
	2	P3: CATCTTCTTGTGGTTCTTCTG P4: TTAGGGTTCAAATGTATACCC	427-448 840-860	434 bp
	3	P5: CTGTGAGGAGTTGGGGGAGGAGATT P6: GGCGAGGGAGTTCTTCTTCTAGGGG	1730-1754 2397-2421	692 bp
	4	P7: GGGTCACCATATTCTTGG P8: CCCTGAGCCTGAGGGCTCCACC	2814-2831 3080-3101	288 bp
	5	P9: CTCCAGTTCAGGAACAGTAAACCC P10: ATAACCTGAAAGCCAAACAGTGGG	67-90 744-766	700 bp
	6	P11: ATTCCTATGGGAGTGGGCCTCAG P12: GCAGCACAGCCTAGCAGCCATGG	779-801 1407-1429	651 bp
	7	P13: CCATACTGCGGAACCTCTAGC P14: CAATGCTCAGGAGACTCTAAGGC	1409-1429 2011-2033	625 bp
	8	P15: GGAGCTACTGTGGAGTTACTCTC P16: CTTCTGCTGCGAGGCGAGGG	1928-1950 2350-2369	442 bp
	9	P17: GGCAGGTCCCCTAGAGAAGAAGT P18: TTGGGATTGAAGTCCCAATCTGG	2383-2406 2960-2982	600 bp
	10	P19: GGGGTGGAGCCCTCAGGCTCAGG P20: CTGTAACACGAGAAGGGGTCCTAG	3072-3094 134-157	300 bp
HDV	1	P1: GGAGACCGAAGCGAGGAGGAAAGCA P2: CGACCTGGGCATCCGAAGGAGGACG	393-417 843-867	475 bp
	2	P3: CTGCAGGGTCCGCGTTCCATCCTT P4: CCAGTGAATAAAGCGGGTTTC	697-720 953-973	277 bp
	3	P5: GACCCGAAGAGGAAAGAAGGACGC P6: GGTGTGAACCCCTCGAAGGTGG	896-919 1143-1165	270 bp
	4	P7: CTTCTGTCGGTGATCCTGCCTCT P8: CCAGCAGTCTCCTCTTTACAGA	631-652 941-962	332 bp

2 min, and cooled to room temperature. Three μ l of the above mixture was then added to the surface of the microarray, which had been immersed in the prehybridisation medium for 1h, and covered with a glass coverslip pretreated with SIGMACOTE® (Sigma, St. Louis, USA) to prevent sample evaporation. The slide was placed in a sealed hybridization box; 10 μ l UPW was added to every well at the two ends of the chamber. After hybridization at 42°C for 5 h, the slide was taken out and washed with: 1) low-stringency washing buffer containing $1 \times$ SSC/0.2% SDS at 42°C for 5 min; 2) high-stringency washing buffer containing $0.1 \times$ SSC/0.2% SDS at room temperature for 10 min; 3) $0.1 \times$ SSC for 1 min; 4) Milli Q water; 5) ethanol; respectively. The chip was then desiccated at room temperature. Scanning was performed using a ScanArray Lite Microarray Analysis System (GSI Lumonics Co).

Results

Preparation of HBV and HDV probes for the microarray

From the 1.5% agarose gel electrophoresis results, we found that that all the respective 10 and 4 primer pairs amplified HBV DNA and HDV cDNA and produced products of the expected size (Fig. 2, Fig. 3).

Confirmation of the DNA obtained from HBV and HDV by sequence analysis By using Blastn and the Genbank database, each sequenced PCR product was confirmed to be a HBV or HDV genome fragment. Fig. 4 and Fig. 5 show one DNA fragment from HBV located at the polymerase gene region and one cDNA fragment from HDV located at the complete genome region, respectively.

Microarray design The clinical diagnostic microarray was prepared by immobilizing the captured target genes of pathogens on a slide, which was treated specifically. The DNA or RNA extracted from the patient's serum was labeled with fluorochrome and hybridized to the target DNA. In this paper, the microarray was prepared by spotting PCR products of the

Fig. 2. Agarose gel electrophoresis of the PCR products of ten fragments by different primer combinations for to prepare the HBV genechip probes.

Fig. 3. Agarose gel electrophoresis of PCR products of four fragments using different primer combinations to prepare the HDV genechip probes.

conserved sequences of HBV and HDV onto the surfaces of glass slides by using a Cartesian 5,500 MicroArrayer. Controls were immobilized at the same time. The control system was composed of three parts: (i) empty controls were DMSO without gene fragments, (ii) negative controls were gene fragments of plants (rice), an eukaryocyte (K562 cell), and a prokaryocyte (*E. coli*), which were not homologous with HBV and HDV, and (iii) positive controls were the reconstructed gene fragments. Ten of the 12×12 microarrays could be simultaneously immobilized on a glass slide.

Analysis of the microarrays hybridized with HBV and HDV DNA by fluorescent scanning From the hybridizing signals obtained using 80% laser energy and 70% GMT on the Gene chip, it was evident that it satisfied the specificity and sensitivity requirements for detecting HBV and HDV, as shown in Fig. 6. The single array shown in Fig. A was hybridized with a sample of HBV, Fig. B was hybridized with a sample of HDV, and Fig. C was hybridized with an

admixture of HBV and HDV. No signal was observed on the empty control spots (Printing 50% DMSO) or negative control spots (Printing gene fragments of eukaryotic cells such as K562 cell, prokaryotic cells such as HIV, and plant genes such as rice. Our institute preserved all gene fragments). The signals of spots hybridized to HBV, HDV, HBV positive controls, HDV positive controls, and an admixture of HBV and HDV were strong and obvious.

Discussion

In China, chronic viral hepatitis is considered a major public health problem. Approximately 150 million individuals have contracted chronic hepatitis B and the incidence rate of HBV infection is 158/1000,00 annually (Jin *et al.*, 2001). Moreover, up to 40% of these individuals will eventually develop serious hepatic complications. HBV infection can cause acute and chronic liver disease including cirrhosis and HCC. The risk of

Fig. 4. Sequence analysis of one of the DNA fragments from HBV.

Fig. 5. Sequence analysis of one of the cDNA fragments from HDV.

Fig. 6. Scanning results of hybridizing signals on Gene chip.

HCC in individuals that have developed chronic hepatitis B is 300 times higher than in normal people (Jin *et al.*, 2001). The hepatitis D virus (HDV), a defective virus, requires the supply of hepatitis B surface antigen (HbsAg) envelope by hepatitis B virus (HBV) for its assembly and transmission. During acute and chronic infection, and HDV infection often leads to more severe disease (Porres *et al.*, 1989).

In the clinical diagnosis of hepatitis virus, antibody-based methods such as ELISA and molecular biologic techniques, such as PCR, and molecular hybridization had been considered practical ways of detecting viral infection. However, such immunoassays suffer from a problem because antibody assays are insufficient for diagnostic purposes due to the brief diagnostic window period and their inability to diagnose patients with hyp immunity. For viral diagnosis, common nucleic acid hybridization may have an excellent specificity, but sensitivity requirements are not often satisfied. In the protocol of PCR, cross contamination, and the incidents of false negative and false positive often occur, and simply producing DNA provides an indication that the right product

has been amplified. DNA microarrays offer a solution to these problems and provide us with a simple and sensitive method of diagnosing hepatitis virus infection. Compared with traditional diagnostic techniques, the technique has a number of obvious advantages—integration, micromation and automatization (Ma *et al.*, 1999). In principle, one could draw on this resource when needed to clinically diagnose different hepatitis virus subtypes and the strain of drug resistance simultaneously on a microarray slide, and the captured DNA could be assayed in a single reaction. The DNA genechip also offers a dependable basis for clinical laboratory diagnosis, treatment, the monitoring of response to therapy, and the occurrence, development and prognosis of hepatotropic viral infections (Petrik *et al.*, 2001; Sun *et al.*, 2003a, 2003b).

The chip we developed is cost-effective, and the procedure required to prepare the chip, straightforward and convenient. When results are verified by molecular hybridization, PCR cross contamination can be overcome, and by incorporating negative and positive controls, we detection results are ensured. Subjective factors in terms of judging results were

reduced greatly by using analytic software (Falus *et al.*, 1998). In addition, the sensitivity of the assay may be enhanced by increasing the amount of captured DNA on the slide or by pretreating the sample DNA.

There are two ways of preparing DNA microarray probes: the first one is the PCR amplification of the DNA fragments with a molecular clone, and the other is the artificial synthesis of oligonucleotide arrays using a DNA synthesizing machine. We preferred the first method, making use of Primer Premier 5.00 to design special HBV and HDV primers. While designing the primers, we tried to make the annealing temperatures of HBV and HDV coincide so that the PCR protocol could be performed easily in the same reaction system. Because of the convenience of PCR, we were able to directly prepare DNA microarray probes after purifying the PCR product. As for the small genome of HBV (3.2 Kb) and HDV (1.7 Kb), the method is practical as it offers speedy, and simple clinical application.

Using Restriction Display-PCR RD-PCR (Ma *et al.*, 1998; Zheng *et al.*, 1998), Professor MA and ZHENG prepared microarray probes on a massive scale, which were nearly the same length, and developed diagnostic gene chips. Their method for preparing gene chip probes is used in our laboratory at present (Li *et al.*, 2002; Mao *et al.*, 2002; Zhu *et al.*, 2002). Further applications of this technique for sample labeling could produce good results and speed up multi-virus detection by microarray technology (Feng *et al.*, 2002; Zhang *et al.*, 2002; Li *et al.*, 2003). By RD amplification of the enzyme digested samples, the sample fragments can be significantly labeled with fluorescent signals, and can produce much stronger hybridizations than conventional labeling methods.

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