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Hepatitis B virus array for genotyping and mutation detection

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KEY MESSAGES

1. A comprehensive hepatitis B virus (HBV) array providing simultaneous analysis of HBV genotypes, mutations of reverse-transcriptase polymerase gene and mutations of S gene was developed.
2. The specificity and sensitivity of the HBV array was validated through analysis of 506 serum samples.
3. The array demonstrated improved sensitivity compared with existing commercial kits, with a detection limit down to ~28 copies/mL of HBV. It also enabled early detection of emergence of drug-resistant mutants.
4. The array may be useful for clinicians in making timely decisions on switching to alternative drugs.

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Introduction

Hepatitis B virus (HBV) infection is a serious global health problem affecting about 2 billion people worldwide, of whom 350 million are chronic HBV carriers. The HBV is the tenth leading cause of death, with about one million deaths annually secondary to chronic hepatitis, cirrhosis, or hepatocellular carcinoma (HCC).[1] The HBV is virtually endemic throughout Asia and Africa, and most chronic HBV infections are acquired perinatally or during childhood. For individuals with HBV infection, antiviral treatment is an effective way to reduce morbidity and mortality, and life-long antiviral therapy is advocated.[2] However, in some patients, resistant viral strains carrying mutations in the B, C, and D domains of the HBV reverse transcriptase (rt) polymerase gene may emerge with prolonged therapy.[3] These patients then have a higher risk of developing HCC than patients without resistant virus. Close monitoring of serum for early detection of any drug-resistant mutant is important to enable timely switching to or adding of other antiviral drugs to achieve adequate viral suppression.

For patients who have undergone liver transplantation for hepatitis B–related end-stage liver disease, prophylaxis against recurrent HBV infection is given by administration of hepatitis B immunoglobulins derived from vaccinated subjects. The emergence of immune escape HBV mutants results in viral persistence despite adequate antibody titres. Identification of such S gene mutations helps devise alternative vaccines or post-transplant prophylaxis.[4] Different genotypes of HBV may predispose to disease complications (such as cirrhosis or HCC) and response to treatment.

Kits for the separate determination of viral mutants and genotypes are commercially available. The design of a comprehensive array to enable simultaneous detection of rt polymerase gene mutations, S gene mutations, and genotypes is useful.

Methods

This study was conducted from November 2006 to October 2008. Oligonucleotide primers pertaining to both the wild type and mutant sequences at the various positions along the rt polymerase and S genes of HBV were spotted onto coated glass slides. Each slide contained two sections of oligonucleotides for analysis of two different test samples. In addition, in each section, the oligonucleotides were spotted six times. Oligonucleotides for the detection of the eight genotypes of HBV were also spotted. Asymmetric polymerase chain reaction (PCR) product of the HBV gene fragment was prepared from a patient's serum DNA and used in an allele-specific arrayed primer extension reaction (AS-APEX) on the spotted array as described previously.[5]

Serum DNA from 100 unrelated HBV patients was analysed. The results were compared with those assayed previously using a commercial kit (Inno-Lipa HBV Genotyping assay; Innogenetics, Ghent, Belgium). Samples with discordant results were sequenced to ascertain the correct mutation.

Sensitivity and specificity of the array were confirmed by analysing serial samples from 45 HBV patients on long-term follow-up at the hepatology clinic of the Queen Mary Hospital. Typically serum samples were collected pre-treatment and five to seven times during therapy over a period of

TABLE. Longitudinal follow-up study of patients receiving antiviral therapy

Patient no: drug (date taken)	Initial	I	II
Patient 1: lamivudine (97), LG (04)	AA67 (11/8/97) (A67)	AA68 (20/5/04)*	AA69 (9/6/04)
Results using commercial kit	424.5 x10^6 c/mL (lamivudine↓, LG ↓)	3.74 x10^6 c/mL (180M, 204V)	3.44 x10^5 c/mL (80I, 80V, 180M, 204I)
Results of both genotype, and mutants detected by the array	C: wild type only	C: L80, 80I, 80V, V84, 84M, L180, 180M, 204I	C: L80, 80I, 80V, V84, 84M, L180, 180M, 204I
Patient 2: lamivudine, LG	AA83 (3/9/97)	AA84 (8/9/04)*	AA86 (12/10/04)
Results using commercial kit	311.3 x10^6 c/mL (Lamivudine↓ LG↓)	28.1 x10^6 c/mL (180M, 204I)	1.63 x10^6 c/mL (80I, 80V, 180M, 204I)
Results of both genotype, and mutants detected by the array	C: wild type only	C: 80I, 80V, L82, 82M, L180, 180M, 204I	C: 80I, 80V, L180, 180M, 204I
Patient 3: adefovir (15/11/03)	50 (13/1/04)*	M1 (14/6/04)	M2 (21/9/04)
Results using commercial kit	8.26 x10^5 c/mL (80I, 180M)	1.2 x10^3 c/mL (80I, 180M, L180, 204I)	1.65 x10^2 c/mL
Results of both genotype, and mutants detected by the array	B, C: S78, T78; 80I, S85, 85A, L179, 179P, 180M, V191, 191I, 204I	B: 80I, 180M, 204I	B: L82, 82M, T128, 128A, V207, 207M
Patient 4	37 (26/4/04)	N1 (3/1/05)*	N2 (5/12/05)
Results using commercial kit	7.9 x10^4 c/mL (80V, 80I)	<2000 c/mL (no polymerase chain reaction product) [10/10/05 11935 c/mL 80I]	1.6 x10^3 c/mL
Results of both genotype, and mutants detected by the array	C: L80, 80I, 128A, L180, 180M, 229V (S gene)	C: 80I, 128A, 204I	C: 80I, 128A, 204I

* Earlier detection of mutants by hepatitis B virus array

1 to 3 years. The results of the array analysis were compared with those detected with the Inno-Lipa kit. Genotypes of these samples were also recorded.

Quantitative PCR based on the TaqMan Technology was used to assess the copy number of HBV mutants in five different HBV serum samples. Each serum sample was then subjected to a serial doubling dilution with the same starting copy number of Eurohep (HBV standard DNA sequence containing the wild-type virus only). These diluted samples were then used for array analysis. The minimum amount (copy number/mL) of mutant strain detectable was calculated. The experiment was repeated twice for each of the five samples.

In addition to analysis of samples from Chinese patients with hepatitis B, 25 samples from patients of other ethnicities were analysed to validate the eight oligonucleotides for the determination of HBV genotypes. These samples were also assayed by the commercial kit (Inno-Lipa HBV Genotyping assay) and sequencing was performed to confirm the genotypes.

Results

Of the initial 100 HBV patients' samples analysed, 56 samples gave concordant results with mutations detected by the array as well as the Inno-Lipa HBV Genotyping assay. In 43 samples, additional mutations were detected on the array. The presence of these mutations was confirmed by sequencing.

Results of samples from most patients were concordant using both the array and the Inno-Lipa kit. In a few patients, the emergence of drug-resistant mutations was detected 3-7 months prior to overt biochemical relapse of hepatitis. This indicates the enhanced sensitivity of the array. In addition, it was possible to assay by array samples containing <60 c/mL of HBV (as the lower limit determined by the commercial quantitative PCR kit). The Table shows the results of four selected patients on longitudinal follow-up.

Serial dilutions of one serum sample with an equal amount (viral copy number) of Eurohep, PCR amplification, and analysis by the array showed that it was possible to detect the mutants down to a minimum of 139 c/mL at a dilution of 1:20 480. By diluting the first PCR product an extra 10-fold from a 1:10 240 dilution (ie final dilution of 1:102 400), it was possible to achieve a detection limit of 28 c/mL. Similar results were obtained for the other four serum samples tested.

Oligonucleotides for the detection of HBV genotypes were validated from 25 serum samples sent from our overseas collaborator. It was possible to validate oligonucleotides for genotypes A, B, C, D,

E, and G with these samples. We failed to find any samples with genotype F and H, possibly because those are extremely rare and not associated with any of the ethnic groups we have studied.

Discussion

The HBV array in the present study has been validated through -5(,)-[(o)-5(f)(a)4(u)-5(c)-10(s)-10(h)-5(T*[(o)-5(f)-5( )-167(d)-5( )-16(n)-5(o)11,)-[(o)-5(f(h)-5(T*[(o)-5(f)-5([([(o)-