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Journal of Clinical Virology 34 Suppl. 1 (2005) S49–S53

JOURNAL OF CLINICAL VIROLOGY

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The DNA-Chip technology as a new molecular tool for the detection of HBV mutants

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Abstract

Genetic variability of the Hepatitis B virus (HBV) strongly impacts the natural history of infection and the efficiency of diagnosis, vaccination and treatment. A genotypic assay able to provide genetic information on all HBV genes would be a very important tool for clinicians and epidemiologists. The DNA-Chip technology has proved to be powerful and convenient for the diagnosis of infectious diseases due to bacteria and viruses. We have designed a genotyping assay based on polymerase chain reaction and DNA-Chip for HBV. Starting from a unique specimen, analysis of polymorphisms at 150 positions along the genome and 383 mutations is possible as well as the determination of the genotype. Preliminary experiments with plasmas from infected patients show that results obtained with this reagent are strongly correlated with those obtained with sequencing.

Keywords: HBV; Mutations; Genotyping; DNA-Chip

1. Introduction

Infection by the Hepatitis B virus (HBV) is usually chronic. The HBV disease can be acute, fulminant, asymptomatic or chronically active. Ultimately, the infection can lead to cirrhosis and liver cancer. It has been suggested that polymorphisms in several HBV genes have an impact on disease evolution (Hunt et al., 2000; Locarnini et al., 2003). Such polymorphisms have been described in the basal core promoter (BCP), in pre-core and core, and pre-S1 and pre-S2 as well as in the X gene. Beside interferon, several antiviral treatments are now available and others will be available soon. These molecules target the DNA-polymerase activity encoded by the *pol* gene. Although these treatments are usually very efficient, they generally select resistant variants wich bear mutations in *pol*. For example, mutations in the YMDD motif of the HBV polymerase are detected in 14–32% of patients after one year of treatment with lamivudine and in up to 70% after treatments longer than 4 years (Lai et al., 2003). A genotypic assay would also be useful to select and adapt treatment by detecting mutations in *pol*. There are 8 HBV genotypes and recent evidence suggests that different genotypes have different severity and sensitivity to treatment (Ganem and Schneider, 2001). Finally, polymorphisms in the *a* determinant of the *s* gene have been associated to vaccine and immune therapy escapes and to failures of HBsAg diagnostic kits to detect infections. Ideally, a genotypic assay dedicated to HBV would be useful to clinicians for the prognosis of the evolution of the disease and for selecting and adapting treatments, to epidemiologists for monitoring and control of the spread of resistant and immune escape variants, and to vaccine and diagnostic industries for improving the formulations of their products. This assay would then need to detect polymorphisms in all HBV genes and to determine genotype.

There are several genotyping technologies that can be adapted for HBV. Allele-specific polymerase chain reaction (PCR) is rapid and easy-to-use but is limited to one or very few polymorphisms. Nitrocellulose-based hybridization assays are sensitive to minor populations. However, it is not possible to analyse all polymorphisms of interest in a single strip. Sequencing is currently the mostly used. Sequencing can analyse the complete genome and detect unknown polymorphisms. However, the sequencing process is complex and time-consuming and the interpretation of electrophoregrams is subjective and may lead to results variability. DNA-Chip reagents can only detect known mutations but can automatically generate a highly reliable analysis of the complete genome.

2. DNA-Chip technology for infectious diseases diagnosis

DNA-Chips are glass surfaces covered by probes that are used as detection tools following extraction and

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1590-8658/ $ – see front matter

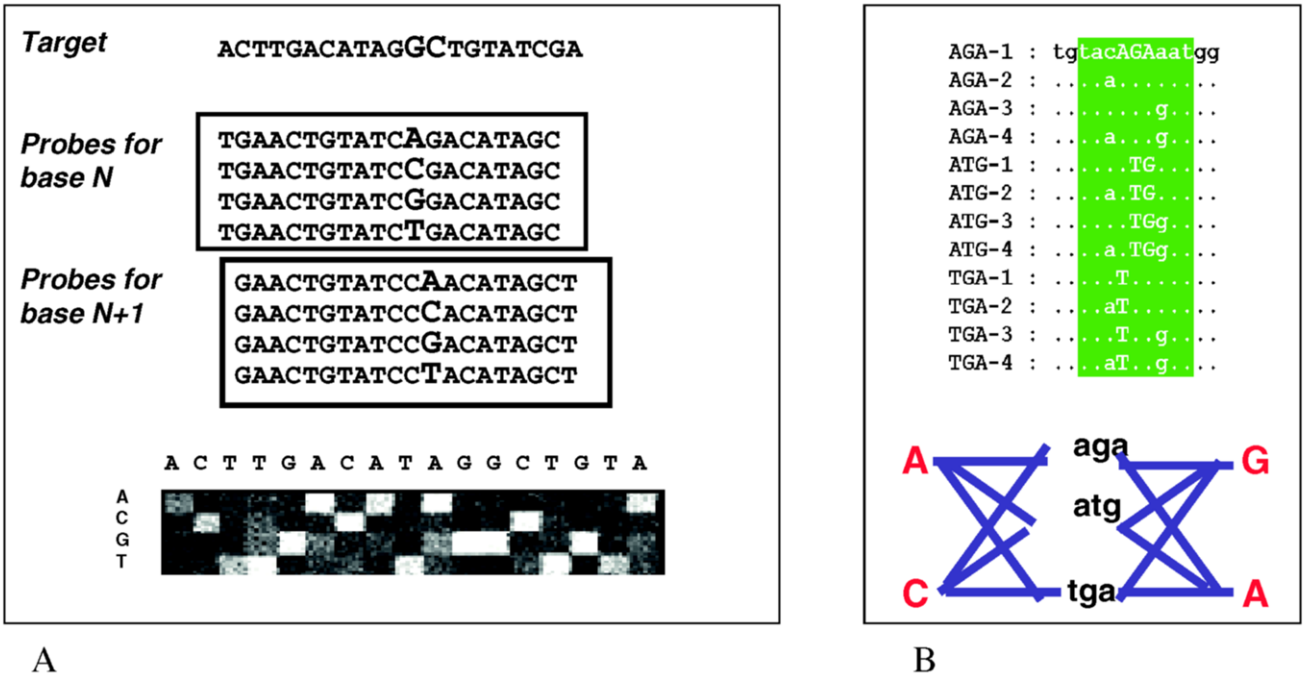

Fig. 1. 4-L tiling resequencing strategy. (A) Series of 4 probe sets are used to determine the base present at each position of the target region. These probes have the same sequence except at the interrogation position where one of the 4 possible bases is present. The probe which generates the highest fluorescence signal is considered as complementary to the target. (B) Additional probe sets are used when natural polymorphisms affect the genetic background of the virus in the vicinity of the analysed position.

amplification of nucleic acids. There are different chip technologies. Assays that require low probe densities, for example for virus identification, can use spotted chips. Oligonucleotides are chemically synthesized or produced by PCR before spotting. PCR-produced probes can contain more than 70 bases and thus tolerate mismatches. However manufacturing processes for spotted chips are difficult to standardize. Oligonucleotides *in-situ* synthesis technology, derived from computer chip photolithography, has been developed by Affymetrix Inc. (Santa Clara, CA, USA) to produce DNA-Chips with a highly standardized manufacturing process (Fodor et al., 1993). Smaller probes (17–25-mer oligonucleotides) can be synthesized but very high densities can be reached, to obtain a broad range of information and to provide robust results. Currently, photolithography masks required for chip synthesis can define $11 \times 11\,\mu m^2$ spots. Each spot contains several million copies of an individual oligonucleotide. A chip with a 1.6 square centimetre surface contains 850 000 spots with individual oligonucleotides. To synthesize 20-mer oligonucleotides, a series of 80 individual masks (4 masks for each oligonucleotide base rank) is required. The design of a new chip or the modification of a chip to add new sequences or to improve the sequence of existing probes (for example, to correct sequence errors or to increase probe length and hybridization efficiency) requires a new mask set, whose price is relatively high. As a consequence, it is very important to build extensive sequence databases and to include probes able to detect the maximum number of polymorphisms, even if some of them have not yet been strongly associated with consequences at the phenotypic level.

Re-sequencing using an Affymetrix chip is obtained with 4L-tiling (Fig. 1A). A series of 4 probes are used to identify the base at a given position in a target of interest. All probes have the same sequence, which is complementary to the expected sequence of the amplicon generated from the target, except at the "interrogation position" corresponding to the base to be analyzed. At that position, the probes differ in that they contain one of the 4 bases. The interrogation position is usually central to the probe. The probe with a perfect match with the amplicon will generate a hybridisation signal significantly ($\geqslant$20%) higher than the signal obtained with the other 3 probes and allows to identify the base in the target at that position. To identify the next base, another series of four probes is used, whose sequences are shifted by one base along the sequence. Thus, to re-sequence a target of 10 bases, at least 40 probes are required. However, in some instances, many more probes are required. HBV, like RNA viruses, is highly genetically variable. Natural polymorphisms in the vicinity of an interrogated position can interfere with hybridisation. Probes with different background sequence must be synthesized, based on exhaustive sequence databases. Figure 1B shows an example of probe sets used to analyse codon 215 of the reverse transcriptaseT gene of human immunodeficiency virus (HIV). Three possible codons can be found at that position, two of them (atg and tga) being associated with resistance to azidothymidine (AZT). In addition, depending on the viral strain, two bases (a or c)