Viral mutant discovery in hepatitis B virus quasi-species in patients undergoing long-term lamivudine treatment

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Introduction

Hepatitis B virus (HBV) infection is a common cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma in Asia. The HBV reverse transcriptase lacks proof-reading activity. As a result, it has a much higher error rate than DNA viral transcriptases, as it replicates through a RNA intermediate. HBV DNA is thus often present in quasi-species in an individual. One or more species may be favourably selected by factors such as host immune clearance and use of antiviral drugs.\(^1\)

HBV variation plays an important role in HBV genotypes and drug-resistant mutations. It is clinically important to detect known and unknown mutations that are associated with or conferred by drug resistance.\(^2\) Current methods are insufficient to detect minor proportions of unknown mutants in an economic and efficient way. The most widely used method to detect novel mutants is direct sequencing. This provides complete sequence information, but it is not sufficiently sensitive for minor mutants present at <20% of the entire HBV population. Cloning and sequencing of virus sequences can also be used to detect minor mutations in HBV quasi-species, but it is costly, time-consuming, and labour-intensive.\(^3\)

Thus, a high-throughput method for detecting virus mutations at minor proportions is needed. Matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) enables high accuracy, sensitivity and specificity. Some research groups have developed specific and sensitive assays based on this technology to detect known mutations.\(^4,5\)

This study evaluated the performance of base-specific RNA cleavage and MALDI-TOF MS in HBV from chronic hepatitis B (CHB) patients undergoing long-term lamivudine treatment.

Methods

This study was conducted from October 2007 to September 2009. A total of 195 serum samples from...
30 CHB patients who received lamivudine treatment for 3 to 4 years, and 104 serial serum samples from 15 patients were analysed. For each patient, a pre-treatment sample and follow-up samples at 3-month intervals were collected. Mutations detected by MALDI-TOF MS were also studied by bi-directional sequencing and investigated randomly by cloning and re-sequencing.

**Results and discussion**

**Base-specific RNA cleavage and MALDI-TOF MS**

This new comparative approach combines the base-specific RNA cleavage reaction and automated MALDI-TOF MS analysis. HBV DNA sequence is amplified in two separate reactions that provide analysis for both strands (Fig 1). T7 promoter sequence tagged at the 5’ end of the primer is incorporated into the target sequence during PCR reaction to initiate single-stranded RNA synthesis from the double-stranded PCR product via in vitro transcription. During in vitro transcription, deoxycytosine was used instead of cytosine (C), and thymidine (T) was substituted by uracil (U). Base-specific cleavage was achieved using RNase A, which ordinarily cleaves single-stranded RNA at each C or U residue. MALDI-TOF MS was applied to the products of each cleavage reaction and extracted a list of signal peaks with masses and intensities. Mass spectra obtained from four cleavage reactions were specific to each of the four bases of the target sequence.

**Establishment of reference sequence**

To monitor the nucleotide changes during antiviral treatment, the follow-up sequences of HBV isolated were compared with the sequence of HBV isolated before treatment. For each patient, the DNA sequence of the pre-treatment sample was obtained by directly sequencing PCR product using the primer pair of 683F and 915R and confirmed by another pair of primers (509F and 915R).

**Detection of viral mutations in HBV quasi-species**

After cleavage reactions, four characteristic patterns of fragments were generated and analysed by MALDI-TOF MS. Discovery of nucleotide changes was based on observing the changes in signal pattern by comparing measured spectra with an expected signal pattern predicted in silico from a reference sequence. Two types of signal changes indicate mutations: emergence of entirely new peaks and intensity change of existing peaks. The emergence of new peaks was the primary consideration for mutation identification because it displayed solid and reliable information on the new peak, whereas the
intensity change was used as supporting evidence. In addition, the surrounding sequence of mutations and concurrence of two mutations would affect the spectra and consequent analysis.

Detection of nucleotide change at position 720 of the HBV sequence in patient L011 was used as a demonstration. Figure 2a shows a portion of the spectrum ranging from 1960 Da to 2460 Da of T-specific cleavage of forward RNA transcript. The spectrum of the pre-treatment sample comprised seven expected signals, reflecting the fragments observed from the reference sequence. Comparison of the spectrum of the post-treatment sample with that of the reference sample yielded two observations: (1) a new signal represented a cleavage product with a composition of C5A1T1 (2100.4 Da), which was localised at position 38 of the amplicon (or nucleotide 720 of HBV sequence); (2) the intensity of the existing peak C6A1T1 (2389.6 Da) derived from the reference sequence decreased in the post-treatment sample. In the T-specific cleavage of reverse RNA transcript, partial replacement of wild type C by mutation T was confirmed. An additional peak at 4533.3 Da was detected while the signal intensity at 4548.8 Da was much lower in the post-treatment sample (Fig 2b). No informative signal could be obtained from the C-specific cleavage of forward RNA transcript because the mutation-specific signal at 1911.2 Da of the post-treatment sample shared the same mass with other fragments in this reaction (Fig 2c). Confirmation was obtained in the spectrum of C-specific cleavage of the reverse transcript for the presence of a new peak at 4345.6 Da (Fig 2d).

The analysis of a mutation point was complicated by the presence and close proximity of a second mutation. For example, limited information could be obtained from the four cleavage reactions in identifying nucleotide changes at position 739 and 741 due to the nature of MassCLEAVE and detection range of MALDI-TOF MS. In addition, in some extreme cases, the detection of certain mutations was restricted by the surrounding DNA sequences.

**Discovery of mutations in HBV quasi-species using MALDI-TOF MS and capillary sequencing**

It is crucial to monitor the nucleotide changes and consequent amino acid substitutions during antiviral treatment. The baseline aminotransferase was 1135 IU/L and HBV DNA was \(10^{4.2}\) copies/mL (Fig 3). Initial treatment with lamivudine had suppressed

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FIG 3. Dynamics of serum aminotransferase (ALT), hepatitis B virus (HBV) DNA levels and amino acid substitutions in patient L011 during lamivudine (LMV) therapy. LMV-resistant mutations (rtM204V/I) were detected 12 weeks after initiation of therapy. Amino acid changes at residues 222 and 229 were accompanied by the emergence of rtM204V/I.

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ALT (IU/L)  
-  +  +  +  +  +  +  +

rtM204V/I  -  +  +  +  +  +  +  +

rtA222T  -  +  +  +  +  +  +  +

rtL229V/C  -  -  +  +  +  +  +  +
the viraemia, resulting in the decline of HBV DNA to $10^7$ copies/mL and serum aminotransferase to 30 IU/L after 12 weeks. Nonetheless, at follow-up time points, viral load had increased back to baseline levels. Seven out of eight samples at consequent time points showed lamivudine-resistant mutations—rtM204V and rtM204I. Additionally, amino acid changes at position 222 (rtA222T) and at position 229 (rtL229V/C) had been present along with the rtM204V/I mutations in this patient since 12 weeks. Early emergence of rtM204V/I was associated with lamivudine resistance, and other amino acid changes along with rtM204V/I may have contributed to antiviral treatment failure.

MALDI-TOF MS, combined capillary sequencing, offered such a high-throughput platform for discovering novel mutations. Its detection of multiple mutations in a single sample was generally good with up to eight mutations detected in patient L050. In addition, MALDI-TOF MS is cheap to perform (around US$4 per sample for four cleavage reactions). Therefore, screening for potential mutations should be by MALDI-TOF MS for most samples, followed by confirmatory sequencing for special fragments in certain samples. This can be achieved with high efficiency and reduced cost.

**Conclusions**

MALDI-TOF MS is an accurate and cheap method to detect novel viral mutations, and has a synergistic effect with capillary sequencing in identifying a minor proportion of viral quasi-species. Further studies of clinical and functional significance of the identified mutations are needed.

**Acknowledgement**

This study was supported by the Research Fund for the Control of Infectious Diseases, Food and Health Bureau, Hong Kong SAR Government (#07060222).

**References**