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Rapid identification of YVDD mutants of hepatitis B virus using smart amplification process with competition probe

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Summary. – Accurate and timely detection of drug-associated viral mutants is important during antiviral therapy. Combining Smart Amplification Process (SMAP) with competition probe, an assay specifically designed to detect point mutation at codon 204 of the hepatitis B virus (HBV) polymerase gene was developed. This assay was sensitive to detect 20 copies of mutant/reaction and recognize as little as 1% of minor mutants in the viral population. The comparison of direct sequencing and SMAP method on 35 clinical specimens showed the concordance in 88% of the cases. This method provides an efficient alternative for rapid identification of HBV mutation associated with lamivudine resistance.

Keywords: hepatitis B virus; YVDD mutant; SMAP; lamivudine

Introduction

Over the past decade, a remarkable progress has been made in the effectiveness of the nucleoside/nucleotide analogs in suppressing HBV infection (Ilioeje *et al*., 2007). Under the selection pressure generated by the presence of an antiviral agent, however, viruses with a mutation that confers a replication advantage are selected and eventually become the predominant viral species (Ghany and Doo, 2009). Because of this, the long-term therapy with nucleotide analogs is associated with the development of antiviral drug resistance (Lok *et al*., 2003; Liaw *et al*., 2004). The emergence of drug resistance becomes a major challenge in the era of antiviral therapy.

As one of the primary antiviral agents, lamivudine is the first oral nucleoside analogue approved for the treatment of chronic hepatitis B (Reinis *et al*., 2001). Resistance to lamivudine occurs rapidly and affects 24% and 70% of chronic carriers after 1 and 5 years of therapy, respectively (Lai *et al*., 2003). The principal mutations identified in HBV as a result of lamivudine treatment are located at codon 204, within the tyrosine-methionine-aspartate-aspartate (YMDD) motif of the virus reverse transcriptase, leading to the replacement of methionine (M) by valine (V) or isoleucine (I) (rtM204V/I) (Ghany and Liang, 2007).

Methodological options for detecting mutations are an increasingly important issue in HBV clinical management (Kim *et al*., 2008). Direct sequencing is the gold method. However, the sensitivity of direct sequencing-based methods is limited, since it is only capable of detecting mutations present in viral quasispecies with a prevalence of ≥20% of the total HBV population (Lok *et al*., 2007). To date, a number of other techniques have been reported for detecting drug-induced mutations of HBV (Yeon, 2008), such as restriction fragment length polymorphism (Jardi *et al*., 1999; Hosseini *et al*., 2006), oligonucleotide microarray technology (Li *et al*., 2005), selective real-time PCR (Lupo *et al*., 2009), matrix-

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Abbreviations: BP = boost primer; CP = competition probe; FP = folding primer; HBV = hepatitis B virus; OP = outer primer; SMAP = Smart Amplification Process; TP = turn-back primer; wt = wild-type; YMDD = tyrosine-methionine-aspartate-aspartate; YVDD = tyrosine-valine-aspartate-aspartate

assisted laser desorption/ionisation time-of-flight mass spectrometry (Kim *et al.*, 2005), as well as pyrosequencing (Lindstrom *et al.*, 2004; Margeridon-Thermet *et al.*, 2009; Solmone *et al.*, 2009). However, these technologies are still relatively time consuming, complicated, and expensive for routine screening. Since drug resistant viral mutants are usually identified after several months of drug treatment with re-elevation of serum HBV DNA levels and flare-ups of serum transaminase levels, it is important to establish a method to detect the lamivudine-resistant HBV mutant as early as possible, when the mutant virus is still a minor fraction of the total HBV population.

SMAP is a novel approach that can detect single nucleotide polymorphism with a high sensitivity and specificity under isothermal conditions (Mitani *et al.*, 2007). This method has been successfully used in the mutation detection of human genes (Watanabe *et al.*, 2007; Hoshi *et al.*, 2007; Aomori *et al.*, 2009; Inoue *et al.*, 2009; Araki *et al.*, 2010). In this study, we tested the applicability of SMAP method to detect tyrosine-valine-aspartate-aspartate (YVDD) mutant virus. The main goal of the research was to develop a reliable and cost-effective method for rapid screening of drug-resistant viral mutations.

Materials and Methods

Serum samples. Thirty-five samples, obtained from the patients receiving lamivudine therapy to treat chronic HBV infection, were included in the study. Patients enrolled in this study had been hepatitis B surface antigen-positive for 6 months or longer and had elevated ALT values, with HBV DNA levels greater than 20,000 copies/ml before commencement of lamivudine therapy. Neither of these patients was positive for anti-hepatitis C virus antibody or anti-human immunodeficiency virus antibody (ELISA, KeHua Bio-Engineering Co., Ltd, China). Informed consent was obtained from all patients, and the appropriate institutional review board approved the study.

Real-time PCR for HBV DNA. HBV DNA was extracted from a 50 µl sample by using extraction reagents of the commercially available HBV DNA real-time PCR detection kit (DaAn Bio-Engineering Co. Ltd., P.R. China), as per the manufacturer′s instructions. HBV DNA levels were determined by DaAn TaqMan real-time PCR assay, with a detection limit of 3 log copies/ml.

Construction of YMDD and YVDD plasmids. To assess SMAP on well-characterized material, previously sequenced clinical samples harboring the rtM204V mutation and the wt were used as templates for plasmid cloning. PCR products were amplified using forward primer 5′-GTATGTTGCCCGTTTGTCCTC-3′ (459–479 nt) and reverse primer 5′-AGCAAACACTTGGC AGAGACC-3′ (1168–1188 nt). Samples were amplified in a 50 µl reaction containing 5 µl of 10× buffer (TaKaRa, Japan), 4 mmol/l $MgCl_2$, 200 µmol/l each dNTP, 0.2 µmol/l each primer, 1.5 U Hot Start Taq DNA polymerase and 2 µl of template DNA. After an initial denaturation for 5 mins at 95°C there followed 40 cycles of 95°C for 45 secs, 58°C for 1 min, and 72°C for 2 mins. A final cycle with an elongation step of 10 mins at 72°C was included at the end. The PCR products were purified by PCR purification kit (Axygen, USA), and inserted into pMD®18-T Vector using the TA Cloning kit (TaKaRa, Japan) according to the manufacturer′s instructions. After the multiplication in *E. coli* DH5a, plasmids were extracted using the MiniBEST Plasmid Purification Kit (TaKaRa, Japan) and analyzed by direct sequencing to confirm the presence of the expected mutations. DNA plasmids were then quantified by SMA1000 UV Spectrophotometer (Merinton, China).

Sequencing. The sequencing assay was adapted from Vincenti *et al.* (2009) with slight modifications. The first-round of nested PCR was performed using forward primer 5′-CCTGCTGG TGGCTCCAGTT-3′ (56–74 nt), and reverse primer 5′-CRTCA GCAAACACTTGGC-3′ (1175–1192 nt). The second-round PCR was performed using forward primer 5′-CTCGTGGTGGAC TTCTCTC-3′ (253–271 nt), and reverse primer 5′-GCAAANC CCAAAAGACCCAC-3′ (1000–1019 nt). Amplification conditions for both the first and the second rounds were the same as those used for the plasmid cloning. Purified PCR products were sequenced using the primers similar to the second round of PCR, according to the manufacturer's instruction of the Amersham Chemistry DYEnamic ET Terminator Sequencing Kit (Amersham Biosciences, USA).

SMAP assay. A SMAP primer set, including the turn-back primer (TP), the folding primer (FP), the boost primer (BP), and two outer primers (OP1 and OP2), suitable to detect allele-specific amplification by SMAP was designed using Primer3 software. For suppression of the background amplification, the 3′-end of FP and the 5′-end of TP were both used to recognize the point mutation. In addition, a competition probe (CP) competing with the FP/TP on initial hybridization events for generating the intermediate products was designed. The primer set was selected as follows: OP1: 5′-TAGTGCCATTTGTTCAGTG-3′, OP2: 5′-ACCCAAA GACAAAAGAAAAT-3′, FP: 5′-GCGAGTCGCTGTTTGGCT TTCAGTTATGTG-3′, BP: 5′-GGCCAAGTCTGTACAACATCT-3′, TP: 5′-TGTGGATGATACAGCGGTAAAAAGGGAC-3′ and CP: 5′-GGCTTTCAGTTATATGGATGATGT-NH2-3′.

SMAP assays were carried out in a total of 20 µl reaction mixture containing 2 µmol/l each of FP and TP, 0.25 µmol/l each of OP1 and OP2, 1 µmol/l BP, 1.4mmol/l dNTP, 1 mol/l Betaine, 20 mmol/l Tris-HCl (pH 8.0), 10 mmol/l KCl, 10 mmol/l $(NH4)_2SO_4$, 8 mmol/l $MgCl_2$, 0.1% Tween® 20, 1/100,000 diluted original SYBR Green I, 8 U of Bst DNA polymerase (New England Biolabs, USA), 1 µl of template DNA. In cases where CP was used, its concentration ranging from 5–12.5 µmol/l in the reaction mixture was tested. SMAP reactions were performed at 60°C and DNA amplification was monitored using a real time PCR instrument, the Opticon System (Biorad, USA). After amplification, the products of the SMAP reaction were cleaved by Bsp14071 (Fermentas, LTU) and run on a 1.8% agarose gel for sizing and comparison.

Results

Testing of CP in the SMAP assay

A general rule to distinguish the wild from mutant strains is to use primers mismatching the wt strain template, which significantly compromises its amplification. At the same time, the mutant strain is selectively amplified by primers perfectly matching with the mutated template. (Lupo *et al.*, 2009). The autocycling amplification of SMAP is mostly mediated by the function of FP and TP (Kawai *et al.*, 2008). For this purpose, the base corresponding to the point mutation at both the 3′-end of the FP, and the 5′-end of TP was used to discriminate the target. Though 3 bp overlapping between the critical ends of these two self-priming primers exists, the exponential background amplification from mispriming occurs frequently in the preliminary assay (Fig. 1). To minimize false positives derived from non-target misamplification, strategy using the CP was adopted. CP was designed to compete with the self-priming elongation site on the intermediate product generated from FP or TP.

To optimize the concentration of CP, YMDD plasmids with the concentration of 10^6 copies/ml were tested in the presence of CP with the level ranging from 0~250 pmol/μl. The amplification of YMDD strains was totally inhibited in SMAP with more than 150 pmol/μl of CP. When using YVDD plasmid as a template, the amplification time was delayed with increasing CP concentration. These results indicated that using CP that has complete homology to the wt sequence is an effective way to suppress the background. CP with the concentration of 150 pmol/μl was used in the following assays.

Specificity and sensitivity of the SMAP assay

After SMAP reaction, the amplified products were detected by eletrophoresis. The results showed the specific ladder-like pattern that is similar to the hairpin induced isothermal amplification technology (Ren *et al.*, 2009). No amplification was obtained for the YMDD sample and the blank control. To confirm the accuracy of the specific amplicon by SMAP reaction, *Bsp*1407 I restriction analysis followed by electrophoresis was performed. As expected, the amplification products digested by *Bsp*1407 I confirmed that the correct target sequence had been amplified (Fig. 2).

Tenfold dilutions (10^7 to 10^1 copies/ml) of YVDD plasmid were used as standards to test the sensitivity on the real-time PCR platform. As shown in Fig. 3a, SMAP reaction rapidly amplifies (less than 30 mins) the YVDD mutants at the concentration of 10^3 to 10^7 copies /ml, whereas the same system was incapable of amplifying wt YMDD strains even after 60 mins. A "no-template" control reaction was also negative after 60 mins. Each assay was done in duplicate. The limit of the detection was 10^3 copies/ml, about 20 copies/reaction. Melting curves for the products were obtained after SMAP amplification for 60 mins and detected using 0.2°C steps, and

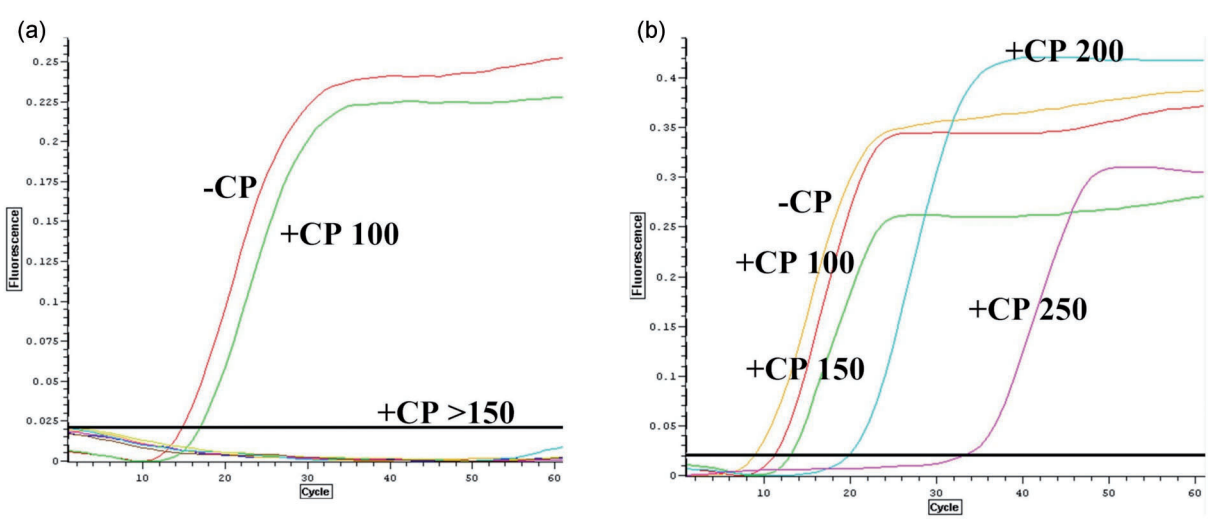

Fig. 1

Effect of CP on the SMAP assay

Effect of 0–250 pmol/μl CP on the SMAP assay of YMDD plasmids (a) and YVDD plasmids (b), both at the concentration of 10^6 copies/ml.

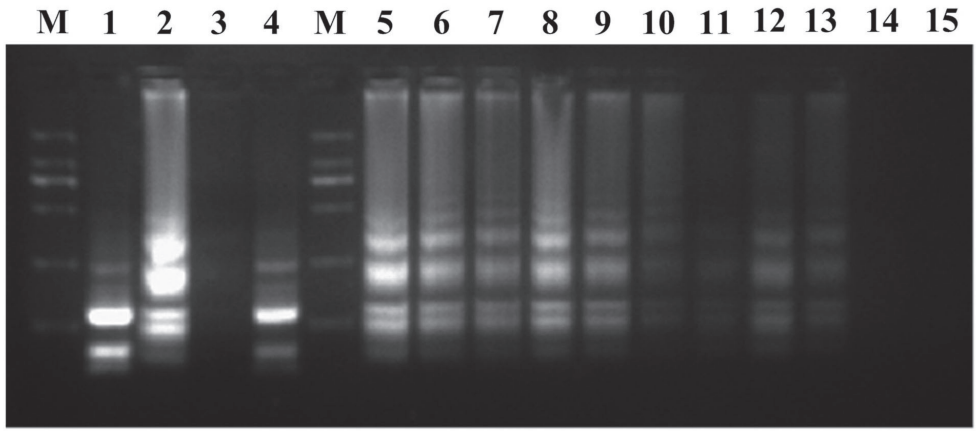

Fig. 2

SMAP assay of YVDD HBV in sera of chronic hepatitis B patients treated with lamivudine

Agarose gel electrophoresis of SMAP products. Positive sera (lanes 2, 5–13), positive sera, the SMAP products digested with *Bsp*14071 (lanes 1 and 4), negative serum (lane 3), DNA size marker (lane M), blanks (lanes14 and 15).

a hold of 1 sec at each step from 65 to 93°C. All isolates had a melting temperature (Tm) of ~85.6°C, indicating similar sequences, and hence similar amplicon.

To test the SMAP assay for the capability of differentiating limited mutants in a background of wt HBV strain, the YMDD and YVDD plasmids were mixed at a ratio of 100:0, 10:90, 1:99, or 0:100, corresponding to a final concentration of 10^5 copies. Similar results were obtained with all of the mutant plasmids tested. As shown in Fig. 3c, SMAP assay clearly identified 1000 copies of YVDD mutant plasmid in a background of 10^5 copies of heterogeneous plasmid. These results suggested that the level of sensitivity of YVDD variant present in the mixture was 1%.

Testing of the SMAP assay on clinical samples

The YVDD-resistant status of the HBV was examined in 35 samples from the patients receiving lamivudine therapy. By using both SMAP assay and direct sequencing in these samples, the two methods gave concordant results (i.e., presence or absence of a mutated strain) in 88% of the cases. In the four samples, the SMAP assay detected the presence of the rtM204V mutation, whereas the direct sequencing assay showed only the YMDD virus. Further sequencing after TA cloning was performed to evaluate the discordant results. Three of these samples were shown to consist of a mixture of viruses. For the remaining case, SMAP assay displayed exponential kinetics of the amplification profile, but with a long delay in appearance when compared to the profile of the sample with similar concentration. After subcloning, 10 randomly picked clones were sequenced and showed to be pure wt.

Discussion

Management of antiviral resistance has been well shaped in recent years (Fournier and Zoulim, 2007; Ghany and Doo, 2009). One concept is that the rescue therapy should be initiated at the time of virologic breakthrough, which is defined as a 10-fold increase in serum HBV DNA from nadir during the treatment in a patient, who had an initial virologic response (Lok and McMahon, 2009). However, there is a time lag between genotypic and phenotypic resistance to lamivudine ranging from 3 to 24 months (Hadziyannis *et al.*, 2000). In this regard, regular monitoring for genotypic resistance throughout treatment is mandatory to ensure early detection and early rescue of drug resistance (Liaw, 2007).

SMAP reaction is a unique genotyping technology that can detect genetic mutation under isothermal condition (Mitani *et al.*, 2007). Unlike the SMAP technology used in the detection of human gene mutation reported previously, this study aims to detect specific mutants in a viral population. Adding to the complexity is that the patients undergoing the antiviral therapy often contain a subpopulation of viral mutants mixed within the wt viral strains.

Compared with other methods for mutation detection, the SMAP technique has certain advantages. First, high amplification efficiency of isothermal amplification technology implies the high sensitivity. It has been reported that the detection using SMAP is at least 10-times more sensitive than PCR amplification and sequencing, which is the usual methodology performed today for mutation detection (Kawai *et al.*, 2008). The YVDD SMAP assay in this study could accurately detect as few as 10^3 HBV copies/ml and could discriminate minor mutant populations that comprised only

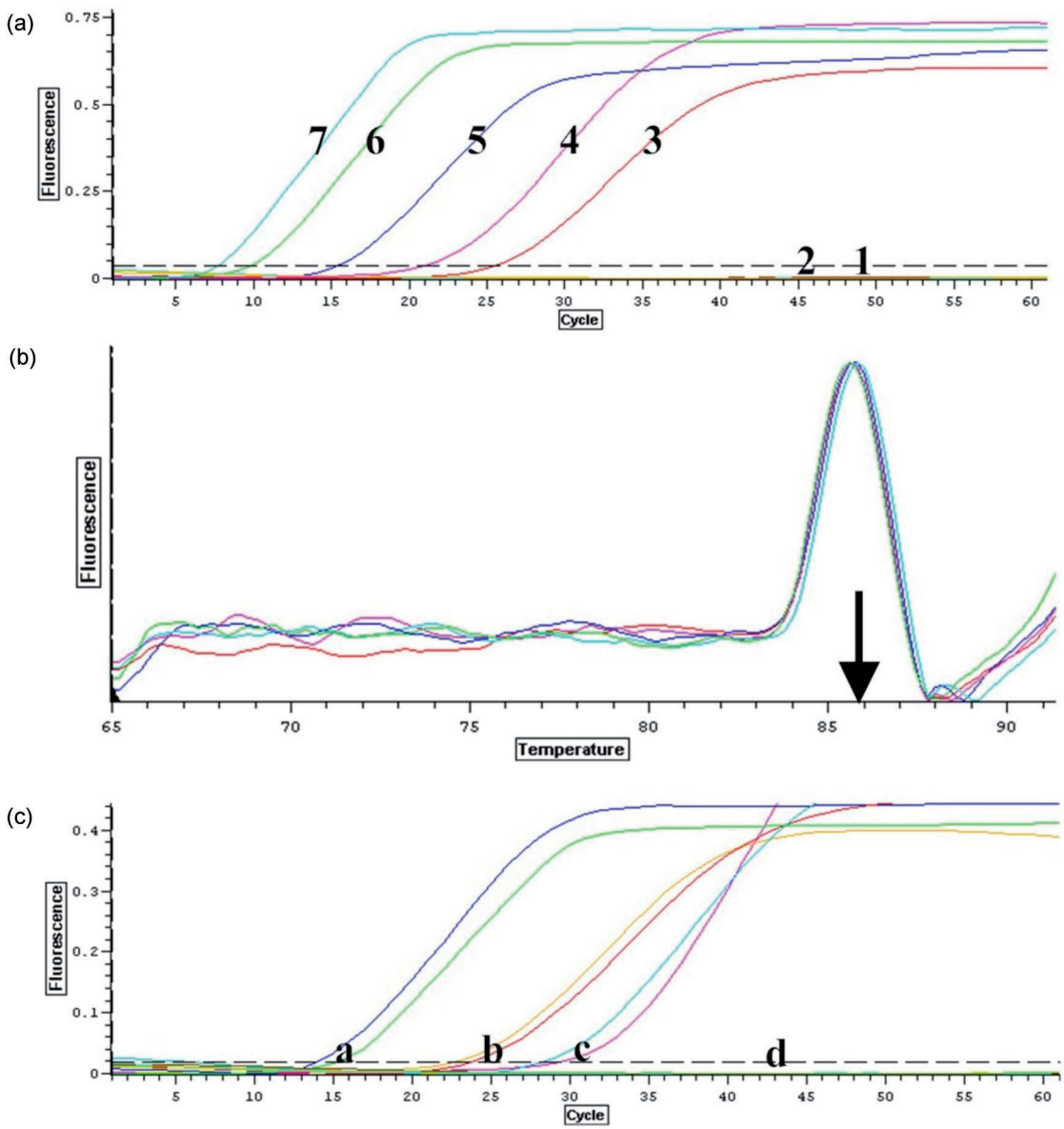

Fig. 3

Sensitivity and specificity of the SMAP assay

(a) The amplification plot from YVDD plasmids in the concentration range from 10^1 to 10^7 copies per ml (curves 1–7). (b) Melting curves of the SMAP products from (a). (c) Amplification plots from various mixtures of YVDD and YMDD plasmids (total 10^5 copies/ml). YVDD plasmid 100% (**a**), 10% (**b**), 1% (**c**), and 0% (**d**).

1% of the wild-type/mutant DNA mixture. This indicates that the YVDD SMAP assay can detect resistant strains at a stage prior to viral breakthrough. Moreover, the YVDD SMAP assay detected more mutations than direct sequencing in clinical samples. This also suggests that the SMAP method is more sensitive than direct sequencing. Second, in terms of specificity, the SMAP method could accurately detect single or mixed populations in both plasmid and patient samples. Nonspecific amplification was not observed, even in the presence of excess background. In addition, SMAP assay described in this study could be accomplished in less than 1.5 hr, while other methods are still time consuming, requiring several steps such as DNA extraction, PCR, electrophoresis, etc. It has been reported that the SMAP assay

can amplify the targets directly from whole blood or blood samples, requiring only a simple heat lysis and denaturation step (Tatsumi *et al*., 2008). Therefore, this method is suitable for routine examination at most medical situation.

The advantage of the SMAP assay is based on its mechanism of multiple strand extension co-functioning and self-priming auto-cycling without temperature change. One limitation of SMAP technology is that it requires careful design of primer sets, however. Though the discrimination primer recognizing the target mutation was simultaneously designed at the 3′-end of the FP, and the 5′-end of TP in SMAP format, the background noise derived from the high amplification efficiency of the SMAP assay still occurred. By using CP, the quality of primer set was improved without compromising amplification efficiency. We have demonstrated complete suppression of the exponential background amplification through the use of CP in YMDD mutation detection, indicating that multiple blocking strategies could be adopted simultaneously to optimize the design process. One sample detected positive by the SMAP assay showed the pure wt virus by sequencing. Though analysis of more clones may detect minor subpopulations of the mutant, this case could not exclude the occurrence of mis-amplification of SMAP reaction. One potential way is to adjust CP. Since the Tm of CP used in this study is 53′, a more rational design is to modify CP by locked Nucleic Acid or peptide nucleic acid to enhance its hybridization efficiency. In addition, the SMAP method can be applied only to known point mutations at fixed positions. With several important drug-related mutations described to date, however, one possible SMAP solving method is using a combination of mutation-specific primer sets, since SMAP assay has been successfully used in many different mutation profiles like point mutations, in-frame deletions, insertions, and duplications, etc. (Watanabe *et al*., 2007; Aomori *et al*., 2009; Miyamae *et al*., 2010).

In conclusion, a novel assay based on SMAP method for identifying YVDD mutant viruses in patient samples has been described. The SMAP method can accurately and sensitively detect lamivudine-resistant mutants in an easy, rapid, and cost-effective way, making the SMAP technology a practical tool for widely use in management of anti-viral therapy.

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