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Rapid and sensitive detection of hepatitis B virus 1762T/1764A double mutation from hepatocellular carcinomas using LNA-mediated PCR clamping and hybridization probes

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Abstract

The 1762T/1764A double mutation of the hepatitis B virus (HBV) basal core promoter has been suggested to be a potential biomarker for hepatocellular carcinoma (HCC) among individuals with chronic HBV infection. In this study, a real-time PCR assay is established using the hybridization probes and an oligonucleotide clamp containing locked nucleic acids (LNAs). The LNA-containing oligonucleotide clamp specific for the wild type HBV is able to suppress the amplification of the wild type HBV templates. In addition, the clamp can inhibit the binding of the WT templates to the fluorescence probes thereby suppress the wild type HBV signals during the melting curve analyses. These effects facilitated the detection of HBV double mutation in the presence of 3000-fold excess of the wild type genome. Thus PCR amplification coupled with the melting curve analyses provides a quick, simple, and highly sensitive tool for the detection of this HBV double mutation.

1. Introduction

Hepatocellular carcinoma (HCC) is a major cause of cancer morbidity and mortality in many parts of the world. One of the major etiological factors associated with HCC development is chronic infection with hepatitis B virus (HBV). The contribution of HBV to the pathogenesis of liver cancer is believed to be multifactorial. In addition, HBV mutants may also participate in the tumorigenesis process (Block et al., 2003; Locarnini et al., 2003; Okuda, 2002). Among the HBV variants, a double mutation comprised of adenine-1762 to thymine transversion and guanine-1764 to adenine transition (1762T/1764A) has been found in 50–85% of HCC tumor tissues (Arbuthnot and Kew, 2001; Baptista et al., 1999; Hsia et al., 1996; Kuang et al., 2004; Kuang et al., 2005). This double mutation appears to be associated with enhanced viral replication, increased severity of liver inflammation, which may ultimately increase the risk of HCC (Baptista et al., 1999; Hou et al., 1999; Laskus et al., 1995; Lindh et al., 1998; Pang

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et al., 2004; Takahashi et al., 1995). Therefore the 1762T/1764A double mutation may be a potential biomarker for HCC in long term HBV-carriers.

Detection of this double mutation can be achieved by PCR amplification of the target sequence followed by direct DNA sequencing (Baptista et al., 1999), PCR followed by restriction enzyme (Sau3AI) digestion (restriction fragment length polymorphism) (Takahashi et al., 1995), mismatched PCR coupled with restriction fragment length polymorphism analysis (Hou et al., 1999), or short oligonucleotide mass spectra analysis (SOMA) (Kuang et al., 2004). These methods are either time-consuming, labor intensive, or insensitive due to the interference by the excessive wild type (WT) HBV DNA molecules in the sample. Methods that rely on densitometry analysis have an intrinsic limit in distinguishing two bands that differ by 20-fold in intensity; thus direct DNA sequencing and restriction fragment length polymorphism require 5–20% total viral population to be mutants (mutant/WT ratio 1:4–20).

In the present study, a real time PCR assay was designed employing a locked nucleic acid (LNA)-containing oligonucleotide as the WT HBV clamp to reduce the interference from the WT HBV, thereby allowing a highly sensitive detection of this double mutation. The hybridization probes used in the real time PCR allows a post-amplification melting curve analysis to distinguish the HBV mutant from the wild type. Thus the PCR amplification and the melting curve analysis can be carried out in a single run, making the detection much quicker and simpler.

2. Materials and methods

2.1. DNA extraction and cloning

Tumor tissue DNA was isolated by proteinase K/SDS treatment followed by phenol/chloroform extraction and ethanol precipitation. The tumor tissues were from HBV-positive individuals as described previously (Zhou et al., 1997). The PCR Script® Amp Cloning kit (Stratagene, LA Jolla, CA) was used to subclone the described HBV PCR fragment. DNA samples were quantified by the absorbance at 260 nm. The copy number of the plasmid is calculated based on the sequence-predicted molecular weight and the Avogadro constant. To minimize the loss of DNA during serial dilution, 200 µg/ml bovine serum albumin (New England BioLabs, Ipswich, MA) and in low-retention siliconized tubes were used to dilute very low concentrations of plasmids.

2.2. Detection of HBV double mutation by PCR and direct DNA sequencing

A 186-bp DNA fragment from the position 1642 to 1827 of the HBV genome was amplified using the Seq Fwd (5'-ccc aag gtc tta cac aag agg-3') and the Seq Rev (5'-gga aaa agt tgc atg gtc ct-3') primers (Figure 1). The PCR reactions were assembled in a final volume of 25 µl containing 0.5 U HotStart Taq (Qiagen), 1X PCR buffer, 200µM of dNTPs, 1 µM of each primer, and 50 ng DNA templates. The cycle profile was 95°C for 10 min to activate Taq polymerase, followed by 95°C 5 sec, 55°C 15 sec, 72°C 10 sec for 40 cycles, and a final extension for 10 min at 72°C. The PCR products were then sequenced in both directions using the Seq Fwd and Seq Rev primers by the DNA sequencing core facility at the University of Pennsylvania.

2.3. Detection of HBV double mutation by LNA clamped real time PCR

The LNA-mediated clamped real time PCR reaction was assembled in a final volume of 10 µl containing 1X reaction buffer, 0.5 unit of LightCycler® FastStart Taq polymerase (Roche Applied Science, Indianapolis, IN), 50 ng of HCC tissue DNA as template, 0.4 µM of each PCR primer (LC Fwd: 5'-ccg acc ttg agg cat act tc-3'; LC Rev: 5'-aaa gtt gca tgg tgc tgg-3'), 2.5 mM MgCl₂, 500 ng/µl BSA, 200µM dNTP, 0.2 µM Sensor (5'-gag att agg tta atg atc ttt gta

cta gg-fluorescein), 0.2 μ M Anchor (5'-LCRed640-ggc tgt agg cat aaa ttg gtc tgt tca-PH), and 2 μ M HBV WT-specific LNA-containing oligonucleotide (5'-GGt tAa AGG TcT TtG t-PH; LNA monomers are indicated in upper case letters). The reaction was incubated in the LightCycler II (Roche Applied Science) at 95°C 10 min to activate the Taq polymerase, followed by amplification at 95°C 5 sec, 65°C 15 sec, 52°C 10 sec, and 65°C 10 sec for 45 cycles. The LNA-oligonucleotide has a predicted melting temperature (T_m) of 72°C, roughly 12°C higher than those of the PCR primers. Both the Sensor and the LNA clamp are phosphorylated at the 3'-end so that they cannot function as primers. The details of the oligonucleotides used in this study are illustrated in Figure 1.

2.4. Melting curve analysis of the PCR products

Immediately after the amplification cycles, the PCR products were denatured at 95°C and then cooled down to 40°C to allow hybridization with the fluorescence-labeled probes. The temperature was then increased from 40°C to 68°C at the speed of 0.1°C/sec. The dissociation of the WT sensor probe from the mutant HBV template is earlier (at a lower temperature) than from the WT HBV template due to the mismatch. Dissociation of the sensor probe terminates the fluorescence resonance energy transfer between the sensor and the anchor, which is recorded automatically by the instrument. The results were analyzed using the LightCycler™ software 3.5 (Roche Applied Science, Indianapolis, IN).

3. Results

3.1. Establishment of LNA-mediated clamped PCR assay for the HBV1762T/1764A double mutation

Plasmids carrying WT HBV DNA and the 1762T/1764A double mutation were constructed using the pPCR-Script Amp SK(+) vector (Stratagene, LA Jolla, CA). These plasmids were used as DNA templates to establish and evaluate the LNA-clamped real time PCR assay. In the absence of HBV WT-specific LNA-containing clamp, the HBV WT DNA showed a melting curve which peaks at around 60°C (Fig. 2; line 2), while those from the HBV double mutation gave a T_m peak at around 56°C (Fig. 2; line 1). When the WT HBV and the mutant DNA were mixed at approximately equal molar ratio in a single PCR reaction, a broader T_m peak was observed (Fig. 2; line 5). In the presence of LNA clamp, however, the T_m peak representing the WT HBV (60°C) disappeared, while the mutation-specific T_m peak was preserved (Fig. 2; line 6). These results indicate that the LNA-containing oligonucleotide clamp is able to specifically suppress the WT HBV signal.

To determine the efficiency of our LNA-oligonucleotide, mixtures of HBV 1762T/1764A plasmid with increasing amount of WT HBV plasmid were used as PCR templates. It was found that 2 μ M LNA clamp was able to suppress the amplification of 30,000 copies WT HBV to allow detection of 10 copies of HBV 1762T/1764A double mutation (mutant/WT ratio of 1:3,000) (Fig. 3A). At higher ratios, the double mutation was not detectable possibly due to primer titration by the excessive WT HBV (data not shown). Thus the detection sensitivity is at a mutant/WT ratio of 1:3,000.

To determine the minimal copy number detectable by the LNA PCR assay, varying amount of plasmid carrying the HBV double mutation was used in the PCR reaction while the WT HBV DNA was kept constant at 30,000 copies. A minimum of 10 copies of the mutant HBV DNA was detected in four out of four experiments. The representing experiment is shown in Figure 3B (line 4).

3.2. Detection of the HBV 1762T/1764A double mutation from HCC samples by the LNA-mediated clamped PCR assay

To validate this one-step LNA-mediated clamped PCR assay for HBV 1762T/1764A double mutation, genomic DNAs extracted from HCC tumor samples were examined by PCR and direct sequencing. The sequencing results are shown in Figure 4. Out of six HCC tissue DNA samples, four (samples 131K, 150K, 169K and 174K) were found to have HBV wild-type sequence, one (sample 8K) contained the 1762T/1764A double mutation, and one (sample 219K) showed mixed bases at 1762 and 1764 which suggest the mixture of the double mutation and the WT (Fig. 4B).

All the 6 HCC DNA samples were subjected to LNA-mediated PCR clamping and post-PCR melting curve analysis. Sample 8K, which was the only sample which showed clear-cut double mutation by sequencing, presented an atypical melting curve which has a lower T_m than that of the double mutation (Figure 5). This is possibly caused by the additional mutation (A to G) at 1752. On the other hand, sample 219K, which showed mixed bases at 1762 and 1764, gave a typical profile of a double mutation. It is possible that the WT signal was completely suppressed by the LNA-mediated PCR clamping, such that only the double mutation signal was revealed. This is reminiscent of the conditions described in Figure 2 (lines 5 and 6) using mixed plasmids. Interestingly, samples 169K and 174K, which was WT by DNA sequencing, gave a typical T_m profile of the 1762T/1764A double mutation. The remaining two samples, 131K and 150K, presented a WT profile in the absence of the LNA clamp (Fig. 5).

3.3. LNA-oligonucleotide clamp increases sensitivity of DNA sequencing

Samples 169K and 174K showed inconsistency between real time PCR and DNA sequencing. The chromatogram sequencing files for these two samples were examined in more detail. In both samples, 1762A and 1764G are the dominant bases, but 1762T and 1764A do exist, although at smaller amount (Fig. 6A, left panel). When the PCR was done in the presence of the LNA clamp, however, both samples showed predominantly 1762T and 1764A (Fig. 6A, right panel). These results confirmed the presence of HBV double mutation in the samples 169K and 174K.

To further prove that the clamp can increase the DNA sequencing sensitivity, mixtures of the double mutation with the WT plasmids were amplified in the presence or absence of the clamp. The amplicons were purified and subjected to direct DNA sequencing. In the absence of the clamp, the 1762T/1764A double mutation is barely detectable at the mutant/WT ratio of 1:4. However, the same mixture gave a clear-cut mutant readout in the presence of the clamp (Fig. 6B, top panel). The mutant signals can even be seen at the ratio of 1:16 in the presence of the clamp (Fig. 6B, lower panel). This data indicates that the clamp is able to increase the sensitivity of DNA sequencing to detect this double mutation by suppressing the amplification of the WT HBV DNA.

4. Discussion

The hybridization probes were used in this study because of their post PCR melting curve analysis capability. The probes consist of an LCR640-labeled Anchor oligonucleotide, and a fluorescein-labeled Sensor oligonucleotide that matches the WT sequence (Fig. 1). Fluorescence resonance energy transfer (FRET) between LCR640 and fluorescein occurs when the two probes anneal to the same template. The slow temperature increase during the melting curve analysis causes a gradual dissociation of the Sensor from the template, resulting in loss of the FRET signal. Mismatch between the Sensor and the mutant template will allow the Sensor to dissociate earlier. In this assay, the double mutation gave a distinct melting curve which peaks at about 56°C. The WT HBV, when amplified in the absence of LNA

oligonucleotide, gave a melting curve which peaks at about 60°C. Thus the melting curve analysis is able to provide additional confirmation in the detection of the HBV double mutation.

The intensity of the FRET signal depends on the optimal probe/template ratio. Too much templates (as a result of over amplification) can cause the Sensors and Anchors to bind to separate templates, resulting in reduced the FRET signal. Therefore the intensity of the FRET signals in the melting curve is not used for quantification purpose but is used for mismatch detection only. It should also be noted that the T_m peak may shift slightly in different experiments due to variations in salt concentrations, but the difference between the WT and the mutant is consistent. Plasmid standards are included in the experiments for comparison purpose.

Real time PCR using similar hybridization probes for HBV 1762T/1764A detection was reported recently, and showed a similar T_m difference ($\sim 4^\circ\text{C}$) between the WT and the double mutation (Zhang et al., 2002). However, the sensitivity of that assay is only at about 1:20 mutant/WT ratio. To improve the sensitivity, a LNA-containing oligonucleotide is used in this study to inhibit the amplification of WT HBV. LNAs are a novel class of bicyclic RNA analogs that have exceptionally high affinity and specificity toward their complementary DNA target molecules (Hertoghs et al., 2003; Singh et al., 1998). The LNA-containing oligonucleotide was designed to have a higher T_m than the amplification primers. During the thermal cycles, the LNA oligonucleotides anneal to the WT HBV templates before the PCR primers, and stay annealed during the 65°C synthesis step, thereby blocking the polymerase extension on the WT DNA template. The suppression of WT HBV amplification is confirmed by DNA sequencing (Fig. 6). In addition, the clamp competes with the Sensor in the real time PCR thereby diminishing the WT signals. This allowed a highly sensitive detection of the double mutation even in the presence of 3,000 fold excess of WT HBV (1:3,000 mutant/WT ratio). In contrast, direct DNA sequencing in the absence of the clamp requires a mutant/WT ratio of $< 1:4$ ($> 20\%$ mutant).

Because HBV virus replicates its DNA genome using a reverse transcriptase that has a high error rate (Pult et al., 2001), mutant viral genomes are frequently found. The additional polymorphic mutation(s) in the targeted area may impact our assay in several ways. First, if significant variations occur in one of the amplification primer regions, PCR amplification will be compromised and detection of the potential double mutation is not possible. To minimize this impact, the 3'-end of both primers are placed at highly conserved regions of HBV genome. Second, when the double mutation is accompanied by additional mutation(s) in the region covered by the sensor probe, the T_m peak will deviate (left shift) from that of the double mutant, but still distinct from the WT HBV. This occurred in the sample 8K (Fig. 5). Thus in the case of an atypical melting curve, DNA sequencing is required for ultimate clarification. Third, certain mutations/ or variants in this region may escape the inhibitory effect of the clamp. A NCBI BLAST was performed to search for the known variant in the clamp region. In addition to 1762T/1764A double mutation, there is only one variant, G1757A, that accounted more than 1% of the total hits in the entire search. A plasmid containing this variant was constructed and subjected to the LNA mediated double mutation assay. G1757A mutation is approximately 50% less prone to the inhibitory effect of the clamp than the WT without the G1757A mutation (data not shown). However, its melting profile is distinct from that of the double mutation (data not shown), thus it will not produce false positivity even if G1757A escapes the inhibitory effect of the clamp in the assay.

In summary, a LNA-clamped PCR assay is reported in this study for the detection of HBV 1762T/1764A double mutation. When coupled with melting curve analysis, it provides a simple, rapid, one-step detection method with high sensitivity and specificity. The capability to selectively amplify mutant HBV is a clear advantage when assaying blood samples where

WT HBV is usually present at high levels. This assay is valuable for further evaluation of the significance of the HBV 1762T/1764A double mutation.

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Figure 1.

The oligonucleotides used in this study. HBV wild type (WT) DNA sequence (GeneBank Accession #X04615) from positions 1631 to 1830 is listed. The position and the direction of the oligonucleotides are shown with line arrows aligned to the HBV WT sequence. The sequence of the LNA-containing oligonucleotide is listed with the LNA monomers in upper-case letters.

Figure 2.

Differentiation of the HBV 1762T/1764A double mutation from the WT by melting curve analysis and the WT clamp. (1) HBV 1762T/1764A (300 copies); (2) WT HBV (300 copies); (3) H₂O only; (4) WT HBV plus LNA clamp; (5) WT:HBV1762T/1764A equal molar mix; (6) WT:HBV1762T/1764A mix plus LNA clamp. Details of real time PCR and melting curve analysis are described in materials and methods section.

Figure 3.

Sensitivity of LNA-mediated clamped real time PCR for the detection of HBV 1762T/1764A double mutation. Panel A, melting curves of (1) 10 copies of HBV 1762T/1764A plus LNA clamp; (2) 30,000 copies of WT HBV; (3) 30,000 copies of WT HBV plus LNA clamp; (4) 10 copies of HBV 1762T/1764A plus 30,000 copies of WT HBV; (5) 10 copies of HBV 1762T/1764A plus 30,000 copies of WT HBV plus LNA clamp; (6) H₂O only. Panel B, amplification curves of HBV 1762T/1764A double mutation at (1) 3,000 copies; (2) 300 copies; (3) 30 copies; (4) 10 copies; (5) 5 copies; (6) 0 copy (H₂O only). The PCR was carried out in the presence of 30,000 copies of WT HBV DNA and 2 μ M WT clamp.

A

	1731	1741	1751	1761	1771	1781
WT	TGGGAGGAGATTGGGGGAGGAGATTAGGTTAAAGGTCTTTGTACTAGGAGGCTGTAGGCAT					
8K	:::::::::::::::::::::::G:::::::::T:A:::::::::::::::::::::::::::::					
131K	:::					
150K	:::					
169K	:::					
174K	:::					
219K	:::N:N:::::::::::::					

B

Figure 4.

Analysis of HCC tumor DNA by PCR followed by direct DNA sequencing. The Seq fwd and Seq rev primers (see Figure 1) were used for amplification and sequencing. The wild type (WT) is from GeneBank Accession #X04615. Bases identical to the WT sequence are indicated by the colon signs. N indicates a base that cannot be determined automatically by the software. In panel B, the chromatogram file for the sample 219K is shown.

Figure 5. Analysis of HCC tumor DNA by LNA-mediated clamped real time PCR and melting curve analysis. Shown here are the melting curves for the HCC tissue samples with the sample names indicated along the curves. Reactions from samples 131K and 150K did not have the LNA clamp.

Figure 6.

Effect of LNA-mediated PCR clamping on DNA sequencing. Regular PCRs were performed with or without the LNA-containing WT HBV clamp (+ or – LNA clamp), and were subjected to direct sequencing. Results for the HCC samples 169K and 174K are shown in panel A. Results from the plasmid mixtures are shown in panel B. DM/WT 1:4 indicates a mixture of the double mutation and WT plasmids at a ratio of 1:4. The chromatogram files are shown with the readout sequence on the top. TTAAAGGTCTT is the WT sequence with 1762A and 1764G underlined. TTAATGATCCTT contains 1762T and 1764A double mutation. Note that 1762 and 1764 positions may contain mixed bases.