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Strategic Assay Developments for Detection of HBV 1762T/1764A Double Mutation in Urine of Patients with HBV-Associated Hepatocellular Carcinomas

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1. Introduction

Hepatocellular carcinoma (HCC) is the 7th most common cancer worldwide and remains the third leading cause of cancer deaths (Yang and Roberts 2010). It has a 5-year survival rate of less than 11% even in developed nations (Garcia, Jemal et al. 2007). The 5-year survival rate drops from 26% to 2% in patients with localized versus metastasized cancer (ACS, 2010). The poor prognosis is due mainly to late detection with the methods currently available. Thus, a better screening method to detect HCC at its early, curative stage is needed to improve its outcome. One of the major etiological factors associated with HCC development is chronic infection with hepatitis B virus (HBV). In developing countries, 59% of liver cancers are attributable to HBV and, in developed countries, 23% of liver cancers are attributable to HBV (Garcia, Jemal et al. 2007).

The contribution of HBV to the pathogenesis of HCC is believed to be multifactorial including the possible role of HBV mutants. A double mutation—an adenine (A) to thymine (T) transversion at nucleotide position 1762 and a guanine (G) to adenine (A) transition at nucleotide position 1764 (1762T/1764A)—in the HBV genome has been found in 50% to 85% of HCC tumor tissues (Hsia, Yuwen et al. 1996; Baptista, Kramvis et al. 1999; Arbuthnot and Kew 2001; Hannoun, Horal et al. 2002; Kuang, Jackson et al. 2004; Kuang, Lekawanvijit et al. 2005). It has been shown that chronic HBV carriers with the HBV 1762T/1764A double mutation have an elevated risk of HCC and that the risk increases with increasing viral load of the double mutation (Yuan, Ambinder et al. 2009). Thus, the HBV 1762T/1764A double mutation is an important risk factor for HCC for people infected chronically with HBV (Chen, Iloeje et al. 2007; Yuan, Ambinder et al. 2009).

This double mutation appears to be associated with enhanced viral replication and increased severity of liver inflammation, which may contribute ultimately to the development of HCC (Laskus, Rakela et al. 1995; Takahashi, Aoyama et al. 1995; Baptista, Kramvis et al. 1999; Hou, Lau et al. 1999; Pang, Yuen et al. 2004). Furthermore, this mutation has been associated with decreased circulating HBV e antigen (HBeAg) and is thought to trigger the host immune response to HBV infected hepatocytes leading to increased apoptosis and regeneration. This could contribute to host liver injury and progression to HCC (Yang, Yeh et al. 2008). Previous studies have shown that the HBV 1762T/1764A double mutation can be detected in plasma samples from patients with HCC (Zhang, Gong et al. 2002; Kuang, Jackson et al. 2004; Kuang, Lekawanvijit et al. 2005; Yang, Yeh et al. 2008); the results of these studies suggest that the HBV 1762T/1764A double mutation is a potential biomarker for the early detection of HCC in long-term carriers of HBV.

Some of the available methods for detection of this double mutation involve PCR amplification of the target sequence followed by direct DNA sequencing(Baptista, Kramvis et al. 1999), PCR followed by restriction enzyme digestion(Takahashi, Aoyama et al. 1995), mismatched PCR coupled with restriction fragment length polymorphism analysis(Hou, Lau et al. 1999), or short oligonucleotide mass spectra analysis (SOMA) (Kuang, Jackson et al. 2004). These methods are not feasible for clinical use as they are either time-consuming, labor intensive, or insensitive due to the excessive HBV wild type DNA molecules in the sample.

The authors (Su, Wang et al. 2004; Su, Wang et al. 2005; Su, Wang et al. 2008) and other researchers(Botezatu, Serdyuk et al. 2000; Serdyuk, Botezatu et al. 2001; Chan, Leung et al. 2008; Melkonyan, Feaver et al. 2008) have shown that urine contains DNA from the circulation. This circulation-derived urine DNA, transrenal DNA, is fragmented mostly into segments of fewer than 300 base pairs (bp) (designated as low-molecular-weight urine DNA) and can be used to detect tumor-derived genetic mutations. The authors have also shown that the preferential isolation of low-molecular-weight urine DNA from total urine DNA and its use as the substrate enhances the sensitivity and specificity of the urine test for detecting tumor-derived circulating DNA markers (Su, Song et al. 2008). Moreover, the authors have shown that the concentration of circulation-derived DNA in urine is comparable to that in blood (Su, Wang et al. 2008).

As mentioned, the HBV 1762T/1764A double mutation was detected in the plasma of patients with HCC (Kuang, Jackson et al. 2004; Kuang, Lekawanvijit et al. 2005; Yang, Yeh et al. 2008). To the authors’ knowledge, none of the previous studies have attempted to detect the HBV 1762T/1764A double mutation in urine. Urine is an advantageous bodily substrate in HCC screening as it proves absolutely noninvasive, opportune for collection in remote geographic areas such as developing countries, and further requires no special facility or equipment apart from sterile collection containers, as compared to the requirements for serum or plasma collection. Thus, it was of interest to see if the HBV 1762T/1764A double mutation sequence could be detected in the urine of patients with HCC who were infected with HBV.

Our study involves development of two PCR-based assays for detecting this HBV 1762T/1764A double mutation. The first assay comprises of a real-time PCR assay (HybProbe_DM) utilizing hybridization probes and an oligonucleotide clamp containing locked nucleic acids (LNAs). This LNA clamp complementary to HBV wild type DNA can reduce interference from HBV wild type to allow for increased sensitivity for detecting the HBV double mutation. However, Sikora et al.(Sikora, Zimmermann et al. 2010) and Shekhtman et al. (Shekhtman, Anne et al. 2009) suggested that polymerase chain reaction (PCR) assays targeting template sequences of 50 nucleotides (nt) or less are necessary to obtain a sensitivity greater than 50% in order to detect DNA of interest in urine derived from the circulation. Thus, a second assay containing a 2-step nested PCR assay (HBVDM_40) with a target template size of 40 bp and incorporation of locked nucleic acid was also developed. Both assays were then tested to detect HBV 1762T/1764A double mutation in urine of HBV-infected patients with HCC. The HBV double mutation was detected in the urine by this two-step PCR assay and suggest that it is necessary to minimize the targeting template size to have sufficient sensitivity to detect circulating DNA markers in urine.

2. Assay design and development

The goal of our study is to develop an assay suitable for urine detection of the HBV 1762T/1764A double mutation. We outline two approaches taken. The first is a real-time PCR-based assay utilizing fluorescent hybridization probes along with a LNA clamp complementary to HBV wild type DNA, as described previously (Ren, Lin et al. 2009). This assay allows real-time quantification of HBV 1762T/1764A double mutation DNA as well as PCR product characterization through melting curve analysis. The LNA clamp increases sensitivity of this assay by inhibiting HBV wild type DNA amplification allowing detection of HBV 1762T/1764A double mutation. However, this assay contains a 133 bp amplicon size and may not be suitable to detect low molecular weight urine DNA. A second assay was developed targeting a template size of 40 bp. This assay does not contain real-time capabilities and is a 2-step nested PCR assay. The use of LNA is also incorporated, but rather than appropriating a wild type clamp LNA was incorporated into the PCR primers to allow for selective amplification of the HBV 1762T/1764A double mutation DNA. The assays' design and development are highlighted below.

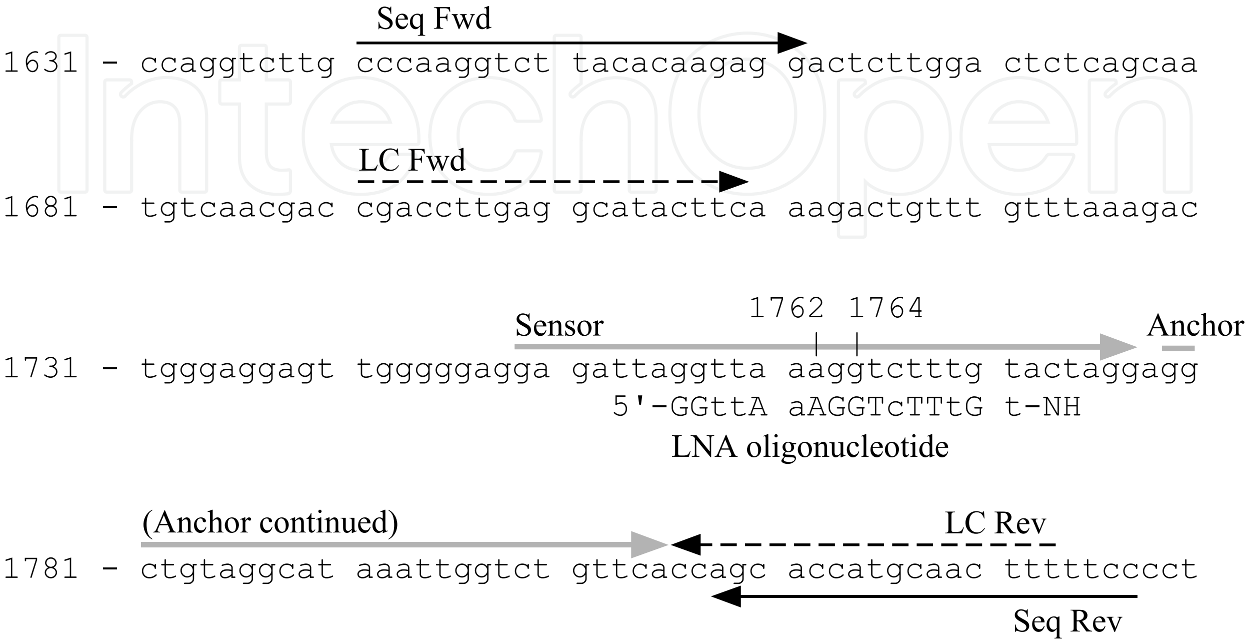
2.1 HybProbe_DM assay

This assay was validated by PCR and direct DNA sequencing. The PCR (186 bp) utilized primers SeqFwd and SeqRev that lay outside of HybProbe_DM assay (Fig 1). The HybProbe_DM assay was designed on a LightCycler® 2.0 platform. The sensor and anchor hybridization probe are labelled below. The LNA clamp is a 16 nt oligonucleotide complementary to HBV wild type DNA and contains 10 LNA (Fig 1).

Plasmid containing 186bp segment (from positions 1642 to 1827 of the HBV genome) HBV wild type DNA and 1762T/1764A double mutation were constructed using pPCR-Script AMP SK(+) vector (Stratagene, La Jolla, Ca). The amount of plasmid DNA was determined using a Nanodrop™ 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) at 260 nm absorbance and confirmed by ethidium bromide staining agarose gel with a known quantity of 100-bp molecular weight DNA standard (New England Biolabs, Ipswich, MA).

These plasmids served as control templates in the testing of these assays. The real time PCR assay 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), 0.4 µM of each PCR primer (LC Fwd and LC Rev), 2.5 mM $MgCl_2$, 500 ng/µl, BSA, 200 µM dNTP, 0.2

uM Sensor, 0.2 uM Anchor, and 2 µM HBV wild type-specific LNA-containing oligonucleotide (Ren, Lin et al. 2009). The reaction was incubated in the LightCycler II (Roche Applied Science) at 95°C 10min to activate the Taq polymerase, followed by amplification at 95°C 5 s, 65°C 15 s, 52°C 10 s, and 65°C 10 s for 45 cycles. Both the Sensor and the LNA clamp are phosphorylated at the 3′ end so that they cannot function as primers.



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1631 - ccaggtcttg cccaaggtct tacacaagag gactcttgga ctctcagcaa

1681 - tgtcaacgac cgaccttgag gcatacttca aagactgttt gtttaaagac

1731 - tgggaggagt tgggggagga gattaggtta aaggtctttg tactaggagg
                                5'-GGttA aAGGTcTTtG t-NH

1781 - ctgtaggcat aaattggtct gttcaccagc accatgcaac tttttcccct
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Fig. 1. HybProbe_DM oligonucleotides design. HBV wild type DNA sequence (GeneBank Accession #X04615) from positions 1631 to 1830. Positions and direction of oligonucleotides are denoted by marked arrows and LNA-containing oligonucleotide is listed with LNA denoted by capital letters.

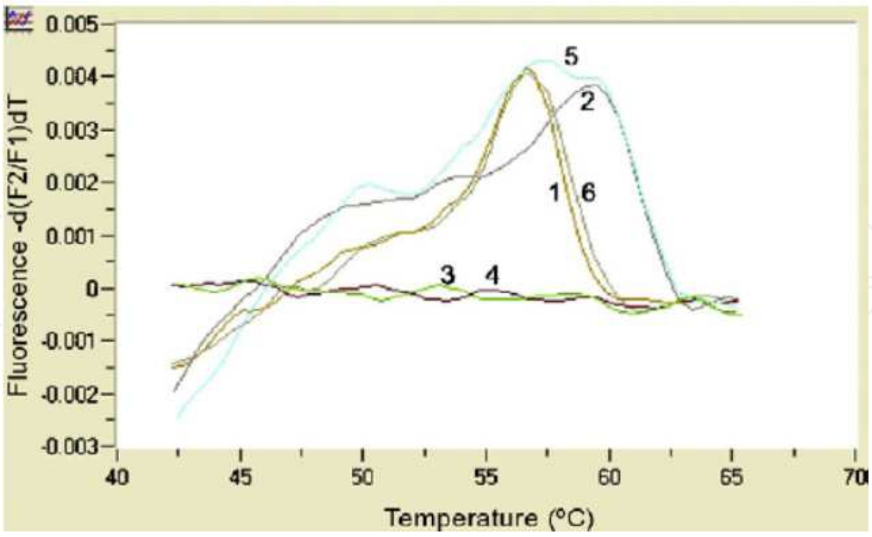

Fig. 2. Differentiation of the HBV 1762T/1764A double mutation from the HBV wild type DNA by melting curve analysis and the wild type clamp. (1) HBV 1762T/1764A (300 copies); (2) HBV wild type DNA (300 copies); (3) H_2O only; (4) HBV wild type DNA plus LNA clamp; (5) wild type: HBV 1762T/1764A equal molar mix; (6) wild type: HBV 1762T/1764A mix plus LNA clamp.

Sensitivity was validated using varying ratios of mutant in HBV wild type DNA background in the presence of LNA clamp. A minimum of 10 copies of mutant HBV DNA was detected (Fig. 2) and a mutant/wild type ratio of 1:3000 (Fig. 3).

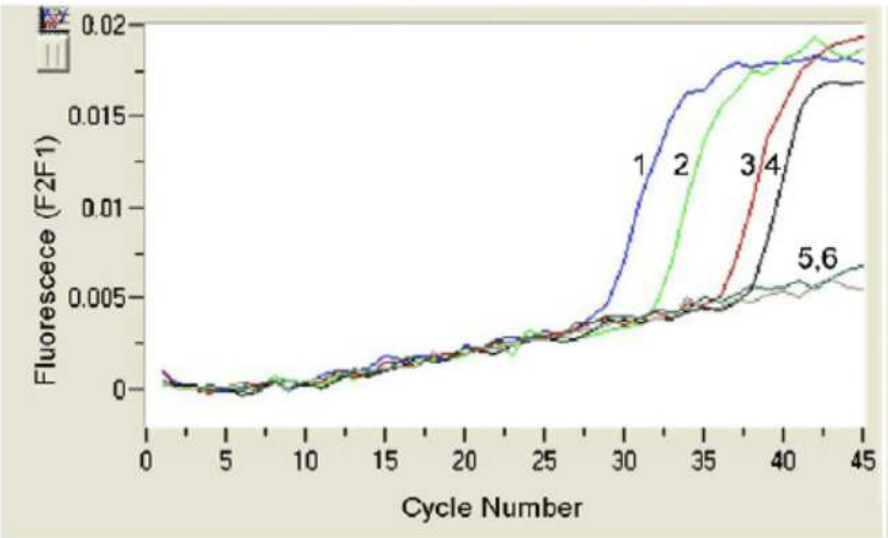

Fig. 3. Sensitivity of LNA-mediated clamped real-time PCR for the detection of HBV 1762T/1764A double mutation. Amplification curves of HBV 1762T/1764A double mutation at (1) 3000 copies; (2) 300 copies; (3) 30 copies; (4) 10 copies; (5) 5 copies; (6) 0 copy (H_2O only). The PCR was carried out in the presence of 30,000 copies of HBV wild type DNA and 2 µM of wild type clamp.

2.2 HBVDM_40 assay

This assay was developed to suit urine detection criterion. To detect HBV double mutation in the small fragmented DNA templates of urine, a two-step nested PCR assay targeting a 40-bp DNA fragment from the nucleotide positions 1743 to 1787 of the HBV genome was developed with the sequences and location of primers displayed in Fig 4. To enhance the amplification specificity of the mutated sequences, two LNAs were incorporated into the forward primer of the first PCR at the mutation sites, positions 1762 and 1764. An artificial tag sequence was added to increase the size of the first amplicon. Thus, the nested primers

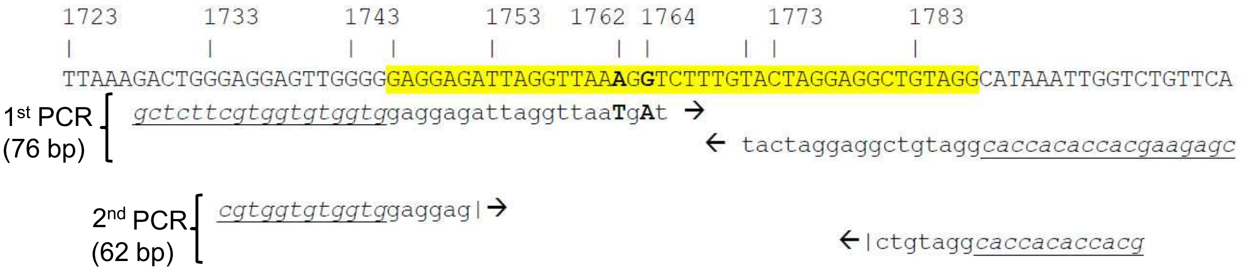

Fig. 4. HBVDM_40 assay showing locations and sequences of the primers for the first and second step PCR reactions. The nucleotide sequence of the HBV wild type genomes (GenBank accession # X04615) from 1723 to 1805 is listed and the target sequence of the assay (40 nt) is highlighted. The LNA nucleotides in the forward primer of the first-step PCR are at positions 1762 and 1764 (indicated by capital letters). The tag sequences of each primer are italicized and underlined.

for the second PCR reaction could be designed to contain most of the tag sequence. DNA isolated from a hepatoma cell line, HepG2 (American Type Culture Collection, Manassas, VA), that is free of any known hepatotropic viruses(Javitt 1990), was used as a negative control for HBV DNA.

To determine the sensitivity and specificity of the HBVDM_40 assay, a serial dilution of HBV 1762T/1764A double mutation positive control plasmid construct ranging from 1,000 copies to 1 copy per µL was prepared. The 1762T/1764A double mutation serial dilution standard, 1,000 copies of wild type plasmid DNA, and 5 ng HBV-negative control HepG2 DNA were then tested using the HBVDM_40 assay. The first PCR was assembled in a final volume of 20 µL containing 0.5 U HotStar Taq (Qiagen, Valencia, CA), 1X PCR buffer, 200 µM dNTPs, 1 µM of each primer, and DNA templates. The cycle profile was 95ºC for 15 min to activate the Taq polymerase, followed by 95ºC for 30 sec, 62ºC for 30 sec, 72ºC for 30 sec for 30 cycles, and a final extension for 4 min at 72ºC. The PCR product of the first PCR reaction was diluted 1 to 1000, and 1 µL of the dilution was used as the template for the second PCR reaction. For the second step of the PCR reaction, the reaction was assembled in a final volume of 20 µL containing 0.5 U HotStar Taq (Qiagen), 1X PCR buffer, 200 µM dNTPs, 0.5 µM of each primer, and DNA templates. The cycle profile was 95ºC for 15 min to activate the Taq polymerase, followed by 95ºC for 30 sec, 55ºC for 30 sec, 72ºC for 30 sec for 35 cycles, and a final extension for 4 min at 72ºC. The PCR product was analyzed in a 12% polyacrylamide gel using the TrackIt™ 25 bp DNA Ladder (Invitrogen, Carlsbad, CA).

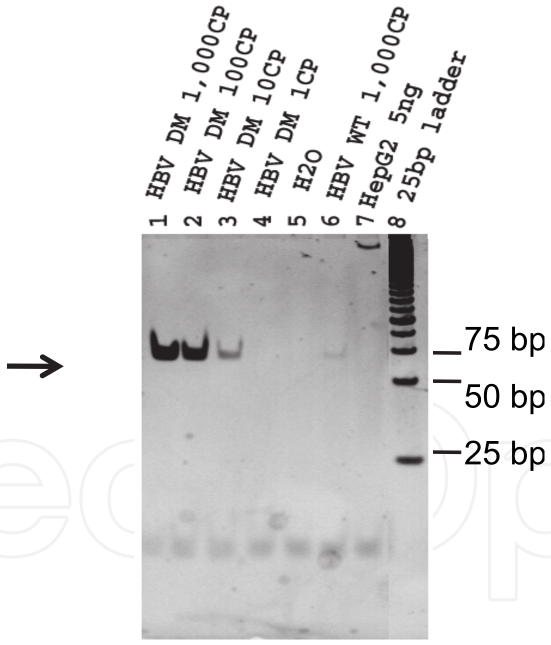

Fig. 5. Sensitivity and specificity of the HBVDM_40 assay. Serial 10-fold dilutions from 1 copy to 1000 copies of HBV 1762T/1764A double mutation positive control plasmid DNA and 1000 copies of the plasmid DNA containing HBV wild type sequences, 5 ng of HepG2 DNA, and H_2O were subjected to the HBVDM_40 assay. PCR products of the second-step PCR were analyzed in 12% polyacrylamide gel, stained with ethidium bromide, and visualized by photographing under a UV-transilluminator. The location of an expected 62-bp amplicon is indicated by the arrow. The TrackIt™ 25 bp DNA Ladder was used to indicate molecular weight markers.

As shown in Fig. 5, this two-step nested PCR assay could detect specifically as few as 10 copies of the HBV 1762T/1764A double mutation. No detectable PCR product was obtained from the water, 1000 copies of HBV wild type plasmid DNA, or 5 ng of HepG2 DNA. Overall, both of these assays display similar sensitivity (10 copies of HBV 1762T/1764A double mutation).

3. Assay comparison in patient testing

The assays were subjected to 15 HCC patient samples. Patient information is listed in Table 1. For a negative control, urine samples from 31 subjects (18 men and 13 women; average

Code	Age	Gender	HBV-infected	HCV-infected	Stage of HCC	Tumor size by Ultrasound or CT	AFP level (ng/mL)
2K	53	M	+	ND	II	5.5 cm	22.04
3K	74	M	-	ND	I	11 cm	12.68
5K	29	M	+	-	II	7 cm	24302
6K	71	F	+	ND	III	3 cm	667.1
7K	65	M	+	-	II	2 cm	6.2
8K	57	M	+	ND	III	16 cm	18032
10K	56	M	+	-	II	2.8 cm	4.68
17K	70	F	-	+	I	2.7 cm	820.6
18K	67	F	+	-	II	5 cm	3.53
19K	64	M	+	ND	III	6 cm	6.98
20K	57	M	+	ND	I	2.5 cm	4.03
21K	64	F	-	+	I	5 cm	6.71
25K	60	M	+	ND	III	5.5 cm	1.78
26K	48	M	+	-	II	3.5 cm	6101
27K	58	F	+	ND	II	2.3 cm	789.8

F, female; HBV, hepatitis B virus; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; M, male; ND, not determined

Table 1. Clinical Information for patients with HCC. Participants with diagnosed HCC were recruited from the National Cheng-Kung University Medical Center liver clinic. Patients with cancer were enrolled from surgical or oncologic services (prior to initiation of chemotherapy, radiation therapy, or surgery). Eligible patients with HCC were tested for detectable hepatitis B surface antigen, and 11 out of 15 patients with HCC were found to be positive. *AFP, alpha-fetoprotein; CT, computerized tomography; F, Female; M, male; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; ND, not determined*

age, 41 years, range 18–73 years) who were negative for any liver disease, including infection with HBV or hepatitis C virus, were collected. These patient samples were obtained under institutional review board approvals. Total urine DNA from each urine sample was isolated and fractionated for the low molecular weight urine DNA fraction using carboxylated magnetic beads (AgentCourt Bioscience Corporation, Beverly, MA) as described previously(Su, Song et al. 2008). DNA isolated from 15 urine matched HCC tissues was tested for HBV wild type, to confirm the pathological information provided and for the appearance of the 1762T/1764A double mutation.

HBV infection status indicated in the patients' records was confirmed by performing the HybProbe_DM assay with HCC tissue samples, in the absence of the LNA clamp, to allow amplification of both wild type and mutated sequences. The tumor tissue DNA was isolated by proteinase K/SDS treatment followed by phenol/chloroform extraction and ethanol precipitation. As expected, of the 15 HCC tissue samples tested, 4 samples (3K, 8K, 17K, and 21K) did not contain any detectable HBV DNA; this result was consistent with the pathological records provided (Table 1). The tissue DNA was then tested to detect the HBV 1762T/1764A double mutation using the HBVDM_40 assay and the HBV double mutation HybProbe assay. The results are summarized in Table 2.

HybProbe_DM assay detected 8 of 11 (72%) and the HBVDM_40 assay detected 9 of 11 (82%) HBV-infected HCC tissue DNA samples for HBV 1762T/1764A double mutation. This is consistent with previous findings whereby 1762T/1764A double mutation was found in 50% to 85% of HBV-infected HCC tissues (Hsia, Yuwen et al. 1996; Baptista, Kramvis et al. 1999; Arbuthnot and Kew 2001; Hannoun, Horal et al. 2002; Kuang, Jackson et al. 2004; Kuang, Lekawanvijit et al. 2005). Subsequently, the low molecular weight urine DNA isolated from 15 corresponding urine samples was subjected to both the HybProbe_DM and HBVDM_40 assays. As expected, the HBV double mutation HybProbe_DM assay, targeting 133 bp of the region of interest, was not sensitive enough to detect the DNA marker that is within fragmented, circulation-derived transrenal DNA (< 300 bp). Only one urine sample (6K) from the 11 patients with HCC who were infected with HBV was positive for the HBV 1762T/1764A double mutation by the HBV double mutation HybProbe_DM assay (data not shown), whereas 8 HBV-infected HCC urine samples were positive for HBV 1762T/1764A double mutation by the HBVDM_40 assay.

Interestingly, there were two urine samples (5K and 18K) that were HBV 1762T/1764A double mutation positive whose matched tissue samples were negative for HBV 1762T/1764A double mutation. It is possible that the 1762T/1764A double mutation detected in the urine samples was derived from the HCC tissue that was not part of the paraffin-embedded tumor tissue sections obtained.

Although the sensitivities of both the HBV LNA-mediated clamped HybProbe_DM assay and the HBVDM_40 assay are similar, 10 copies per assay, the HBVDM_40 assay is more suitable for detecting HBV 1762T/1764A double mutation when the urine DNA substrate was used, due to the smaller target template size. The maintenance of similar sensitivity in such a short amplicon is attributed to the LNA molecules incorporated at the sites of the mutations in the forward primer to amplify selectively the 1762T/1764A double mutation sequences. This substitutes the role of the 16nt LNA clamp in the HybProbe_DM assay that used in order to inhibit amplification of wild type sequences. Through this approach the target template size can be shortened and suitable for urine detection of circulation derived DNA markers.

		HBV Double Mutation HybProbe Assay		HBV Double Mutation_40 Assay	
Subject	**HBV**	**Tissue**	**Urine**	**Tissue**	**Urine**
2K	+	DM	-	DM	DM
3K	-	-	-	-	-
5K	+	WT	-	DM	DM
6K	+	DM	DM	DM	DM
7K	+	DM	-	DM	DM
8K	-	-	-	-	-
10K	+	WT	-	-	-
17K	-	-	-	-	-
18K	+	WT	-	-	DM
19K	+	DM	-	DM	-
20K	+	DM	-	DM	DM
21K	-	-	-	-	-
25K	+	DM	-	DM	-
26K	+	DM	-	DM	DM
27K	+	DM	-	DM	DM

[a]Each DNA sample was tested twice. Positivity is defined as both assays positive.
DM, HBV DNA 1762T/1764A double mutation sequence; WT, wild-type sequence.

Table 2. Detection of HBV double mutations in HCC tissues and matched urine[a]

4. Discussion

Individuals infected with HBV are a high-risk group for developing HCC. They need to be screened regularly for HCC. However, the latent period between contracting HBV infection and developing HCC ranges from a few to 30 years (Kim, Lee et al. 2000). Currently, the alpha-fetoprotein blood test and ultrasonography are the only screening methods available. Given the cost and the technical expertise required for blood collection and ultrasonography, a noninvasive, urine-based screening method is highly preferable and likely to have higher compliance than serum assays or imaging. It has been suggested that the HBV 1762T/1764A double mutation is a potential predictive marker for HCC because of its association with 50% to 85% of HBV-associated HCC and its detection in the circulation of patients infected with HBV who were either diagnosed with HCC at the time the blood was collected or who developed HCC a few years later (Kuang, Lekawanvijit et al. 2005).

To the authors' knowledge, this is the first report of the detection of HBV 1762T/1764A double mutation in the urine of patients with HCC and infected with HBV. This finding was achieved by developing a PCR assay that targets a template size of 40 bp optimized for use with transrenal DNA and is the first step toward developing a urine test for screening for HCC in individuals infected with HBV. The source of the HBV 1762T/1764A double mutation DNA detected in the urine of patients with HCC is unclear. It is possible that the HBV 1762T/1764A double mutation DNA is derived from the breakdown of DNA from the HBV infectious particles in circulation, from the closed circular form of the HBV genome, or from the integrated HBV genome released from infected hepatocytes. Because the DNA substrate is the low molecular weight urine DNA fraction (less than 1 Kb), the authors do not think that the HBV 1762T/1764A double mutation DNA detected was derived from infectious HBV virions in urine or in the circulation. Previous studies by the authors have shown that low molecular weight urine DNA co-migrated with DNA of 1 to 2 nucleosomes isolated in serum, which suggests that the source of this low molecular weight urine DNA is mostly from apoptotic cells (Su, Wang et al. 2004). It has been suggested that the turnover of hepatocytes in liver infected with HBV is increased, mostly by apoptosis. The authors thus speculate that the HBV 1762T/1764A double mutation detected in the urine of patients with HCC who were infected with HBV might originate from apoptotic infected hepatocytes.

In this small pilot study, the association of the detection of HBV double mutation sequences with the size of the tumor (Tables 1 and 2) was not found, perhaps because the sample size was too small. In this cohort, only 1 (20K) of 11 HCCs from patients infected with HBV was a stage I tumor. Interestingly, HBV double mutation was detected in this stage I tumor (20K, tumor size 2 cm), but not in 3 higher stage tumors [10K (stage II, tumor size 2 cm), 19K (stage III, tumor size 6 cm), and 25K (stage III, tumor size 5.5 cm)]. A larger number of samples are needed for this comparison. As discussed earlier, the amount of tumor DNA in urine could be due to the amount of apoptosis of the tumor. With heterogeneity of HCC, the rate of apoptosis might vary from tumor to tumor. Other physiological conditions such as hydration of body could also contribute to the concentration of circulation-derived DNA in urine. Nevertheless, it is encouraging that 9 of 11 urine samples from patients with HCC who were infected with HBV were positive for HBV double mutation by the HBVDM_40 assay, suggesting that this assay could be included in a urine test for HCC screening for patients infected with HBV. The assay developed in this study is not robust enough to be used in clinical settings for routine screenings. Nevertheless, the HBV 1762T/1764A double mutation was detected in the urine by this two-step PCR assay; this study showed that it is necessary to minimize the targeting template size to have sufficient sensitivity to detect the circulating DNA marker in urine. The development of a high-throughput quantitative assay for this marker to translate this finding for clinical use is in progress.

It would be of great interest to compare the detectability of the HBV double mutation DNA in urine with that of the current screening methods, the serum level of alpha-fetoprotein and ultrasound. Although the sample size is small (n=15), 3 (7K, 18K, 20K) of 6 urine samples from AFP-negative (< 20 ng/mL) patients with HCC who were infected with HBV were positive for the HBV double mutation by HBVDM_40 assay. The HBV 1762T/1764A double mutation would need to be evaluated in a large-scale study to determine how early in the disease process of HCC this marker appears in the urine by screening the urine from patients with hepatitis, cirrhosis, or HCC who were infected with HBV. Thus, the specificity and sensitivity of the marker alone for HCC screening can be determined.

The use of urine for cancer detection shows promise for potential clinical applications in HCC screening. Current literature has implicated other HBV mutations as potential HCC screening markers. A mutation in the precore region is of interest. Not only has this mutation been shown to be genotype specific but also to play a role in HBeAg secretion by forming a premature stop codon. Precore variants can be first detected around the time of HBeAg seroconversion and potentially prevent HBeAg synthesis without prohibiting production of infectious HBV virions (Kao 2003; Yang, Yeh et al. 2008). A particular precore region mutation involves position 1896 a G to A substitution (G1896A), however it depends on the nucleotide present at position 1858 as it forms a stem structure. As a result, this G1896A mutation rarely occurs in HBV genotype A due to pairing with dominate C1858 not T1858 as seen in other genotypes. The impact of this mutation is still in question. Studies have shown this mutation to be associated with a more aggressive disease such as reactivation of chronic HBV while others have shown the opposite where there is a better prognosis with the occurrence of this mutation (Kao 2003; Yang, Yeh et al. 2008).

Other mutations at position C1653T in the enhancer II region and position 1753 T to C/A/G in the core promoter region have also been found. However, the mutation at 1753 was not found to be significantly associated with HCC development, but the core promoter mutation at 1753 were found to correlate with HCC development. The C to T mutation at 1653 alters the X protein possibly contributing to hepatocarcinogenesis and shows increased frequency with progression from chronic hepatitis to cirrhosis (Yuen et al., 2008). Although the link between the core promoter mutations and development of HCC is still unclear, it is thought that these mutations play a role in altering pregenomic secondary structure that enhances viral replication and this has been demonstrated by increased HBV DNA levels in patients with core promoter mutations compared to those with no core promoter mutations.

Lastly, mutations in the PreS/S have been of interest. S mutations involving glycine to arginine substitution at position 145 (G145R) have been shown to play a role in immune escape by altering HBV envelope proteins. PreS deletions play a role in forming the hepatocyte binding site, transportation out of the hepatocyte and are essential for virion assembly (Llovet and Lok 2008; Nguyen, Law et al. 2009). These mutations are thought to occur during pathogenesis of HBV infection and transmitted vertically. Thus there is a potential for these mutations to serve as HCC screening markers.

In HCC screening, it is important to take into consideration the HBV genotypes as they have a role in the clinical outcome, treatment efficacy, prevalence patterns of HBV mutants and the geographic spread of HCC. There are known genotypes A through H with specific geographic distribution. HBV genotypes B and C are predominant in Southeast Asia. Genotype C has been found to be associated with more severe liver disease with higher frequency of HBeAg, higher serum HBV DNA levels, delayed HBeAg seroconversion, higher frequency of basal core promoter mutation, and lower response rate to interferon therapy compared to genotype B. However genotype B is associated with development of HCC in younger populations. Genotype A which is frequent in North America Europe, India, and parts of Africa holds a predominant nucleotide at position 1858 and as a result incur less frequent precore mutations. While genotypes B, C, and D which hold a predominant nucleotide T at position 1858 is more frequent in the Mediterranean and Asia have a much higher frequency of precore mutations. Genotypes F is found in South America while genotypes E (Africans), F (aboriginal populations of South America), H (Amerindian populations of Central America), and G (HBV carriers in France, Georgia, USA, and Germany) are less well studied (Kao 2003; Yuen, Tanaka et al. 2008). Furthermore, in Caucasian and Indian populations genotype D is associated with greater risk of HCC development compared to genotype A indicating host factors in combination with genotype can play a role in HCC risk.

Moreover, HCC, like other cancers, is extremely heterogeneous and has a complex etiology, even within the HBV-infected group (Buamah, Gibb et al. 1984; Bressac, Kew et al. 1991; Arbuthnot and Kew 2001; Block, Mehta et al. 2003; Boyault, Rickman et al. 2007; Aravalli, Steer et al. 2008; Hoshida, Nijman et al. 2009). Any single DNA marker is unlikely to have sufficient sensitivity and specificity to detect HCC such that it can be used as a stand-alone diagnostic test. Thus, a panel of multiple genetic and epigenetic markers, such as HCC-associated HBV mutations, as discussed above, mutations in p53 and CTNNB1 genes (Bressac, Kew et al. 1991; de La Coste, Romagnolo et al. 1998; Miyoshi, Iwao et al. 1998; Huang, Fujii et al. 1999; Legoix, Bluteau et al. 1999; Terris, Pineau et al. 1999; Wong, Fan et al. 2001; Taniguchi, Roberts et al. 2002; Edamoto, Hara et al. 2003; Kirk, Lesi et al. 2005; Zhang, Rossner et al. 2006; Boyault, Rickman et al. 2007; Hussain, Schwank et al. 2007; Cieply, Zeng et al. 2009; Jain, Singhal et al. 2010) and aberrant methylation of p16, APC, and GSTP-1 genes (Lee, Lee et al. 2003; Jicai, Zongtao et al. 2006; Katoh, Shibata et al. 2006; Su, Lee et al. 2007; Chang, Yi et al. 2008; Gao, Kondo et al. 2008; Harder, Opitz et al. 2008; Su, Zhao et al. 2008; Feng, Stern et al. 2010; Hernandez-Vargas, Lambert et al. 2010; Jain, Singhal et al. 2010) could be assembled that cumulatively result in a level of sensitivity and specificity for HCC screening that is superior or complimentary to alpha fetoprotein and its L3 glycoform or to ultrasound imaging, the methods currently used for HCC screening.

5. Conclusion

With the current technology in assay development, it is feasible to design assays targeting clinically relevant mutations in HBV for HCC screening. Given the heterogeneity of HCC and the genotypes of HBV, the challenge lies in developing a panel of assays which can then give a better performance than current screening tests. Also, given the long window period between HBV infection and HCC development, it is important that the test be as noninvasive and cost-effective as possible. The urine based tests can meet both of these demands and are also suitable for developing nations with fewer resources. The next step should be to test-drive the performance of the assays for HBV mutations in a larger study population. This will allow evaluation of these mutations as diagnostic biomarkers and investigate their association with HCC development, prognosis, treatment efficacy and overall survival.

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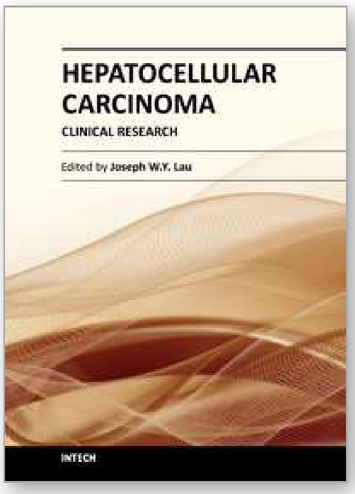

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This book covers the clinical aspects of hepatocellular carcinoma. This book is a compendium of papers written by experts from different parts of the world to present the most up-to-date knowledge on the clinical aspects of hepatocellular carcinoma. This book is divided into three sections: (I) Diagnosis / Differential Diagnosis; (II) Surgical Treatment; (III) Non-surgical Treatment. There are 19 chapters covering topics from novel diagnostic methods to hepatic lesions mimicking hepatocellular carcinoma, from laparoscopic liver resection to major hepatectomy without allogeneic blood transfusion, from molecular targeted therapy to transarterial radioembolization, and from local ablative therapy to regional therapy. This volume is an important contribution to the clinical management of patients with hepatocellular carcinoma. The intended readers of this book are clinicians who are interested in hepatocellular carcinoma, including hepatologists, liver surgeons, interventional and diagnostic radiologists, pathologists and epidemiologists. General surgeons, general physicians, trainees, hospital administrators, and instruments and drug manufacturers will also find this book useful as a reference.

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