

Evaluation of peptide nucleic acid array for the detection of hepatitis B virus mutations associated with antiviral resistance

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Received: 17 February 2011 / Accepted: 3 May 2011
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Abstract A major problem of long-term antiviral therapy in chronic hepatitis B patients is the emergence of hepatitis B virus (HBV) mutations associated with drug resistance. Recently, a new array using peptide nucleic acids (PNAs), which are synthetic nucleic acid analogues, was developed for the detection of HBV mutations at six different codon positions associated with lamivudine (LAM) and adefovir (ADV) resistance. We compared the PNA array with direct sequencing and reverse hybridization (INNO-LiPA) in 73 samples obtained from chronic hepatitis B patients. The PNA array detected mutations associated with LAM and/or ADV resistance in 60 (82.2%) of the 73 samples. The overall

concordance rate of PNA array and INNO-LiPA compared with direct sequencing was 99.5% and 98.2%, respectively. The rate of complete concordance between PNA array and INNO-LiPA was 92.7%. The PNA array assay results were comparable with INNO-LiPA for detection of HBV mutations associated with antiviral resistance.

Introduction

Chronic hepatitis B virus (HBV) infection, which afflicts nearly 350 million people worldwide, is a serious health problem that can result in cirrhosis and hepatocellular carcinoma (HCC) [1, 2]. Although HBV cannot be completely eliminated from patients due to the persistence of covalently closed circular DNA in the infected hepatocytes, the goals of antiviral therapy in chronic hepatitis B patients are to prevent progression to cirrhosis, liver failure and HCC, and improve survival by means of viral suppression [3, 4]. Since lamivudine (LAM) was approved as the first oral nucleoside analogue for the treatment of chronic hepatitis B in the United States in 1998 and in Europe in 1999, the availability of several oral nucleotide/nucleoside analogues, including LAM, adefovir (ADV), entecavir, and telbivudine, has improved the management of chronic hepatitis B infections by maintaining maximal suppression of viral replication [5, 6]. However, treatment of chronic hepatitis often requires the long-term administration of the antiviral agent, because of the high relapse rate when antiviral therapy is discontinued [6]. Unfortunately, a major problem of long-term antiviral therapy is the emergence of drug resistance associated with viral mutation, which proves challenging for hepatitis chemotherapy [7, 8]. Treatment failure of antiviral therapy due to drug resistance can result in lower rates of hepatitis B e antigen

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loss and seroconversion, virological and biochemical relapse, and an increased rate of liver disease progression [6]. The early detection of antiviral resistance permits earlier alteration of appropriate alternative therapy or add-on therapy for preventing adverse clinical outcomes due to drug resistance [3, 9].

Various assays have been developed to detect mutations in HBV that are associated with resistance to antiviral drugs, including direct sequencing of PCR products, real-time PCR with specific probes, reverse hybridization methods such as the line probe assay, and restriction fragment length polymorphism (RFLP) [2, 10]. The currently available commercial tests for antiviral resistance mutations are direct sequencing sequencing (TRUGENE HBV and Affigene HBV DE/3TC Assay) and reverse hybridization (INNO-LiPA HBV DR v2, hereafter referred to as INNO-LiPA). Direct sequencing has the advantage of enabling the identification of emerging mutations, but it cannot detect mutations that are a minor fraction (<20%) of the overall viral population [6]. The reverse hybridization assay is more sensitive for the detection of mutations, but it suffers in terms of specificity and operational complexity [9, 11].

Recently, a new array using peptide nucleic acid (PNA) probes (for research use only; PANArray™ DR HBV Chip Kit; PANAGENE, Daejeon, Korea) was developed for the detection of HBV mutations associated with antiviral resistance. PNAs are synthetic nucleic acid analogues that have more favorable hybridization characteristics than standard DNA-based probes because of their uncharged nature and thermal stability [11, 12]. We evaluated the PNA array in comparison with DNA sequencing and reverse hybridization assay in terms of its ability to detect HBV mutations associated with antiviral resistance.

Materials and methods

Patients and sample collection

A total of 73 serum samples were obtained from chronic hepatitis B patients who were receiving continuous antiviral treatment at the Department of Internal Medicine, Soonchunhyang University Cheonan Hospital. The patients had been positive for HBs Ag for at least 6 months and had received oral nucleoside analogue therapy for at least 5 months (median, 38.3 months; range, 5.5–55.7 months). The 73 patients included 55 males and 18 females (mean age, 46; range, 19–72 years). Quantification of HBV levels was performed previously using the Cobas Amplicor HBV Monitor test (Roche Diagnostics, Branchburg, NJ, USA), and HBV levels ranged from 2.8 to 9.1 log₁₀ copies/mL

(Table 1). Serum samples for the detection of HBV mutation were stored at −80°C until use.

HBV DNA purification

HBV DNA was extracted using a QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA) and QIA Cube (QIAGEN) from 400 µL of serum in accordance with the manufacturer's instructions. The DNA was eluted from columns with 50 µL of RNase-free water.

PNA array assay

Specific PNA probes were designed by considering the position of a potential point mutation and the melting temperature (T_m) value of PNA probes on the basis of viral resistance-specific sequence motifs. After optimization, nucleotide-specific PNA probes were applied as 16 amino-acid-specific probes (grouping all probes corresponding to the same amino acid together) on a slide. The specific and internal control PNA probes were printed as shown in Fig. 1. The PANArray™ DR HBV Chip Kit covers the most important amino acid variations at six different codon positions associated with LAM and ADV resistance: codons 80, 173, 180, 181, 204, and 236 of HBV reverse transcriptase.

For the PNA array, purified DNA was amplified by nested PCR according to the manufacturer's instructions. Briefly, 5 µL of DNA sample was added to the PCR mixture containing the primers HBPr950 (5'-biotin-CGTGGTGGACTTCTCTCAATTTTC-3') and HBPr952 (5'-biotin-CAGAAAGGCCTTGTAAGTTGGCGA-3'). The PCR conditions were 5 min at 94°C; 40 cycles of 30 sec at 94°C, 2 min at 55°C, and 1 min at 72°C; and 10 min at 72°C. After the first-round PCR, nested PCR was

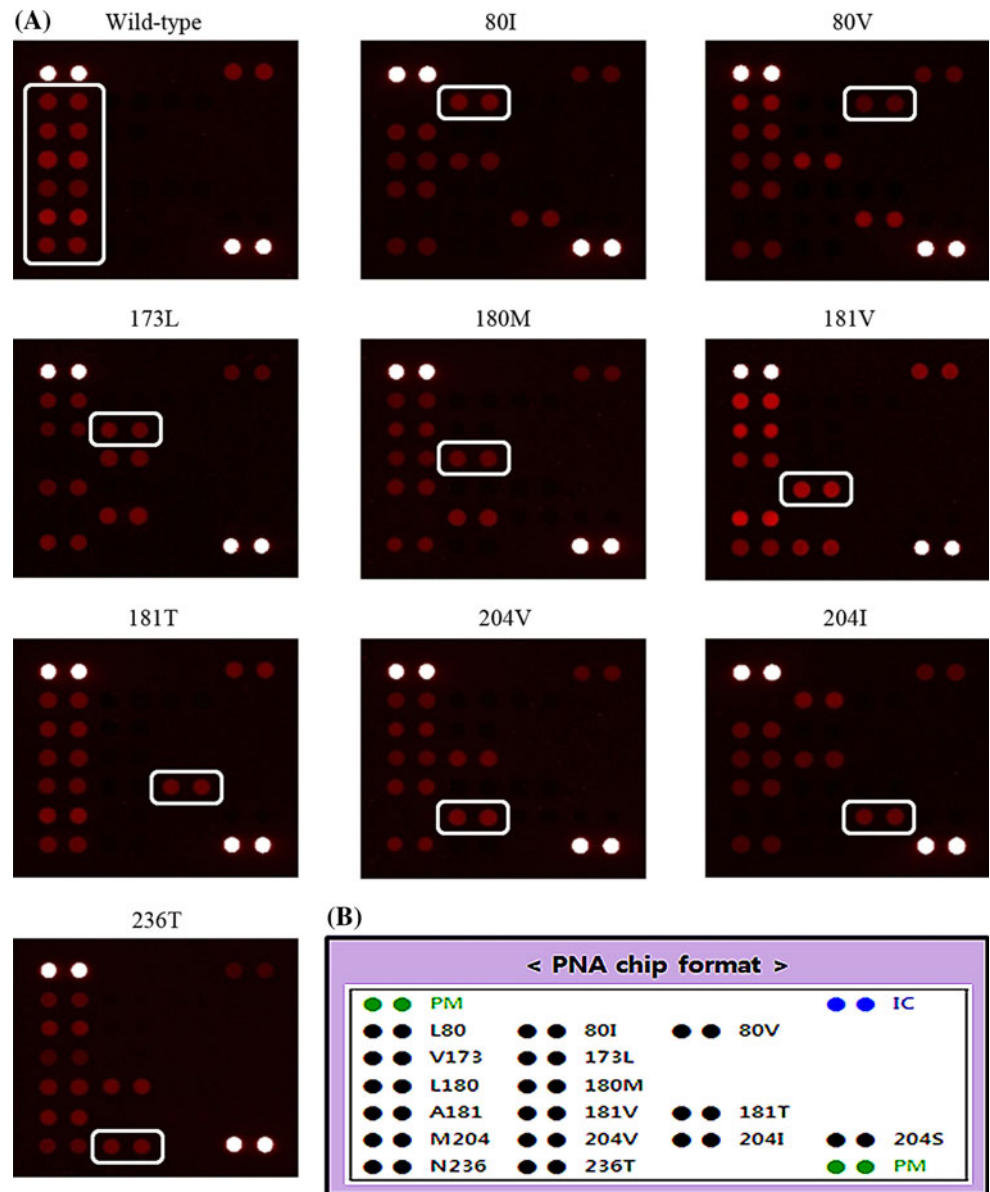
Table 1 Baseline characteristics of patients

No. of patients (males/females)	73 (55/18)
Age (years)	46 ± 10.9 (19–72)
HBe Ag positive	66
ALT (IU/mL)	46.8 ± 49.2 (8–338)
HBV DNA (log ₁₀ copies/mL)	5.0 ± 1.6 (2.8–9.1)
Antiviral treatment	
Rescue therapy with viral breakthrough during	
LAM or CLV monotherapy	50
LAM-ADV sequential therapy	9
LAM-ETV sequential therapy	2
Antiviral therapy without viral breakthrough	12

Data expressed as mean ± SD (range) or number

ADV adefovir, ETV entecavir, CLV clevudine, LAM lamivudine

Fig. 1 Scanning images of PNA array by hybridization of specific PNA probe and layout image of the PNA array. (A) Scanning images of PNA array, (B) Layout image of the PNA array. PM, position marker; IC, internal control



performed under the same conditions using two sense primers, HBM977 (5'-biotin-TGGCCAAAATTCGCA GTCCC-3') and HB-F3 (5'-biotin-CCTTCGGACGAAA CTGCA-3'), and one antisense primer, HBPr (5'-biotin-GCAAAGCCCCAAAAGACCCAC-3'). The PCR product was subjected to hybridization with the PNA array. Briefly, 70 μ L of hybridization mixture containing 5 μ L of PCR product and 65 μ L of the hybridization buffer was hybridized to specific PNA probes printed on the reaction slide at 50°C for 1 h. Washing and scanning were performed as described previously [11]. The fluorescence signals were obtained by scanning at 635 nm, using GenPro Pix 6.0 software (Axon Instruments, Union City, CA, USA); the intensities of the fluorescence signal indicated the hybridization signals of the probe-target duplexes.

Reverse hybridization assay

For the reverse hybridization assay, purified DNA was amplified in a single-round PCR with the INNO-LiPA HBV DR v2 (Innogenetics, Gent, Belgium) primers as described previously: sense primer HBPr950 (HBV nucleotides 255–278) and antisense primer HBPr952 (HBV nucleotides 1121–1099) [9, 13]. The samples were subjected to reverse hybridization by the INNO-LiPA HBV DR v2 strip according to the manufacturer's instructions. The strip is designed for the detection of the most important amino acid variations associated with LAM and ADV resistance at six different codon positions. Hybridization and color development were performed as described previously [9, 13], and the results obtained using the reaction

strips were interpreted according to the manufacturer's instructions.

Comparative analysis with sequencing assay

In an effort to evaluate the performance of the PNA array, direct sequencing of all samples was performed. Purified DNA was amplified in a nested PCR with outer primers (sense primer HBPr950 and antisense primer HBPr952) and inner primers (sense primer HBM977 and antisense primer HBPr). PCR was performed as described for the PNA array. The PCR products were purified using a LabopassTM PCR purification kit (CosmoGenetech, Seoul, Korea) according to the manufacturer's instructions. Purified products were sequenced with selected amplification primers using an ABI 3730xl sequencer (PE Applied Biosystems, Foster City, CA, USA). The results were compared with those of the PNA array and reverse hybridization assay.

Results

Distribution of mutations

On the basis of the PNA array results, mutations associated with LAM and/or ADV resistance were detected in 60 (82.2%) of 73 samples. Mutations associated with primary and/or compensatory LAM resistance occurred in 53 samples. The mutations associated with LAM resistance in five samples were combined with a mutation at position rt181. Mutation in the YMDD motif at reverse transcriptase position 204 (rt204; M204V/I) occurred in 52 samples either with (48 samples) or without (four samples) other compensatory mutations. Mutation rtM204I was detected in 34 samples, and mutation rtM204V occurred in 31 samples. In 13 samples, with mutation rt204, a mixed population of mutation rtM204I plus rtM204V, was detected. Compensatory mutations at positions rt180 and rt80 occurred in 43 and 22 samples, respectively. In all samples with these mutations, the mutation was combined with a mutation at position rt204. A mutation at position rt173 occurred in 13 samples, of which one was not combined with a mutation at position rt204. Mutations associated with ADV resistance occurred in 12 samples. The mutation at position rt181 occurred in 12 samples, and a mutation at position rt236 was detected in six samples. In four samples, the A181T mutation was combined with mutations at positions rt180 and rt204. The different mutations and their frequencies are listed in Table 2.

PNA array versus sequencing

In an effort to compare the PNA array with direct sequencing, both the results of PNA array and sequencing

for six different codons (80, 173, 180, 181, 204, and 236) were obtained from 73 clinical samples. The results were considered completely concordant if both direct sequencing and PNA array showed identical results. The results were considered partially concordant if PNA array showed a mixture of wild-type and mutant sequences while sequencing showed only a wild-type or a mutant sequence. If the PNA array showed a mixed population of two or more mutations and sequencing showed only a mutant sequence, the results were considered partially concordant. Also, the results were regarded as partially concordant if sequencing showed a mixture of wild-type and mutant sequences and the PNA array only showed mutant sequences. However, the results were considered partially discordant if sequencing showed a mixture of wild-type and mutant sequences while the PNA array only showed a wild-type sequence. The results were regarded as completely discordant if one test showed mutant sequences and the other test showed a wild-type sequence.

Complete concordance between results of PNA array and direct sequencing was observed for 390 (89%) of 438 codons analyzed. The complete concordance rate was lowest for position rt180 (78.1%) and highest for position rt236 (97.3%). Overall concordance was observed for 436 (99.5%) of 438 codons analyzed. The concordance rate was 98.6% at codons 173 and 180, and 100% at codons 80, 181, 204 and 236. These findings indicate that the concordance rate for primary mutations associated with resistance to LAM (rtM204V/I) and ADV (rtA181V/T or rtN236T) was 100%. In partially concordant results for 46 codons, the PNA array revealed an additional 19 mutations associated with antiviral resistance in 16 samples when compared to sequencing. Completely discordant results between PNA array and direct sequencing were not observed. Partially discordant results were observed for two (0.5%) of 438 codons analyzed: one each codon at positions rt173 and rt180 was associated with compensatory mutations of LAM resistance (Table 3).

INNO-LiPA versus sequencing

Among 73 samples tested by reverse hybridization assay with INNO-LiPA, indeterminate results that were negative for both wild type and mutant type were initially obtained in eight samples. Retesting revealed that seven samples were positive for wild type or mutant type at six different codon positions, while the result for the remaining sample was indeterminate for position rt173. On the basis of the INNO-LiPA results, mutations associated with LAM and/or ADV resistance were detected in 61 (83.6%) of 73 samples. According to sequence analysis, mutations associated with LAM and/or ADV resistance were detected in 58 (79.5%) of 73 samples. Complete and partial

Table 2 Frequency of the various mutations at six codon positions by PNA array

Mutations at codon						No. of mutations
80	173	180	181	204	236	
				M204V		1
		L180M		M204V		9
		L180M	A181T	M204V		1
	V173L	L180M		M204V		6
L80I		L180M		M204V		1
				M204V, M204I		1
		L180M		M204V, M204I		3
		L180M	A181T	M204V, M204I		1
	V173L	L180M		M204V, M204I		2
L80I		L180M		M204V, M204I		3
L80I	V173L	L180M		M204V, M204I		2
L80I, L80V	V173L	L180M	A181T	M204V, M204I		1
				M204I		2
		L180M		M204I		2
		L180M	A181T	M204I		1
	V173L			M204I		1
L80I				M204I		3
L80I		L180M		M204I		7
L80V		L180M		M204I		3
L80I, L80V				M204I		1
L80I, L80V		L180M		M204I		1
			A181V			1
			A181V		N236T	3
			A181T			1
			A181T		N236T	2
	V173L		A181T		N236T	1
Sum						60

concordance between INNO-LiPA and direct sequencing were observed for 376 (86%) and 53 (12.1%) of 437 codons analyzed, respectively. Concordance rates were 100% (73/73) at codons 80, 204, and 236, 97.2% (70/72) at codon 173, and 97.3% (71/73) at codon 181. The lowest concordance rate was 94.5% (69/73), at codon 180. The concordance rate for primary mutations associated with resistance to LAM (rtM204V/I) was 100%, but the concordance rate for mutations associated with resistance to ADV (rtA181V/T or rtN236T) was 98.6%. In partially concordant results, the INNO-LiPA assay revealed 21 additional mutations associated with LAM resistance in 16 samples when compared to sequencing. A completely discordant result between INNO-LiPA and direct sequencing was observed in one sample at codon 181. Partially discordant results were evident for seven (1.6%) of 437 codons analyzed, and the partial discordance rates were 5.5% (4/73) at codon 180, 2.8% (2/72) at codon 173, and 1.4% (1/73) at codon 181 (Table 3).

PNA array versus INNO-LiPA

Since the PNA array and INNO-LiPA are not regarded as standard methods, different criteria for making decisions of partial concordance and partial discordance were considered. The results were considered partially concordant if one test showed a mixed type and the other test showed a mutant type. If one test showed a mixed population of two or more mutations while the other test showed only a mutant sequence, the results were considered partially concordant. However, the results were regarded as partially discordant if one test showed a mixed type and the other test showed a wild type.

Complete concordance between the results of PNA array and INNO-LiPA was observed for 405 (92.7%) of 437 codon positions analyzed. The complete concordance rate was highest for position rt236 (100%) and lowest for positions rt180 and rt204 (86.3%). Partial concordance was observed for 14 (3.2%) of 437 codons analyzed. Partially

Table 3 Results of PNA array and INNO-LiPA compared with direct sequencing for six different codons

Codon	No. (%) of samples by PNA array				No. (%) of samples by INNO-LiPA			
	Concordance	Concordance		Sum	Concordance	Concordance		Sum
		Complete	Partial			Complete	Partial	
80	73 (100%)	64 (87.7%)	9 (12.3%)	73	73 (100%)	64 (87.7%)	9 (12.3%)	73
173	72 (98.6%)	69 (94.5%)	3 (4.1%)	73	70 (97.2%)	69 (95.8%)	1 (1.4%)	72 ^a
180	72 (98.6%)	57 (78.1%)	15 (20.5%)	73	69 (94.5%)	51 (69.9%)	18 (24.7%)	73
181	73 (100%)	70 (95.9%)	3 (4.1%)	73	71 (97.3%)	68 (93.2%)	3 (4.1%)	73
204	73 (100%)	59 (80.8%)	14 (19.2%)	73	73 (100%)	53 (72.6%)	20 (27.4%)	73
236	73 (100%)	71 (97.3%)	2 (2.7%)	73	73 (100%)	71 (97.3%)	2 (2.7%)	73
Total	436 (99.5%)	390 (89.0%)	46 (10.5%)	438 (100%)	429 (98.2%)	376 (86.0%)	53 (12.1%)	437 (100%)

^a Result of INNO-LiPA in one sample was indeterminate for position rt173

discordant results with the PNA array showing mixed type were observed for 11 (2.5%) codons, whereas partially discordant results with INNO-LiPA array showing mixed type were observed for seven (1.6%) codons. Partially discordant results for primary mutations associated with resistance to LAM and ADV were obtained with two samples. Complete discordance between the results of PNA array and INNO-LiPA was not observed (Table 4).

Discussion

Due to the extremely high rate of HBV replication and the lack of a proofreading function of the RNA-dependent RNA polymerase, mutations occur frequently during the replication of HBV [14–16]. Antiviral resistance, which is an important cause of treatment failure in patients with hepatitis B, is generally related to worsened clinical outcomes [6, 10]. Sensitive and early detection of mutant strains is useful for monitoring the emergence of drug resistance during antiviral treatment and the selection of antiviral treatment regimens [3, 9, 17]. With advances in genomic technologies, many methods have been developed to detect antiviral resistant mutations, including direct sequencing of PCR products, INNO-LiPA, RFLP, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) based restriction fragment mass polymorphism, and ultradeep pyrosequencing [6, 10].

Since the introduction of the PNAs by Nielsen et al. [18], PNAs have become widely used as diagnostic devices in molecular biology and biotechnology [12, 19, 20]. PNAs are synthetic DNA analogs that are capable of forming very stable and highly sequence-specific helix invasion complexes with target sites on duplex DNA [19]. Since the negatively charged ribose-phosphate backbone is replaced by repetitive units of N-(2-aminoethyl)-glycine in PNAs, the PNAs backbone is uncharged [21]. These properties of PNAs allow the formation of strong PNA/DNA duplex showing thermal stability. The T_m of PNA-DNA duplexes is 2.5–8.5°C higher per base pair than the T_m of the corresponding DNA/DNA duplex [11, 22]. Also, PNAs exhibit biological resistance to enzymatic degradation and chemical stability due to their synthetic backbone [12, 19]. PNAs have a greater destabilizing effect in single-base mismatches, enabling detection of single nucleotide polymorphisms and point mutations [12]. The high thermal, biological, and chemical stability of PNAs, coupled with their specific binding affinity, increases the reliability of diagnostic molecular biological applications that use them [19]. Regarding the applications of PNAs for detecting HBV mutations, PNA-mediated PCR clamping was first used to detect mutations in the YMDD motif of the HBV

Table 4 Comparison between PNA array and INNO-LiPA results for six different codons

Codon	No. (%) of samples						
	Complete concordance	Complete concordance			Partial concordance	Partial discordance	
		Wild type	Mutant type	Mixed type		Mixed type by PNA array, wild type by INNO-LiPA	Mixed type by INNO-LiPA, wild type by PNA array
80	69 (94.5%)	49	4	16	2 (2.7%)		2 (2.7%)
173	67 (93.1%)	60	2	5	1 (1.4%)	4 (5.6%)	
180	63 (86.3%)	27	7	29	2 (2.7%)	5 (6.8%)	3 (4.1%)
181	70 (95.9%)	61	1	8	1 (1.4%)	2 (2.7%)	
204	63 (86.3%)	19	24	20	8 (11.0%)		2 (2.7%)
236	73 (100%)	67		6			
Total	405 (92.7%)	283	38	84	14 (3.2%)	11 (2.5%)	7 (1.6%)

^a Result of INNO-LiPA in one sample was indeterminate for position rt173

DNA polymerase gene [23, 24]. Recently, a new array using PNA probes was developed for the detection of HBV mutations associated with LAM and ADV resistance.

In this study, the use of this recently developed PNA array assay in 73 clinical samples showed a high concordance with direct sequencing for the detection of mutations associated with LAM and ADV resistance. For primary mutations associated with resistance to LAM and ADV, the concordance rate of the PNA array in comparison to direct sequencing was 100%. For six different codon positions, the overall concordance rate of 99.5% between the PNA array and direct sequencing was slightly higher than the 98.2% concordance rate of INNO-LiPA. Also, the overall concordance rate of the PNA array was slightly higher than those in previous studies using a PNA array [11]. The partial concordance rate of PNA array and INNO-LiPA compared to direct sequencing was 10.5% and 12.1%, respectively. A high rate of partial concordance was found at codon positions 80, 180 and 204. A high proportion of a mixed population of wild type and mutant type at those positions may influence the concordance rate. Complete discordance of the PNA array in comparison to direct sequencing was not observed, but a completely discordant result of the INNO-LiPA compared to direct sequencing was detected in one sample at rt181. These findings suggest that the INNO-LiPA assay may be not as robust for detecting mutations at position rt181 associated with ADV resistance. In our study, the performance of INNO-LiPA assay for the detection of mutations associated with LAM and ADV resistance was slightly lower than those in previous studies [9, 13]. In a study using an updated line probe assay, Degertekin et al. [25] suggested reasons for the lower concordance rate: many samples with mutations at more than one position and minor populations of mutant type in some samples. Similarly, we presume that INNO-LiPA performance may be affected by differences in viral

load and different proportions of mutant virus populations in samples.

Recently, it was reported that the detection limit of the PNA array assay for the detection of antiviral-resistant HBV is 10^2 copies/mL [11]. In previous studies [9, 25], the detection limit of the INNO-LiPA assay was about 10^3 – 10^4 copies/mL. Direct sequencing cannot detect mutations that are present at <20% of the total viral population, while the PNA array and INNO-LiPA assay can detect mutant populations that represent only 5% of the overall population [3, 6, 9, 11, 25]. Due to their higher sensitivity than direct sequencing, the PNA array and INNO-LiPA assay can provide additional information about mutations compared to sequencing. In our study comparing PNA array with sequencing, the PNA array assay revealed 19 additional mutations associated with antiviral resistance, while sequencing revealed only two additional mutations. A comparison between INNO-LiPA and sequencing revealed that INNO-LiPA identified 21 additional mutations, while sequencing identified eight additional mutations. In a comparison between PNA array and INNO-LiPA, partially discordant results of the PNA array showing a mixed type were observed for 11 (2.5%) codons, whereas partially discordant results of INNO-LiPA array showing a mixed type were observed for seven (1.6%) codons. These results revealed that the PNA array identified 11 additional mutations and INNO-LiPA identified seven additional mutations. Therefore, it is reasonable to assume that the PNA array, in practice, can supply more information about mutations.

In conclusion, the PNA array assay is comparable with the INNO-LiPA assay for the detection of HBV mutations associated with LAM and ADV resistance. We suggest that the PANArrayTM DR HBV Chip Kit may be a useful diagnostic tool as a sensitive, specific, and easily applicable assay for the detection and monitoring of HBV mutations

associated with LAM and ADV resistance. Further studies may be necessary to validate the assay, involving comparison with more sensitive assays such as molecular cloning and sequencing, MALDI-TOF MS, or ultradeep pyrosequencing.

Acknowledgments We thank Tae Young Lee and Young Il Park for excellent technical assistance.

Conflict of interest None declared.

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