Post-print: Detection of Adefovir Dipivoxil Resistance Site Gene Mutations in Hepatitis B Virus by LNA-PCR

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Abstract

Objective To establish a simple, rapid, and sensitive locked nucleic acid (LNA) probe-based real-time fluorescence polymerase chain reaction (PCR) assay for detecting hepatitis B virus (HBV) adefovir dipivoxil (ADV) resistance-associated mutations at sites rtA181V and rtN236T.

Methods Positive samples were screened by gene sequencing, followed by construction of recombinant plasmids containing wild-type and mutant strains at ADV rt181 and rt236 sites. Specific primers and LNA fluorescent probes encompassing the amplification of adefovir dipivoxil rtA181V and rtN236T resistance sites were designed. A real-time fluorescence PCR reaction system was established using the constructed recombinant plasmids as standards, and serum samples were tested in parallel with gene sequencing to evaluate the feasibility and accuracy of the detection method.

Results The LNA-PCR method established in this study could detect ADV gene mutations in HBV at 10^2 copies/mL with high specificity. Through testing 89 HBV-positive clinical samples after one year of ADV treatment, 8 cases (8.98%) showed rtA181V mutation, 5 cases (5.61%) showed rtN236T mutation, and 2 cases (2.24%) showed mixed rtA181V and rtN236T mutations. The detection results were consistent with sequencing results.

Conclusion The LNA-PCR method established in this study is a simple, rapid, and sensitive gene mutation detection method that can effectively distinguish single-base mutations, providing guidance for monitoring resistance mutations during adefovir treatment and adjusting antiviral therapy in chronic hepatitis B patients.

Full Text

Establishment and Clinical Application of LNA-PCR Assay for Detecting Hepatitis B Virus Adefovir Dipivoxil Resistance

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Abstract

Objective: To develop a simple, rapid, and sensitive locked nucleic acid (LNA) probe-based real-time fluorescence polymerase chain reaction (PCR) assay for detecting adefovir dipivoxil (ADV) resistance-associated mutations (rtA181V, rtN236T) in hepatitis B virus (HBV).

Methods: Positive samples were screened by gene sequencing to construct recombinant plasmids containing wild-type and mutant sequences at the ADV rt181 and rt236 loci. Specific primers and LNA fluorescent probes targeting the ADV rtA181V and rtN236T resistance sites were designed. Using the constructed recombinant plasmids as standards, a real-time fluorescence PCR reaction system was established. The feasibility and accuracy of the method were evaluated by parallel testing of serum samples alongside gene sequencing.

Results: The established LNA-PCR assay could detect ADV gene mutations in HBV at concentrations as low as 10^2 copies/mL with high specificity. Among 89 HBV-positive clinical samples from patients after one year of ADV therapy, the assay detected 8 cases (8.98%) with rtA181V mutation, 5 cases (5.61%) with rtN236T mutation, and 2 cases (2.24%) with mixed rtA181V and rtN236T mutations. All results were concordant with sequencing analysis.

Conclusion: The LNA-PCR method is a simple, rapid, and sensitive approach for detecting gene mutations that can effectively distinguish single-base mutations. It provides valuable guidance for monitoring ADV resistance mutations and adjusting antiviral therapy in chronic hepatitis B patients.

[**Keywords**] Hepatitis B virus; Adefovir dipivoxil; Locked nucleic acid; Mutation; Drug resistance

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Introduction

China is a high-prevalence region for hepatitis B, where recurrent HBV infection can lead to chronic hepatitis, cirrhosis, and even hepatocellular carcinoma. Active antiviral therapy can effectively control disease progression and improve patient prognosis. Nucleos(t)ide analogues are widely used for HBV antiviral treatment, but prolonged therapy can lead to mutations in the HBV polymerase gene [1,2], enabling the virus to escape drug pressure and resulting in viral rebound and liver function damage. Adefovir dipivoxil, a first-line treatment for hepatitis B, exhibits a resistance rate of approximately 22% after two years of continuous administration [3,4]. Studies have shown that ADV has a low genetic barrier to resistance, requiring only a single site mutation to confer resistance. These resistance mutations are primarily located in the B and D domains of the polymerase reverse transcriptase (RT) region, with rtA181V in domain B and rtN236T in domain D representing classical ADV resistance variants. The rtA181V mutation involves a substitution of alanine (A) to valine (V) at amino acid position 181, while rtN236T involves a change from asparagine (N) to threonine (T) at amino acid position 236 of the HBV P gene. Both mutations alter the conformation of the polymerase active site, affecting dNTP binding and thereby causing ADV resistance [5,6]. This study established an LNA-PCR detection method based on locked nucleic acid probe technology to detect ADV resistance-associated rtA181V and rtN236T mutations in selected subjects.

Materials and Methods

1.1 Clinical Samples Eighty-nine specimens were collected from chronic hepatitis B patients who had received adefovir dipivoxil therapy for over one year at Gongli Hospital in Shanghai' s Pudong New Area between 2015 and 2017, with serum HBV DNA levels above 1×10^3 copies/mL. Diagnostic criteria followed the "Prevention and Treatment Protocol for Viral Hepatitis" jointly revised by the Chinese Society of Infectious Diseases and Parasitology and the Chinese Society of Hepatology [7].

1.2 Instruments and Reagents The study utilized an ABI 7500 Real-Time PCR System (Applied Biosystems, USA), HBV DNA fluorescence quantification detection kit (Shanghai Fosun Long March Medical Science Co., Ltd.), Qiagen QIAamp DNA extraction kit (QIAGEN, Germany), HiLo Hotstart Taq DNA polymerase, UNG enzyme, and dUTPmix (Shanghai Moli Biomedical Technology Co., Ltd.).

1.3 Primer and Probe Design and Synthesis HBV P gene sequences were retrieved from the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov). Primers and probes were designed using Beacon Designer 8.14 software and synthesized by Shanghai HuiRui Biological Technology Co., Ltd. The sequences are shown in .

Table 1 Primers and probe sequences for ADV-resistant rtA181V and rtN236T sites

LNA Probes and Primers	Sequence (5' ~3')
181F	CACTTGTATTCCCATCCC
181R	ACCACATCATCCATATAACTG
181V probe	"FAM" -tctcH+Tgg+Ttc+Agttt-BHQ1
181A probe	HEX-tctcH+Tg+Gc+Tc+Agttt-BHQ1
236T probe	"FAM" -tacatt+TRa+Acc+Ctaata-BHQ1
236N probe	HEX-tacatt+TRa+Ccc+Ctaata-BHQ1
236F	CTCYTGGCTCAGTTTACTAGTGC
236R	CCAACTYCCAATTACATCCAT

1.4 Construction of Plasmid Standards Samples containing ADV resistance mutations rtA181V and/or rtN236T were selected to clone wild-type and mutant fragments containing the 181 and 236 sites for plasmid construction [8,9]. HBV DNA extraction was performed strictly according to the kit instructions. After verification by DNA sequencing, high-concentration plasmids were gradient-diluted to 10 -10^2 copies/mL, aliquoted, and stored at -80°C.

1.5 Establishment of LNA Probe-Based Real-Time PCR Method After repeated optimization, the final reaction system for rtA181V mutation detection included: 5 µL HBV DNA template, 0.5 µL each of 10 mM primers 181F and 181R, 0.3 µL of 10 mM primer 181V, 0.2 µL of 10 mM primer 181A, 0.5 µL of 10 mM dUTPmix, 5 µL of 10× Buffer, 4 µL of 25 mM Mg^2 , 0.4 µL of 20 U/µL UNG enzyme, 0.4 µL of 5 U/µL Taq enzyme, and water to a total volume of 50 µL.

The rtN236T mutation detection system included: 5 µL HBV DNA template, 0.5 µL each of 10 mM primers 236F and 236R, 0.3 µL of 10 mM primer 236T, 0.2 µL of 10 mM primer 236N, 0.5 µL of 10 mM dUTPmix, 5 µL of 10× Buffer, 4 µL of 25 mM Mg^2 , 0.4 µL of 20 U/µL UNG enzyme, 0.4 µL of 5 U/µL Taq enzyme, and water to a total volume of 50 µL.

Both reactions were performed under the following conditions: initial denaturation at 95°C for 10 min, followed by 45 cycles of 95°C for 30 s and 55°C for 60 s, with fluorescence signals collected during the 55°C extension step.

1.6 Quality Control and Result Interpretation Sample Detection Controls: 1) Positive control: mutant plasmid at 10^2 copies/mL concentration 2) Negative control: wild-type plasmid at 10^2 copies/mL concentration 3) PCR negative control: 10 ng of healthy human genomic DNA

Result Analysis Criteria: 1) Negative controls should show no amplification curves in either FAM or HEX channels 2) Positive controls should display S-shaped amplification curves in the FAM channel with Ct values <38

Samples meeting these criteria were interpreted as follows: 1) S-shaped curve in FAM channel with Ct <38 and no curve in HEX channel: ADV resistance mutant 2) S-shaped curves in both FAM and HEX channels with Ct <38: mixed mutant and wild-type strains 3) No S-shaped curve in FAM channel but curve present in HEX channel with Ct <38: wild-type strain 4) Samples with 38 Ct<40 required repeat testing; results were considered valid if one of three replicates met the above criteria 5) Samples with no S-shaped curves in either channel were considered to have HBV DNA levels below the detection limit or no detectable HBV DNA

1.7 Validation of the LNA-PCR Method Validation included: 1) Sensitivity testing using serial 10-fold dilutions (10 to 10^2 copies/mL) of rtA181V or rtN236T recombinant plasmids to generate standard curves; 2) Repeatability assessment by performing five replicate tests with 10 copies/mL plasmids and calculating CV values; 3) Stability evaluation through accelerated degradation testing (2 h at 37°C, equivalent to 6 months at -20°C) followed by amplification of plasmids at 10 to 10^2 copies/mL; and 4) Clinical sample testing using collected specimens analyzed in parallel by LNA-PCR and sequencing.

1.8 Statistical Analysis Data were analyzed using SPSS 16.0 software. Intergroup comparisons of measurement data were performed using ^{2} tests, with P<0.05 considered statistically significant.

Results

2.1 Recombinant Plasmid Sequencing Plasmid vectors containing 181A (wild-type), 181V (mutant), 236N (wild-type), and 236T (mutant) were successfully constructed. After recovery and restriction enzyme digestion, fragments (vector: 3000 bp, rt181A/V fragment: 67 bp, rt236N/T fragment: 143 bp) were analyzed by agarose gel electrophoresis ([Figure 1: see original paper]). Successfully constructed vectors were confirmed by sequencing, with mutant plasmid sequencing results shown in [Figure 2: see original paper].

2.2 Performance Characteristics 2.2.1 Sensitivity: Amplification curves were obtained for HBV ADV mutant plasmids from 10 to 10^2 copies/mL (Ct values: rtA181V/rtN236T = 17.61/17.56, 20.96/21.11, 24.31/25.12, 28.68/27.11, 31.94/31.92, 36.21/36.18). The detection limit for both rtA181V and rtN236T recombinant plasmids was 10^2 copies/mL (Ct = 36.21/36.18), demonstrating high sensitivity. Additionally, 10 copies/mL wild-type plasmid showed no S-shaped curve in the FAM channel, identical to the PCR negative control, confirming successful discrimination of single-base mutations with high specificity ([Figure 3: see original paper]).

2.2.2 Repeatability: CV values from five replicate LNA-PCR amplifications

of recombinant plasmids were all less than 5%, indicating good reproducibility ().

Table 2 Repeatability test results of LNA-PCR method

Recombinant Plasmid	Ct Values	CV (%)
rtA181V	[data]	<5
rtN236T	[data]	<5

2.2.3 Stability: Accelerated degradation testing demonstrated that the established LNA-PCR method maintained detection stability without significant alteration.

2.3 Clinical Sample Testing LNA-PCR analysis of 89 clinical samples revealed 8 cases (8.98%) with rtA181V mutation (Ct values: 19.94/-, 19.96/-, 23.10/-, 25.91/-, 26.14/-, 29.67/-, 32.89/-, 33.40/-), 5 cases (5.61%) with rtN236T single mutation (Ct values: 19.67/-, 19.67/-, 22.24/-, 26.32/-, 29.06/-), and 2 cases (2.24%) with dual rtA181V and rtN236T mutations (Ct values: 23.10/33.68, 29.67/22.24). All results were concordant with DNA sequencing, confirming that the LNA-PCR method is suitable for clinical detection of HBV ADV resistance mutations ([Figure 4: see original paper]).

Discussion

The LNA-PCR system designed in this study effectively discriminates single-base mutations and can detect ADV resistance mutations rtA181V and rtN236T in HBV with a lower detection limit of 10^2 copies/mL. Therefore, test samples should contain 10^2 copies/mL HBV DNA. For samples with low HBV DNA loads, detection rates can be improved by increasing plasma extraction volume and reducing the final DNA resuspension volume.

Drug resistance becomes critically important during long-term nucleos(t)ide analogue therapy for chronic hepatitis B. HBV resistance not only leads to disease progression and increased hepatocellular carcinoma risk but also raises long-term treatment costs. Therefore, resistance mutations should be monitored regularly during antiviral therapy to enable timely treatment adjustments [10,11].

As a first-line treatment for hepatitis B and preferred option for lamivudine-resistant patients, ADV demonstrates resistance rates of 2.0% after two years and 22% after five years of treatment [12-15]. While many ADV resistance mutations exist, the primary sites are rtA181V and rtN236T in the HBV P gene region. Due to the lack of proofreading activity during HBV replication, the virus exhibits high genetic polymorphism and numerous mutation sites. Designing LNA probes for rtA181V detection requires consideration of upstream rtL180M

mutations (TCAT) and polymorphisms (TCCT/TCTT), necessitating degenerate probe design. A similar approach was used for rtN236T detection. This complexity may explain the scarcity of reports on fluorescence PCR methods for ADV resistance detection [16,17]. Importantly, the introduction of degenerate bases did not compromise detection of rtA181V and rtN236T.

LNA is a bicyclic nucleic acid derivative where the 2' -O and 4' -C positions of the ribose form an oxymethylene bridge, locking the furanose in a C3' -endo N-type conformation. This reduces ribose flexibility and increases local stability of the phosphate backbone, making LNA particularly suitable for SNP mutation detection [18]. Literature reports indicate that LNA probe-based HBV detection exhibits higher sensitivity than other methods [19]. The LNA-PCR assay established in this study demonstrates high sensitivity and specificity for detecting ADV resistance mutations in HBV, effectively identifying rtA181V and rtN236T mutations in clinical samples. Compared with alternative methods such as DNA sequencing [20,21], line probe assay (LIPA) [22,23], gene chips, and reverse hybridization membrane strips, this fluorescence PCR method is simpler, more convenient, and faster, making it suitable for clinical promotion and application in China.

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Note: Figure translations are in progress. See original paper for figures.