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REVIEW

- 1651 Microbiome-liver crosstalk: A multihit therapeutic target for liver disease
Kirundi J, Moghadamrad S, Urbaniak C
- 1669 Radiological findings in non-surgical recurrent hepatocellular carcinoma: From locoregional treatments to immunotherapy
Ippolito D, Maino C, Gatti M, Marra P, Faletti R, Cortese F, Inchingolo R, Sironi S
- 1685 Factors affecting the quality of bowel preparation for colonoscopy in hard-to-prepare patients: Evidence from the literature
Shahini E, Sinagra E, Vitello A, Ranaldo R, Contaldo A, Facciorusso A, Maida M

MINIREVIEWS

- 1708 Gut microbiome therapeutic modulation to alleviate drug-induced hepatic damage in COVID-19 patients
Ahsan K, Anwar MA, Munawar N

ORIGINAL ARTICLE**Basic Study**

- 1721 Locked nucleic acid real-time polymerase chain reaction method identifying two polymorphisms of hepatitis B virus genotype C2 infections, rt269L and rt269I
Kim K, Choi YM, Kim DH, Jang J, Choe WH, Kim BJ

Retrospective Cohort Study

- 1735 Risk factors predict microscopic extranodal tumor deposits in advanced stage III colon cancer patients
Jhuang YH, Chou YC, Lin YC, Hu JM, Pu TW, Chen CY

Retrospective Study

- 1745 Outcomes of ABO-incompatible liver transplantation in end-stage liver disease patients co-infected with hepatitis B and human immunodeficiency virus
Tang JX, Zhang KJ, Fang TS, Weng RH, Liang ZM, Yan X, Jin X, Xie LJ, Zeng XC, Zhao D

CASE REPORT

- 1757 Eosinophilic enteritis requiring differentiation from chronic enteropathy associated with *SLCO2A1* gene: A case report
Kimura K, Jimbo K, Arai N, Sato M, Suzuki M, Kudo T, Yano T, Shimizu T

ABOUT COVER

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Basic Study

Locked nucleic acid real-time polymerase chain reaction method identifying two polymorphisms of hepatitis B virus genotype C2 infections, rt269L and rt269I

Kijeong Kim, Yu-Min Choi, Dong Hyun Kim, Junghwa Jang, Won Hyeok Choe, Bum-Joon Kim

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The presence of two distinct hepatitis B virus (HBV) Pol RT polymorphisms, rt269L and rt269I, could contribute to the unique clinical or virological phenotype of HBV genotype C2. Therefore, a simple and sensitive method capable of identifying both types in chronic hepatitis B (CHB) patients infected with genotype C2 should be developed.

AIM

To develop a novel simple and sensitive locked nucleic acid (LNA)-real time-polymerase chain reaction (RT-PCR) method capable of identifying two rt269 types in CHB genotype C2 patients.

METHODS

We designed proper primer and probe sets for LNA-RT-PCR for the separation of rt269 types. Using synthesized DNAs of the wild type and variant forms, melting

temperature analysis, detection sensitivity, and endpoint genotyping for LNA-RT-PCR were performed. The developed LNA-RT-PCR method was applied to a total of 94 CHB patients of genotype C2 for the identification of two rt269 polymorphisms, and these results were compared with those obtained by a direct sequencing protocol.

RESULTS

The LNA-RT-PCR method could identify two rt269L and rt269I polymorphisms of three genotypes, two rt269L types ['L1' (WT) and 'L2'] and one rt269I type ('I') in single (63 samples, 72.4%) or mixed forms (24 samples, 27.6%) in 87 (92.6% sensitivity) of 94 samples from Korean CHB patients. When the results were compared with those obtained by the direct sequencing protocol, the LNA-RT-PCR method showed the same results in all but one of 87 positive detected samples (98.9% specificity).

CONCLUSION

The newly developed LNA-RT-PCR method could identify two rt269 polymorphisms, rt269L and rt269I, in CHB patients with genotype C2 infections. This method could be effectively used for the understanding of disease progression in genotype C2 endemic areas.

Key Words: Hepatitis B virus; Genotype C2; Polymerase; rt269; Locked nucleic acid-real time-polymerase chain reaction; Chronic hepatitis B

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Core Tip: Hepatitis B virus (HBV) genotype C2 infections have distinct clinical or virological traits, including a higher risk of hepatocellular carcinoma, lower response rate to interferon or prolonged hepatitis B e antigen-positive phase. We recently reported that the presence of two HBV Pol RT polymorphisms, rt269L and rt269I, contributed to unique traits of HBV genotype C2. Here, instead of time- or labor-consuming direct sequencing, we developed a new locked nucleic acid (LNA)-real time-polymerase chain reaction (RT-PCR) method for the separation between rt269L (L1 and L2) and I type from Korean chronic hepatitis B patients of genotype C2. The newly developed LNA-RT-PCR could be effectively used for the understanding of epidemiology and disease progression in genotype C2 endemic areas.

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INTRODUCTION

Although vaccines and therapeutic agents are currently available against hepatitis B virus (HBV), HBV infection is still a high-risk global health issue. More than 350 million people are chronically infected, and approximately 786000 patients die annually worldwide due to HBV-related diseases, including cirrhosis and hepatocellular carcinoma (HCC)[1,2].

HBV belongs into hepadnaviridae and is an enveloped and partially double-stranded DNA virus. Its genome is approximately 3.2 kb in length and contains 4 overlapping open reading frames: Surface antigens (S), core proteins (C), polymerase (Pol), and X proteins (X)[3]. The HBV reverse transcriptase can lead to HBV mutations of higher frequency than that of other DNA viruses due to its lack of proofreading ability[4,5]. This results in the failure of antiviral therapy with nucleos(t)ide analogs and liver disease progression *via* persistent infections[5-9]. According to the criteria of an 8% divergence in HBV genome sequences, HBV has been grouped into 10 genotypes as A-J[10-12]. A number of studies on HBV genotypes have reported that they play significant roles in the development of different disease profiles during chronic hepatitis B (CHB) infection as well as distinct geographic and ethnic distributions[13,14]. Of note, genotype C, particularly C2, *vs* genotype B showed a higher HBV replication capacity and higher tendency of chronicity and more frequently developed into liver cirrhosis (LC) and HCC in CHB patients of HBV endemic Asian nations, such as China, Japan and South Korea[11,15-19]. In addition, incomplete response to interferon (IFN) therapy and higher levels of mutations were also reported in genotype C2 infections[18,20-22]. However, thus far, which factor can explain several

distinct characteristics in clinical and virological aspects found in genotype C2 infections remains elusive.

As one likely answer to this issue, we have recently reported that the presence of two HBV Pol RT polymorphisms, rt269L and rt269I, that are found only in HBV genotype C could affect viral phenotypes and clinical outcomes and cause worse responses to IFN therapy in genotype C2 infections. In particular, we showed that the wild rt269L type infection that is distinct in genotype C *vs* the rt269I type is more strongly related to higher HBV replication and hepatitis B e antigen (HBeAg) positive serostatus, which are two distinct traits of genotype C infections[23-25]. This suggests that the presence of RT polymorphisms, particularly the wild rt269L type, could at least partly contribute into clinical or virological traits that are distinct in genotype C infections. However, our previous study has limitations in exploring the distribution of rt269 polymorphisms in CHB patients due to use of a conventional nested polymerase chain reaction (PCR) based direct sequencing protocol, which could underestimate genuine HBV quasispecies in patient sera[23]. A locked nucleic acid (LNA) is a nucleic acid analog containing a methylene bridge that connects the 2'-oxygen of ribose with the 4'-carbon[26,27]. The real time PCR method using a LNA-based probe capable of improving the hybridization affinity for complementary sequences shows strong mismatch discriminatory power[28,29]. Therefore, without the application of nested PCR, it could discriminate HBV mutations from CHB patients with high sensitivity and specificity.

Therefore, in this study, for the first time, we sought to develop a novel simple and sensitive locked nucleotide probe (LNA probe)-based RT-PCR (LNA-RT-PCR) method that is capable of separating two different rt269 polymorphisms, the wild-type rt269L (CTC/A) and rt269I type (ATC), in CHB patients of genotype C2.

MATERIALS AND METHODS

Patient sera, HBV DNA extraction and genotyping

For this study, serum samples from 94 patients who visited Seoul National University Hospital (2005-2007), met the inclusion criteria of hepatitis B surface antigen (HBsAg) positivity and HBV DNA positivity (for more than 6 mo), and were lamivudine, adefovir dipivoxil, entecavir, telbivudine, tumor necrosis factor, and peg-IFN treatment-naïve were used. All patients had negative tests for hepatitis C virus, human immunodeficiency virus and markers for coexisting autoimmune liver disease and did not have an alcohol or drug addiction. HBV DNA was extracted from 200 µL of serum samples using the QIAamp DNA Blood Mini Kit (QIAGEN Inc, Hilden, Germany). To analyze the genotyping, a nested PCR-based sequencing protocol targeting partial HBsAg sequences was used as previously described [30]. This study was approved by Seoul National University Hospital (IRB-1012-131-346).

Synthesis of positive control DNAs for variants at the HBV rt269 codon

We prepared six positive control DNAs for L1 [CTC, wild type (WT)] and the variants I (ATC) and L2 (CTA) at the HBV rtL269I locus. The DNAs were synthesized based on the HBV C2 polymerase sequence by Integrated DNA Technologies, Inc. They were 473 bp long and included the three variant sequences, with one of the 'A/G' polymorphisms near the variant sequence (Figure 1, Supplementary Table 1). These were used for the development of the methods for the application of LNA real-time PCR to a rapid differential and quantitative identification of the WT and variants. We used these DNAs to intentionally mix DNA templates with WT control DNA and variant control DNA in different ratios in a range of amounts to mimic clinical samples. We also used positive controls for melting temperature (T_m) analysis, detection sensitivity, and endpoint genotyping and for the construction of quantification standard graphs for LNA-RT-PCR to estimate the quantity of HBV WT and variant DNA in clinical samples.

Primer and LNA probe design

Primers were designed using LightCycler Probe Design Software 2.0 (LC PDS 2.0) Version 1.0.R.36 (Roche). The primers were designed to have high melting temperatures (> 65 °C) and to be highly conserved in the target DNA region of HBV. We used LC PDS (version 2.0) software for the probe design and referred to the design guidelines of the LNA manufacturer (Integrated DNA Technologies). The potential presence of cross-complementarities among all the primers and LNA probes was checked by using LC PDS 2.0 software. The LNA probes were purchased from Integrated DNA Technologies, and primers were purchased from Macrogen.

RT-PCR

A LightCycler Version 96 system (Roche) was used for LNA-RT-PCR, and three channels were used for the experiment. An optimal reaction mixture was established for the sensitive and specific detection of target sequences. A 10-µL reaction mixture was prepared for each sample as follows: 1 µL PCR buffer for *Taq* (Ex *Taq* HS, Takara), 2 mmol/L MgCl₂, 0.2 mmol/L deoxynucleoside triphosphate mixture

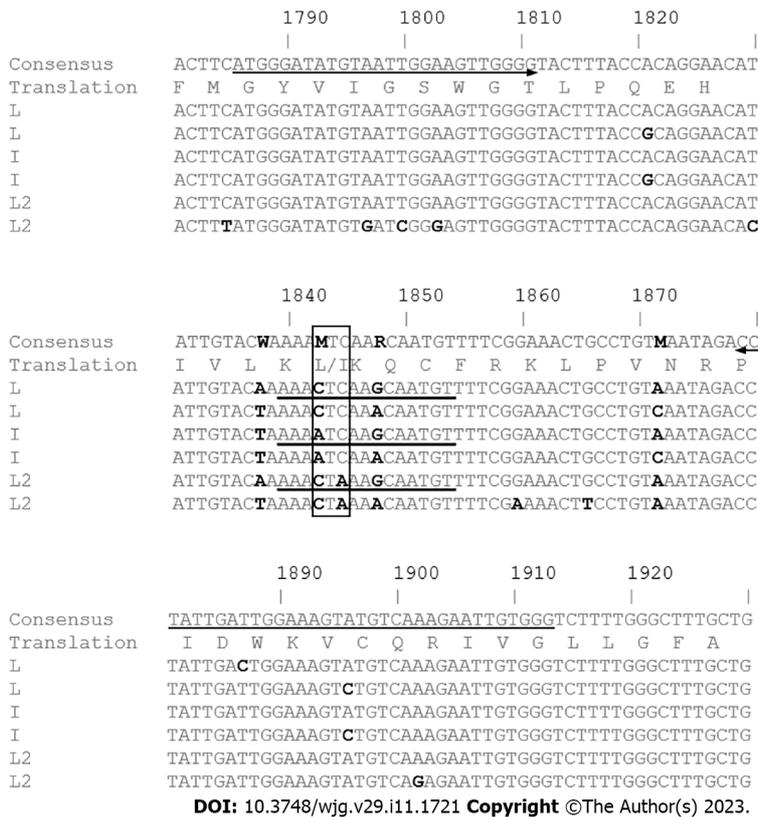


Figure 1 Primer and locked nucleic acid probe positions designed for the detection of three genotypes of polymorphisms in the rt269 codon, ‘L1’, ‘I’ and ‘L2’. Arrows indicate the primer positions. Underlines indicate the probe positions. The numbers designate the nucleotide position on the hepatitis B virus *P* gene sequence. Boldface bases denote the different bases. The box represents the codon and amino acid sequences of rtL269 variants. This single nucleotide difference is the basis of their discriminative identification by locked nucleic acid probes in this study. The amino acid sequence is shown as one-letter amino acid symbols.

(Takara), 0.2 μM forward primer, 0.8 μM reverse primer, 0.4 μM LNA FAM probe (L_CTC), 0.4 μM LNA Hex probe (I_ATC), 0.2 μM LNA Cy5 probe (L2_CTA), 0.25 u Ex *Taq* HS (Takara), 1 mg/mL bovine serum albumin (Ambion, ThermoFisher), 2 μL template DNA, and PCR-grade water (Roche). The cycling conditions were as follows, with default ramping speed rates if not specified: 60 s at 95 °C; four cycles of 10 s at 95 °C, 10 s at 58 °C, and 25 s at 72 °C with a 2.2 °C/s ramp; 46 cycles of 10 s at 95 °C, 10 s at 58 °C (with a single fluorescence acquisition), 25 s at 72 °C with a 2.2 °C/s ramp, and melting-curve analysis with 10 s at 95 °C, 60 s at 53 °C, and 1 s at 80 °C with a 0.08 °C/s ramp under continuous fluorescence acquisition at a rate of 4 readings/°C.

Identification of the WT and variant forms

Identification of the WT and variant forms ‘I’ and ‘L2’ at the rt269 codon in a sample was performed based on the three different LNA probe-specific *T_m* measurements at their own specified channels. To establish the diagnostic *T_m* range for the WT and variant forms, the control DNAs of the WT form, the variant forms and their mixtures at a variety of ratios were tested to observe melting peak formation and measure the specific *T_m* values for the WT and variant forms.

Construction of standard quantification curves

Six types of standard quantification curves for the WT and variant forms were generated with known amounts of positive control DNAs for their application to the estimation of the amount of the target DNAs in unknown samples. The standard curves were produced by duplicate LNA real-time PCR for each target DNA with known amounts (4.0E + 08 to 4.0E + 01 copies) of control DNAs. The R² correlation for all the standard curves was greater than 0.99. The limit of detection and limit of quantification of the WT and variant forms were determined among the series of diluted copies. These standard curves were applied to the quantification of DNA samples in a pure form and dominant type of variants in a mixed form.

Construction of standard genotyping plots to determine a dominant type in a mixture sample

To determine a dominant type of rtL269I variant in a mixture of a sample, standard genotyping plots were constructed using LNA real-time PCR with positive control DNA mixture sets in various ratios

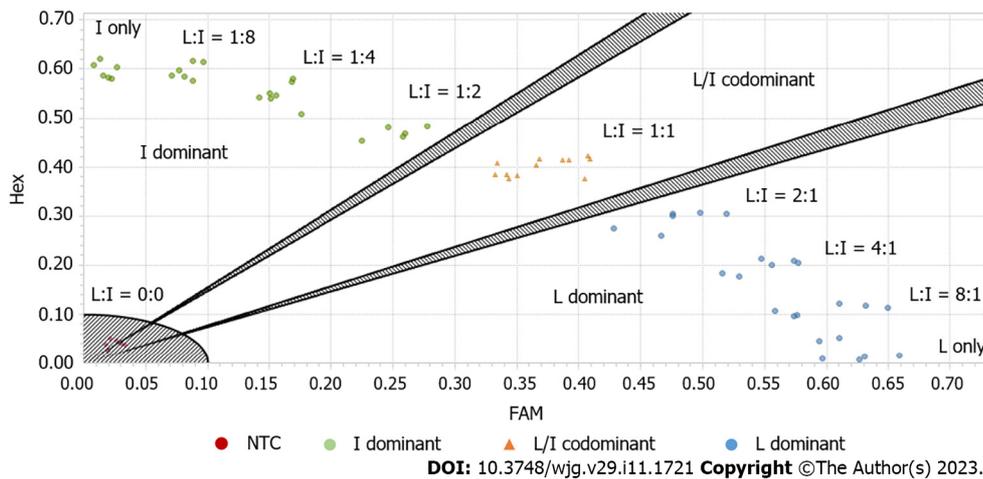


Figure 2 Differentiation of dominant hepatitis B virus rtL269 genotype variants using an endpoint genotyping method (LC96 software). L plus I genotype mixtures were prepared with known amounts of the genotypes in various ratios. I-dominant mixtures were positioned closer to axis Hex with higher Hex-fluorescence values, whereas L-dominant ones were located closer to axis FAM with higher FAM-fluorescence values. NTC: Nontemplate control.

and the endpoint genotyping tool of LC 96 system software. These plots were based on the endpoint fluorescence (EPF) values at the two channels for comparison. These plots were applied to determine the dominant type in the clinical samples (Figure 2).

Application of LNA-RT-PCR to clinical samples

The DNA of a total of 94 human sera was tested for the identification of the WT and 'I' and 'L2' variant forms of the HBV RT gene by LNA-RT-PCR. The quantification cycle (C_q), EPF, and T_m produced by the WT- and variant-targeting LNA probes with sample DNA were measured. Identification of the WT and variant forms was determined by comparing their T_m values obtained from their specific channel (FAM for WT, Hex for 'I', and Cy5 for 'L2') with their diagnostic T_m ranges obtained from standard assays.

Comparison of LNA-RT-PCR and direct sequencing for identification of WT and variant DNA

A total of 94 clinical samples were tested for the comparison of the LNA-RT-PCR method and direct sequencing method in the accurate identification of the rt269 variant and WT DNA. Direct sequencing was performed using the same primer sets producing the 128-bp LNA-RT-PCR amplicon.

RESULTS

Primer and probe design for LNA-based RT-PCR

First, we investigated the full-length HBV reverse transcriptase sequences from 131 treatment-naïve Korean patients chronically infected with HBV genotype C2 (GenBank No CH patients (GenBank Nos: KX264864-KX264922) and HCC patients (GenBank Nos: KX264792-KX264863)[30]. SeqMan II software Version 5.03 (DNASTAR) was used to search for appropriate primer sequences for LNA-based RT-PCR that are highly conserved to first obtain the shortest possible amplification product of the rt269 codon for efficient PCR (Figure 1).

We found three distinct sequence types in the rt269 codon from 131 patients, two types in rt269L, CTC (designated L1) and CTA (designated L2), and one rt269I type, ATC (designated I). Therefore, we designed three different LNA probes for specific simultaneous detection in a single reaction of the 'L' (WT), 'I', and 'L2' variants of HBV. The sequences of primers and LNA probes are shown in Table 1 and Figure 1.

Determination of the diagnostic T_m range for the identification of the WT and variant forms

Identification of the three sequence types, "L1", "I" and "L2", was accomplished by LNA-RT-PCR melting curve analysis by observation of their melting peak formation and their specific T_m measurement at their specified channel (Table 2, Figure 3). LNA-RT-PCR with samples of WT control DNA (n = 68) in amounts ranging from 4.0E + 00 to 4.0E + 08 copies resulted in a 100% positive detection rate and 100% specificity. A distinct melting peak formation at the FAM channel in all the tested WT control DNA samples with T_ms of 62.4 ± 0.4 °C for 'L' and 58.0 ± 0.2 °C for 'L' was observed, but no significant melting peak formation at the other channels (Hex and Cy5) was observed. LNA-RT-PCR with samples of the 'I' positive control DNA (n = 76) also resulted in a 100% positive detection rate and 100% specificity. A distinct melting peak formation in the Hex channel, 60.2 ± 0.7 °C for 'I' and 56.6

Table 1 Primers and locked nucleic acid probes developed for the identification of the hepatitis B virus L/I/L2 variants by multiprobe locked nucleic acid real-time polymerase chain reaction

Primer/probe	Sequence (5' to 3') ¹	T _m (°C) ²	Target	CH
Primers (product: 128 bp)				
Forward	ATGGGATATGTAATTGGAAGtGGGG	65-67	HBV P gene	
Reverse	CCCACAATTCtTGACATACTTTCCAATCAATAGG	67-69	HBV P gene	
LNA Probes				
L_CTC	5' 6-FAM-AAA+C+T+CAAR+CA+ATGT - 3' IABkFQ	61-64	L (WT)	FAM
I_ATC	5' HEX-AAA+A+T+CAAR+CAA+T+GT - 3' IABkFQ	61-64	I	HEX
L2_CTA	5' CY5-AAA+C+T+AAAR+CAA+T+GT - 3' IABkFQ	60-63	L2	CY5

¹Locked nucleic acid nucleotides are written +A, +C, +T or +G.

²Primer T_m was calculated by using LC PDS software version 2.0, and probe T_m was calculated by <https://www.exiqon.com/ls/pages/exiqontmpredictiontool.aspx>.

T_m: Melting temperature; CH: Channel; HBV: Hepatitis B virus; WT: Wild type; LNA: Locked nucleic acid.

± 0.2 °C for 'I', was observed, but no significant melting peak formation in the other channels (FAM and Cy5) was observed. LNA-RT-PCR with samples of the 'L2' positive control DNA (*n* = 52) also resulted in a 100% positive detection rate and 100% specificity. A distinct melting peak formation at the Cy5 channel, 64.6 ± 0.1 °C for 'L2' and 61.2 ± 0.2 °C for 'L2', was observed, but no significant melting peak formation at the other channels (FAM and Hex) was observed.

LNA-RT-PCR with samples (*n* = 320) of the 'L' WT positive control DNA plus 'I' control DNA or 'L2' plus 'I', mixed in different ratios (1:1, 1:2, 1:4, 1:8, 2:1, 4:1, and 8:1) in amounts ranging from 4.0E + 01 to 4.0E + 08 copies resulted in a nearly 100% positive detection rate (only three samples undetected in the smallest amount of DNA) for both the variant and WT DNA. A distinct melting peak formation at the FAM, Hex, and Cy5 channels in all the mixed DNA samples with detectable T_ms was observed. The measured T_ms were shifted slightly downward from the range of the T_ms measured only with nonmixed DNAs, as shown in Table 1. These slight changes did not affect the identification of the sequence types in the samples.

Application of LNA-RT-PCR to clinical samples and comparison with the results of the direct sequencing protocol

Of the 94 clinical samples tested by our LNA-RT-PCR method, 87 samples (92.6% sensitivity) were positively identified as 'L1' (WT), 'I', and 'L2' variants in single or mixed forms. Among the positively identified samples (*n* = 87), all samples produced a distinct melting peak or peaks with a T_m or T_ms within the diagnostic T_m range for the WT form "L1" or the two variant forms "I" and "L2". Of the 87 positively detected samples, 63 (72.4%) and 24 samples (27.6%) were identified either singly or in a mixed manner, respectively. Of the 63 samples identified singly, the prevalence of the 'L1' type, 'I' type and 'L2' type was 82.5% (*n* = 52), 12.7% (*n* = 8) and 4.8% (*n* = 3), respectively (Table 3). Of the 24 mixed form samples (27.6%), the prevalence of samples with almost the same ratio of L1 and I (codominant cases) was 29.2% (*n* = 7). The prevalence of L1 (L1 + I or L1 + L2) and I dominant (L1 + I) cases was 54.2% (*n* = 13) and 16.7% (*n* = 4), respectively. Given that the dominant cases included the respective exclusive cases, of the 87 positively detected samples, the prevalence of L, I and coinfection with L and I was 78.2% [*n* = 68, L1(65) + L2(3)], 13.8% (*n* = 12), and 8.0% (*n* = 7), respectively. PCR direct sequencing using the same primer set used in the LNA-RT-PCR method enabled the successful separation between the L1, L2 and I sequence types in all 94 clinical samples (100% sensitivity). Comparison between results obtained by both direct sequencing and LNA-RT-PCR protocols showed that of the 87 samples identified by LNA-RT-PCR, all (86 samples, 98.9% specificity) but one sample (SNU3-479) produced completely identical results between the two protocols (Figure 4). A mismatched sample was identified as I dominant (L1:I = 1:4) by LNA and exclusive I type by the direct sequencing protocol. The distinct results between both protocols may be due to the difference in sensitivity between the protocols. All seven samples not detected by the LNA-RT-PCR method were demonstrated to have mutations in their respective probe binding sequences by a direct sequencing protocol, which could interfere with normal LNA-RT-PCR (Table 4).

Table 2 Measurement of melting temperatures of the L/I/L2 variants by multiprobe locked nucleic acid real-time polymerase chain reaction

Genotype of positive control DNA [copies, (4.0E + 00)-(4.0E + 08)]	Target sequence ¹	Measured T _m (°C) in channel ²								
		Min ³	Max ³	mean ± SD (detection) ³	Min ⁴	Max ⁴	mean ± SD (detection) ⁴	Min ⁵	Max ⁵	mean ± SD (detection) ⁵
L (n = 34)	AAACTCAA G CAATGTT	61.6	63.1	62.4 ± 0.4 (34, 100%)	-	-	-(0, 0%)	55.2	56.2	55.8 ± 0.2 (26, 76.4%)
L' (n = 34)	AAACTCAA <u>A</u> CAATGTT	57.8	58.7	58.0 ± 0.2 (34, 100%)	-	-	-(0, 0%)	-	-	-(0, 0%)
I (n = 42)	AAAATCAA G CAATGTT	-	-	-(0, 0%)	59.0	61.6	60.2 ± 0.7 (42, 100%)	-	-	-(0, 0%)
I' (n = 34)	AAAATCAA <u>A</u> CAATGTT	-	-	-(0, 0%)	56.3	57.1	56.6 ± 0.2 (34, 100%)	-	-	-(0, 0%)
L2 (n = 34)	AAACTAA A GCAATGTT	-	-	-(0, 0%)	-	-	-(0, 0%)	64.4	64.8	64.6 ± 0.1 (26, 100%)
L2' (n = 18)	AAACTAA <u>A</u> A CAATGTT	-	-	-(0, 0%)	-	-	-(0, 0%)	60.9	61.4	61.2 ± 0.2 (18, 100%)

¹Bold, target codon; underline, A/G polymorphism.

²Bold, target specific melting temperature.

³FAM.

⁴Hex.

⁵Cy5.

Bold words represent genotype-specific T_ms. T_m: Melting temperature; -: No significant melting temperature; SD: Standard deviation.

DISCUSSION

LNA-based RT-PCR assays have been widely applied to viral single-nucleotide polymorphism analysis as well as simple viral detection in clinical settings instead of the less sensitive traditional RT-PCR or nested RT-PCR assays prone that are to cross-contamination[31,32]. In particular, it has recently been reported that this method could successfully identify YMDD mutations of HBV from Korean patients with chronic HBV infections[30,33]. In the present study, we developed an LNA-RT-PCR assay using melting curve analysis for the identification of two polymorphisms within codon 269 of HBV Pol, rt269L and rt269I (three genotypes, rt269L1, rt269L2 and rt269I), with the advantages of easy performance and a low likelihood of cross-contamination. The clinical application of the LNA-RT-PCR assay was also compared in parallel with a direct sequencing protocol using clinical samples. Our data showed that the LNA-RT-PCR assay can separate the two polymorphisms in the rt269 codon of HBV Pol in clinical specimens with high sensitivity (92.6%, 87/94 samples) and specificity (98.9%, 86/87 samples) (Table 3). Of note, this assay can determine an almost exact ratio between two types within specimens from mixed cases (23/24 cases), suggesting its feasibility in the analysis of quasispecies distribution in mixed samples (Table 3, Figure 4).

Our LNA-based RT-PCR assays showed that the WT 'L1' type (n = 65, 74.7%) was found at the highest frequency in our cohort, followed by the 'I' type (n = 12, 13.8%) and 'L2' type (n = 3, 3.4%) (Table 3). This finding suggests that the 'L1' type is responsible for the majority of HBV infections in South Korea and that the WT form is prevalent in genotype C2 infections. Additionally, these findings suggest that the I type may be a variant of L1 rather than an independent polymorphism. Indeed, our

Table 3 Rates of positive detection of the hepatitis B virus L//L2 variants in a total of 94 clinical samples by locked nucleic acid real-time polymerase chain reaction

Type of detection	No. of samples	Percentage
Clinical samples	94	100
Single	63	67.0
L	55	58.5
I	8	8.5
Mixed	24	24.5
L + I (1:1)	7	7.4
L dominant	13	4.3
I dominant	4	12.8
Unidentified	7	7.4
Inconsistent with direct sequencing	1	1.1

Table 4 Samples which cannot be identified by locked nucleic acid real-time polymerase chain reaction assay

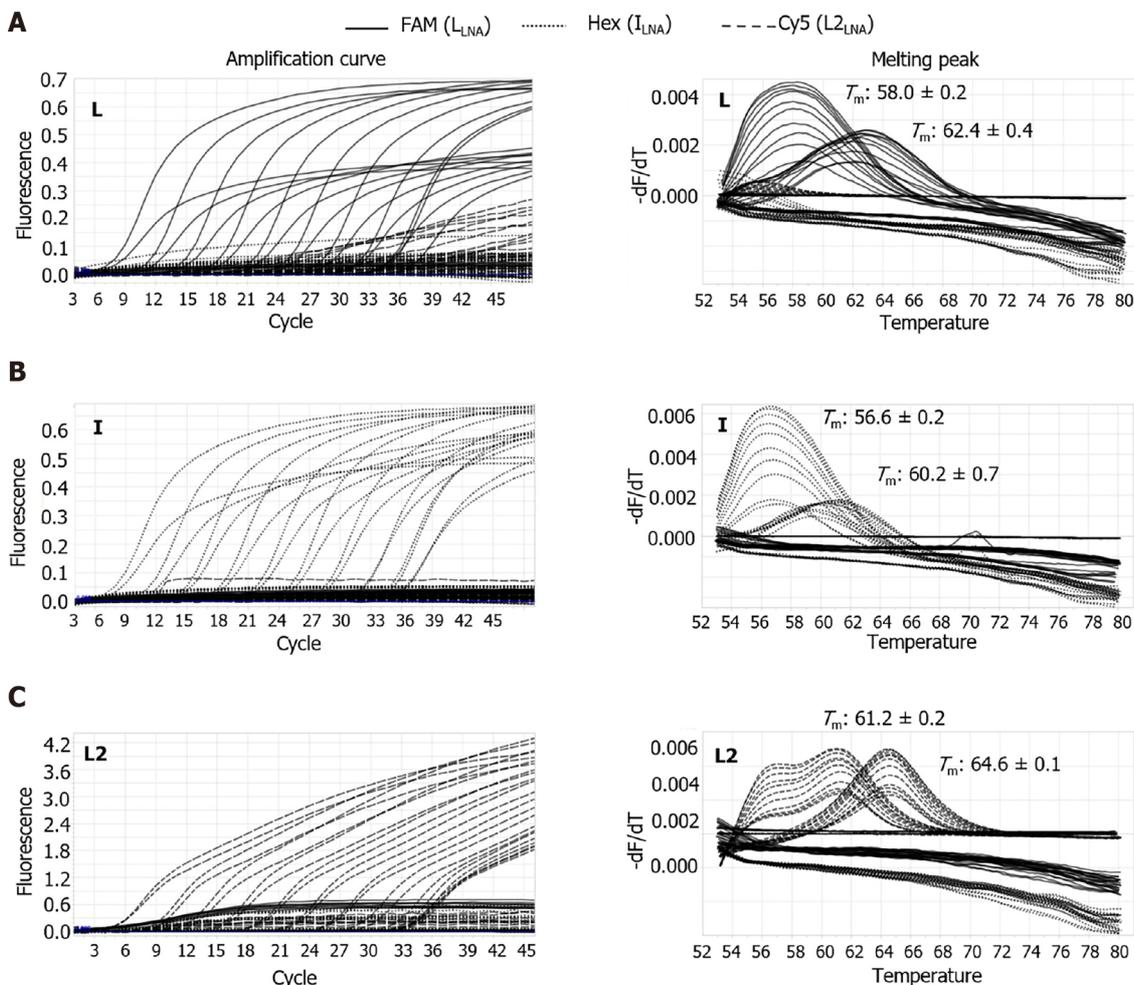
No.	Patients	Direct sequencing (AAACTCAARCAATGT)	Type	LNA-RT-PCR
1	SNU3 30 HCC	AAAATCAAGCACTGT	I	Not detected
2	SNU3 70 HCC	AAAATTAAGCAATGT	I	Not detected
3	SNU3 82 CH	AAAATCAAACATGT	I	Not detected
4	SNU3 123 HCC	AAACTTAAGCAATGT	L	Not detected
5	SNU3 31 CH	AAAATCCAGCAATGT	I	Not detected
6	SNU3 355 LC	AAAATTAAGCAATG	I	Not detected
7	SNU3 388 LC	AAACTTAAGCAATGT	L	Not detected

Bases in bold indicate the different ones from the target probe sequence. LNA-RT-PCR: Locked nucleic acid real-time polymerase chain reaction; HCC: Hepatocellular carcinoma; CH: Chronic hepatitis; LC: Liver cirrhosis.

previous study based on a direct sequencing protocol also showed that the 'L1' type *vs* the 'I' type is more closely related to higher HBV replication, higher HBsAg levels and HBeAg positive serostatus [23], suggesting that the majority of the 'L1' type infections in our cohort may be due to its enhanced viral infectivity. Therefore, it is tempting to speculate that the 'L1' type uniquely found in genotype C2 infections may contribute to some distinct traits of the genotype C2 infections, including an enhanced duration of the HBeAg-positive stage[34-36], higher infectivity[37,38] and a higher prevalence of occult infection *via* vertical transmission[33,39,40]. Since our LNA-based RT-PCR assays can identify L1 of higher infectivity and other variants (L2 or I type) related to disease progression from large serum samples without time-consuming or labor intensive sequencing procedures, it could help in the management or treatment of chronic patients in genotype C2 endemic nations, including China, Japan and South Korea.

In 7 (7.4%) of the 94 samples, despite successful amplification, our LNA-based RT-PCR assays failed to separate the two polymorphisms in the rt269 codon (Table 4). Comparison with the direct sequencing protocol revealed that all seven samples amplified but not identified by LNA-based RT-PCR assays had one more mismatch mutation that was different from the probe binding sequences. This was enough to interfere with normal detection due to the lower melting temperature than the respective probe. Therefore, in the samples amplified but not identified by our LNA-based RT-PCR assays, a further direct sequencing protocol should be recommended for the identification of the two polymorphisms.

A total of 24 (27.6%) of the 87 positively detected samples were identified in a mixed manner, and L1, in most cases of mixed infections, was dominant or codominant over I or L2. These findings further support our hypothesis that I or L2 may be a variant of the L1 type rather than an independent polymorphism. However, to clarify whether mixed infection in a patient is due to simple mutation of L1 to L2 or I type or superinfection of another type, further quasispecies analysis should be investigated in the future.



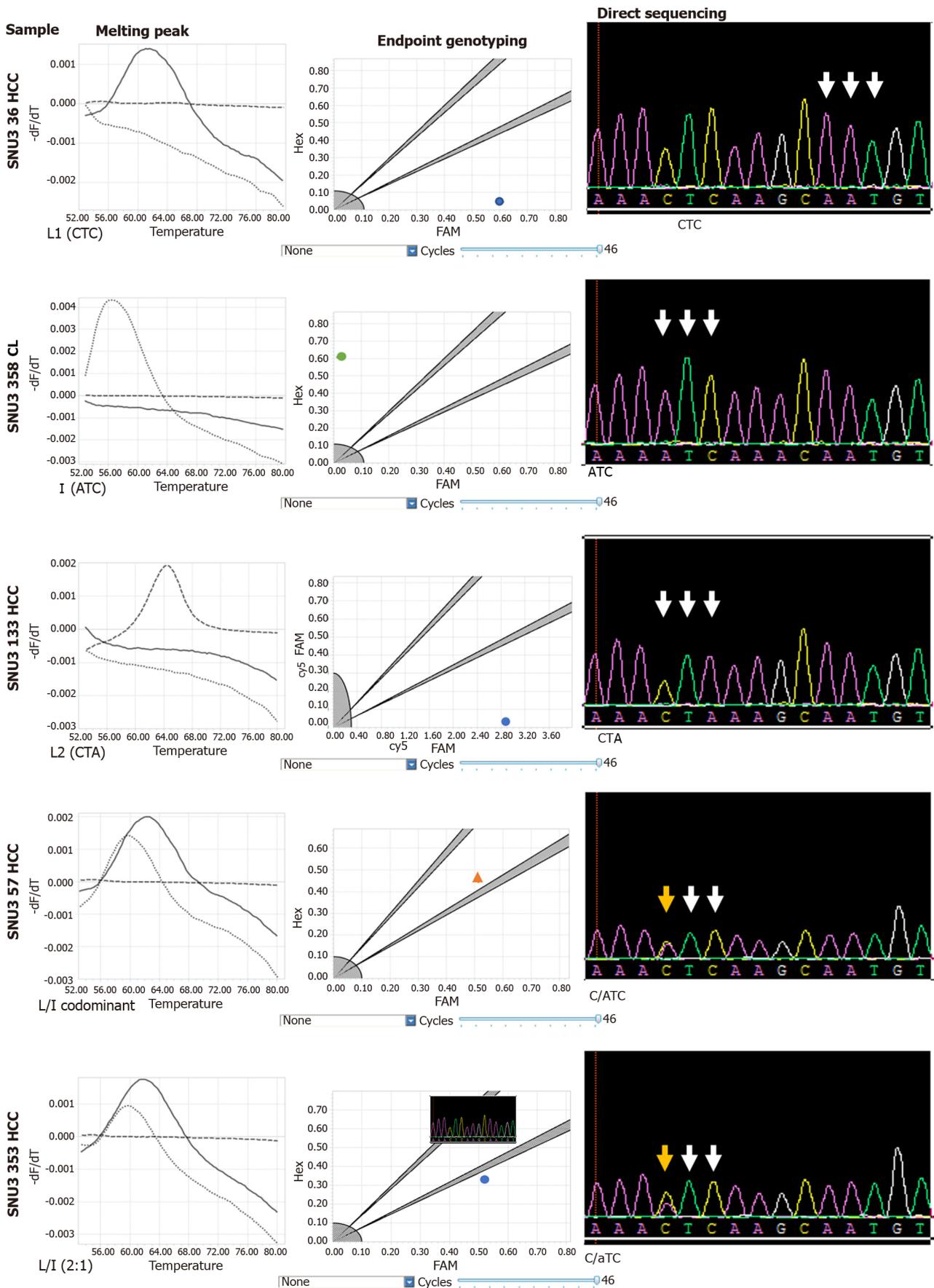
DOI: 10.3748/wjg.v29.i11.1721 Copyright ©The Author(s) 2023.

Figure 3 Multiprobe locked nucleic acid real-time polymerase chain reaction for discrimination among three types of polymorphisms in the rt269 codon. Amplification curves are shown on the left, and melting peaks are shown on the right. A: With L1 wild-type DNA templates, L1-type specific signals in the FAM channel (solid) were detected, showing their dominant amplification and distinct melting temperatures (T_m), with minimal cross signals of amplification and melting peaks generated by weak cross hybridizations of the other probes (I and L2) detection; B: For I variant-type DNA templates, I-type specific signals in the Hex channel (dotted) were detected, showing their exclusive amplifications and distinct T_m values, with no cross signals; C: For L2 variant-type DNA templates, amplification curves showed weak cross signals, but melting peaks were distinct with no cross signals.

The limitation of this study is that all the samples included were obtained from patients at the initial stage of drug use and are from one medical institution. To determine the exact clinical significance of L1, L2 and I infections or mixed infections in genotype C2-infected chronic patients, our LNA-based RT-PCR assays should be applied to a larger population-based cohort of multicenter registries in future studies.

CONCLUSION

In conclusion, our data showed that the LNA-RT-PCR method developed in this study can successfully identify two different polymorphisms, rt269L (L1 and L2) and rt269I, in the rt269 codon of HBV Pol from CHB patients with genotype C2 infections. The wildtype 'L1' form is more prevalent than the rt269I form in Korean CHB patients with genotype C2 infections, which is possibly due to its higher infectivity. Therefore, our LNA-RT-PCR method enables the separation of rt269 types and could be effectively used for a deeper understanding of epidemiology and disease progression in genotype C2 endemic areas.



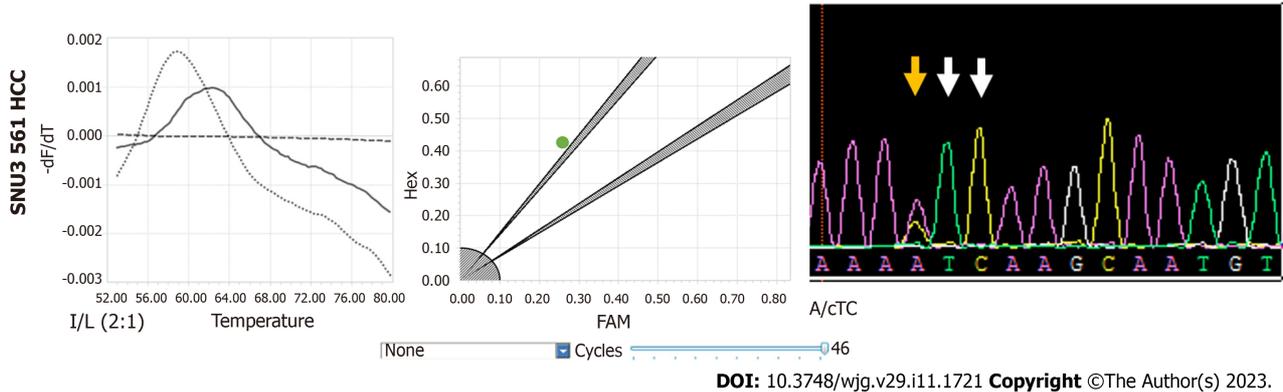


Figure 4 Confirmation of multiprobe locked nucleic acid real-time polymerase chain reaction identification results of hepatitis B virus rtL269 variants by direct sequencing. Nucleotide bases are shown in the parentheses. Lowercase letters represent the base present in a lower amount relative to the dominant variant. Bold indicates the dominant amino acids and bases. Arrows represent the codon sequence positions for leucine or isoleucine; yellow, mixed bases.

ARTICLE HIGHLIGHTS

Research background

Hepatitis B virus (HBV) genotype C infections has distinct clinical or virological traits including higher risk of hepatocellular carcinoma, lower response rate to interferon or prolonged hepatitis B e antigen-positive phase. As a likely answer to this issue, we have recently reported that the presence of two HBV Pol RT polymorphisms, rt269L and rt269I could contribute to unique traits of HBV genotype C.

Research motivation

For the identification between two rt269 types from chronic patients of genotype C2 endemic areas instead of time or labor consuming direct sequencing protocol, we sought to develop a novel simple and sensitive locked nucleotide probe based real-time polymerase chain reaction (LNA-RT-PCR) method capable of separating two rt269 types, rt269L type encoding leucine, 'L' (L1: CTC, L2: CTA) and rt269I type encoding isoleucine (ATC) from chronic hepatitis B (CHB) genotype C2 patients.

Research objectives

To develop a novel simple and sensitive LNA-RT-PCR method capable of identifying two rt269 types in CHB genotype C2 patients.

Research methods

We designed appropriate primer and probe sets for LNA-RT-PCR for the separation of rt269 types. The developed LNA-RT-PCR method was applied to a total of 94 CHB patients of genotype C2 for the identification of two rt269 polymorphisms, and these results were compared with those obtained by a direct sequencing protocol.

Research results

The LNA-RT-PCR method could identify two rt269L and rt269I polymorphisms of three genotypes, two rt269L types ['L1' (WT) and 'L2'] and one rt269I type ('I') in single (63 samples, 72.4%) or mixed forms (24 samples, 27.6%) in 87 (92.6% sensitivity) of 94 samples from Korean CHB patients.

Research conclusions

The newly developed LNA-RT-PCR method could identify two rt269 polymorphisms, rt269L and rt269I, in CHB patients with genotype C2 infections. This method could be effectively used for the understanding of disease progression in genotype C2 endemic areas.

Research perspectives

The newly developed LNA-RT-PCR method could identify three rt269 types, L1, L2 and I from CHB patients of genotype C2 with high-sensitivity and specificity. It could play a relevant role in the clinical management of CHB patients of genotype C2 infection.

FOOTNOTES

Author contributions: Kim K and Kim BJ contributed to study conception and design, and designed and performed experiments; Choe WH contributed to collection of clinical data; Kim K, Choi YM, Kim DH, Jang J, Choe WH, and Kim BJ contributed to data acquisition, data analysis and interpretation; Kim K, Choi YM, Choe WH, and Kim BJ contributed to writing of article, editing, reviewing and final approval of article.

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