Original Article

Different methodological approaches for interleukin 28B genotyping

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ABSTRACT

Background and Objectives: Treatment responsiveness to pegylated interferon-α and ribavirin against hepatitis C virus genotype 1 has strongly been associated with two single nucleotide polymorphisms (rs8099917 and rs12979860) in the region of the interleukin 28B (IL28B) gene. The aim was to perform three genotyping methods and evaluate their specificity, technical characteristics, and costs. In addition, the distribution of both polymorphisms in an Uruguayan population was assessed. **Materials and Methods:** One hundred DNA samples were genotyped by allele-specific polymerase chain reaction (AS-PCR), real-time PCR high resolution melting (RT-HRM), and Sanger sequencing methods. **Results:** The rs12979860 CC genotype, followed by the CT, was the most prevalent (52% and 39%, respectively). For rs8099917, the TT genotype was the most common (61%) followed by the GT (34%). AS-PCR and RT-HRM assays were specific for both IL28B genotypes determinations and showed a total concordance with Sanger sequencing results. **Conclusions:** Any of three genotyping methods is suitable for IL28B genotyping. The choice of the assay will depend on costs, special equipment availability, turnaround time, and specialized human resources.

Key words: Genotyping, interleukin 28B, polymorphism

INTRODUCTION

Hepatitis C virus (HCV) infection is one of the most important causes of chronic liver disease globally.[1] The highest response rates to therapy have been managed using the combination of pegylated interferon-α and ribavirin (pegIFN/RIB), but only 50–70% of patients achieve a sustained virologic response.[2]

Two single nucleotide polymorphisms (SNPs) rs12979860 and rs8099917, located near the region of the interleukin 28B (IL28B) gene, were associated with individual's responsiveness to pegIFN/RIB.[3-6]

Gene distribution of 100 samples was obtained using allele-specific polymerase chain reaction (AS-PCR), real-time PCR high resolution melting (RT-HRM), and Sanger sequencing. In addition, technical characteristics, costs, reagent, and equipment were compared.

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MATERIALS AND METHODS

The study was conducted with genomic DNA isolated from peripheral blood of 100 clinical samples using a commercial kit (The PureLink™ Genomic DNA, Invitrogen, USA) according to the manufacturer's protocol. The DNA concentration was quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific, USA). This study was approved by the Bioethical Board of the our institution. Study procedures were in accordance with the Helsinki Declaration of 1975 as revised in 2000.

Different amplification reactions (PCR) were performed using primers designed with the Primer3 software[7] or web-based AS-PCR assay designing tool.[8] The final version of primer includes some modifications, and they are listed in Table 1.

The AS-PCR was carried out with 1× PCR buffer, 0.2 μM of each primer, 0.8 nM of deoxynucleotides (dNTPs), 50 ng of genomic DNA, 5% of dimethyl sulfoxide (DMSO), and 0.3U *Taq* DNA polymerase (Invitrogen, Brazil) in a final volume of 20 μL. Final concentrations of $MgCl_2$ for rs12979860 or rs8099917 were 1.0 mM or 1.2 mM, respectively. The thermal cycler program was set to 95°C for 5 min, thirty cycles of 95°C 30 s, 50°C or 54°C 30 s (for rs12979860 or rs8099917, respectively) and 72°C 30 s, with a final extension for 5 min at 72°C. PCR products were separated by electrophoresis through a 6% polyacrylamide gel for 1 h at 100V with a 50 bp molecular weight marker (Thermo Scientific, USA). Genotypes were deduced after silver staining.

RT-HRM was performed in a Rotor-Gene 6000 (Corbett Life Science, Qiagen, USA). The reactions were performed separately for each SNPs or simultaneously. The monoplex reaction was set up with 1 μM of each primer (860HRMF–860HRMR or 917HRMF–917HRMR) and 30 ng of DNA in an HRM-PCR master mix (Type IT HRM Kit, Qiagen, USA) in a 10 μL reaction mixture. The optimized thermal cycling conditions included an initial hold at 95°C for 10 min, followed by forty cycles of 95°C for 10 s, 55°C for 15 s, and 72°C for 10 s. Subsequent HRM analysis was performed with a continuous fluorescence acquisition mode from 73°C to 89°C at a ramp rate of 0.3°C/s. The normalized melting regions for rs12979860 or rs8099917 were 83–87°C or 75–78°C, respectively. A tetra-primer amplification was also performed with 30 ng of DNA, 770 nM of each primer (860HRMF, 860HRMR, 917HRMF, and 917HRMR) in a 13 μL reaction mixture using HRM-PCR master mix (Type IT HRM Kit, Qiagen, USA). Cycling conditions included 95°C 10 min, followed by forty cycles of 95°C 10 s, 55°C 15 s, and 72°C 10 s. Subsequent HRM analysis was performed with a continuous fluorescence acquisition mode from 73°C to 89°C at a ramp rate of 0.3°C/s. Parameters used to normalize the melting curves were manually adjusted to obtain clearly genotype groups.

IL28 region amplification to be sequenced was performed in a total volume of 20 μL containing 50 ng of genomic DNA, 1.2 mM $MgCl_2$, 0.8 mM of each dNTP, 0.2 mM of each sequencing primer, 5% DMSO, and 0.3U *Taq* DNA polymerase (Invitrogen, Brazil). The amplification reaction conditions were 95°C 5 min, thirty cycles of 95°C 30 s, 54°C or 56°C 30 s (for rs12979860 or rs8099917, respectively), and 72°C 30 s with a final extension for 5 min at 72°C. PCR products were purified from a 2% agarose gel using a commercial kit (QIAquick Gel Extraction Kit, Qiagen, USA). Sequencing reactions were performed using a Big Dye Terminator Cycle Sequencing kit and analyzed on an ABI Prism 3130 sequencer.

Direct counting method was used to calculate the genotype and allele frequencies. Hardy–Weinberg equilibrium was tested for each SNP using Court calculator.[9]

Table 1: Primer sequences used for interleukin 28B genotyping. Underlined bases indicate the additional mismatch included

	Primer	Sequence (5-3×)	Fragment size (bp)
AS-PCR primers			
rs12979860	860SeqF	GCTTATCGCATACGGCTAGG	157
	860Rwt	AGTGCAATTCAACCCTGGTTAG	
	860Rmut	AGTGCAATTCAACCCTGGTTAA	
rs8099917	917SeqR	AACCCCACCTCAAATTATCC	195
	917Fwt	GTTTTCCTTTCTGTGAGCAACT	
	917Fmut	GTTTTCCTTTCTGTGAGCAACG	
Sequencing primers			
rs12979860	860SeqF	GCTTATCGCATACGGCTAGG	292
	860SeqR	CACAATTCCCACCACGAGAC	
rs8099917	917SeqF	AGTCTTGTATTTCACCTCCTGG	248
	917SeqR	AACCCCACCTCAAATTATCC	
HRM primers			
rs12979860	860HRMF	CCTGGACGTGGATGGGTAC	154
	860HRMR	CGCGGAGTGCAATTCAAC	
rs8099917	917HRMF	CACTGTTCCTCCTTTTGTTTTCC	138
	917HRMR	CATAAAAAGCCAGCTACCAAACTG	

AS-PCR: Allele-specific polymerase chain reaction, HRM: High-resolution melting

RESULTS

The AS-PCR profiles obtained after a polyacrylamide gel electrophoresis are presented in Figure 1. The genotyping of IL28B performed by HRM analysis either with one or two sets of primers simultaneously is shown in Figure 2. These assays were specific in both genotype determinations and showed a 100% concordance with Sanger sequencing results. The electropherograms obtained from the three possible genotypes, for both SNPs, are shown in Figure 3.

Allele and genotype frequencies were calculated using 100 clinical samples, and results are showed in Table 2.

Genetic frequencies of both polymorphisms were in Hardy–Weinberg equilibrium (rs12979860 $P = 0.666$, and rs8099917 $P = 0.925$).

DISCUSSION

To the best knowledge of the authors, this is the first study that involves the determination of IL28B gene polymorphisms in Uruguay. The distribution of these genotypes in an Uruguayan population was analyzed. The rs12979860 CC genotype was the most prevalent followed by the CT genotype. For rs8099917, the TT genotype was the most common followed by the GT genotype. After comparing these genetic distributions with those reported from neighbor countries, they are similar to Argentina data[10] but differ from those of Brazil.[11]

Sanger sequencing is considered the gold standard for SNP determinations. However, a major problem in developing countries is it high cost. As a cheaper alternative, we optimized AS-PCR and RT-HRM for the genotyping and compared the results with those from Sanger sequencing.

The results of the three genotyping technologies here tested showed a complete concordance for all samples, which means that any of them was suitable for the IL28B polymorphism determination. Then, the decision on which technique to apply will depend on costs, specialized equipment availability, turnaround time, and training. AS-PCR does not require labeled reagents, and equipment is mostly found in any molecular biology laboratory. Although it is a cost-effective technique and involves a single PCR reaction followed by a polyacrylamide gel electrophoresis, this assay needs a laborious laboratory manipulation. Although RT-HRM assay involves an easier manipulation and consumes less time than AS-PCR, more expensive reagents, thermal cycler, and a further analysis using specific software is required. Finally, DNA sequencing is the most

Table 2: Allele and genotype distribution of rs12979860 and rs8099917 single nucleotide polymorphisms calculated from results obtained from the three assays (see text)

rs12979860					rs8099917				
Genotype frequency (%)			Allele frequency		Genotype frequency (%)			Allele frequency	
CC	CT	TT	C	T	TT	GT	GG	T	G
52	39	9	0.715	0.285	61	34	5	0.780	0.220

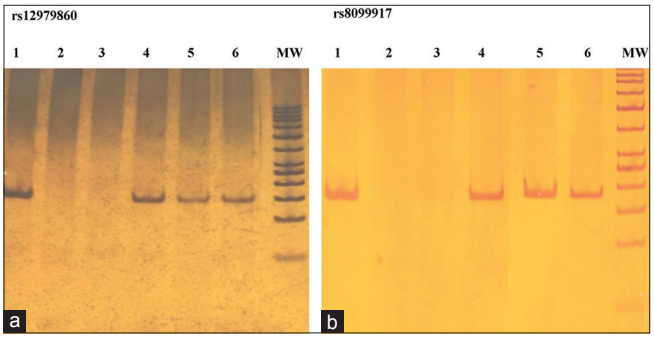

Figure 1: Electrophoresis pattern of the allele-specific polymerase chain reaction. Lanes 1, 3, and 5: Amplified reaction for the wild type allele, Lanes 2, 4, and 6: Amplified reaction for the mutant allele. MW: 100 bP DNA molecular weight. (a) rs12979860 (b) rs8099917

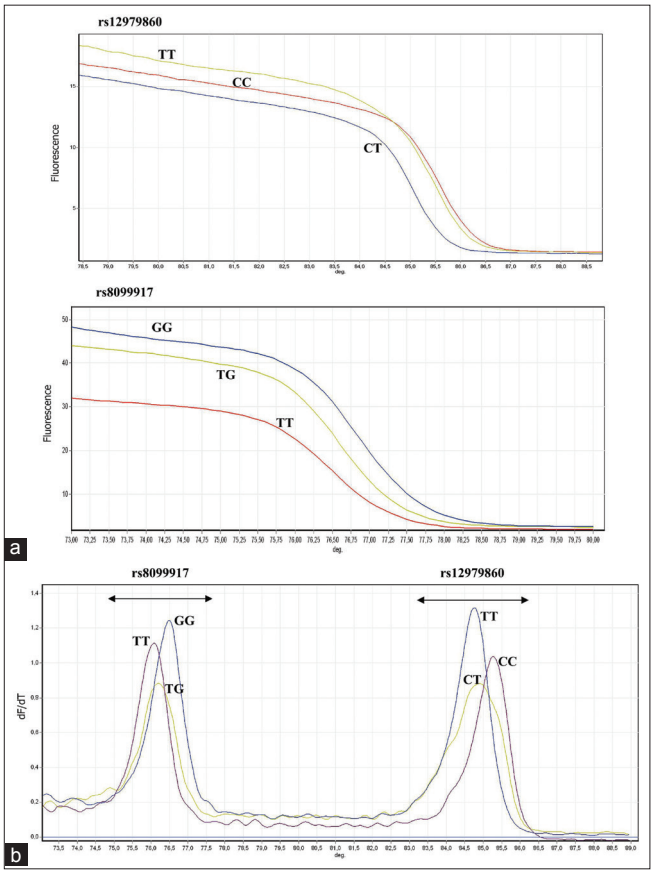

Figure 2: Real-time high resolution melting normalized melt curve. (a) Normalized melting ranges for rs12979860 and rs8099917 after individual amplification reaction. (b) Normalized melting ranges for rs12979860 and rs8099917 for duplex amplification reaction

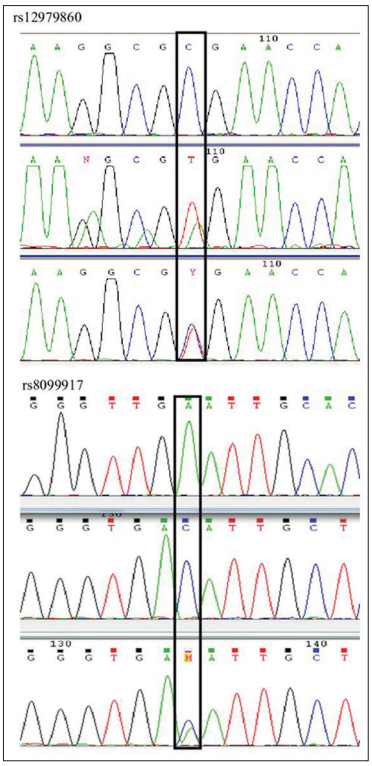

Figure 3: Sanger sequence chromatograms of rs12979860 and rs8099917. The boxes indicate single nucleotide polymorphism positions

laborious platform and is not cost-effective, requires a bioinformatic analysis, and has a longer turnaround time than required for previously mentioned assays. In terms of costs for a single reaction, the more expensive technique is DNA sequencing followed, in decreasing order, by RT-HRM and AS-PCR.

CONCLUSIONS

This pharmacogenetic approach to the treatment of HCV found equivalent results regardless of which of the three analyzed genotyping assays was applied. After an analysis of advantages and disadvantages of each method, the final choice only will depend on the individual convenience of each laboratory based on the characteristics discussed above.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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