IL28B genotyping, SNPs rs12979860 and rs8099917, by MELT-MAMA PCR analysis; a useful tool for prediction of therapy response in hepatitis C patients

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Several studies have identified associations between SNPs occurring near the IL28B gene and response to antiviral treatment among HCV patients. Here, we describe a reliable MELT-MAMA PCR-based genotyping method for IL28B which can be used in the management of HCV patients, helping to better define the course of therapy.

Keywords:
MELT-MAMA PCR, IL28B, Genotyping, Hepatitis C virus.
Hepatitis C virus (HCV) is a positive-polarity, single-stranded RNA virus belonging to the genus hepacivirus in the family Flaviviridae (12). Worldwide, an estimated of three million new infections occur annually and approximately 130 million people have been infected, the vast majority resulting in chronic infections (4). Moreover, a significant number of infected patients develop severe liver disease, including cirrhosis and hepatocellular carcinoma (7, 9, 17). Currently, the first-line of HCV antiviral therapy is based on administration of pegylated IFN-alpha (PEG-IFN-α) and ribavirin (RBV). Unfortunately, this therapeutic strategy is only effective in around 50% of patients infected with HCV genotype 1, although higher rates are reached in individuals infected with other viral genotypes (2, 20). A number of adverse effects to the PEG-IFN-α/RBV therapy are known such as depression, hematological ‘cytopenias’, thyroid dysfunction and skin rash, making the treatment not well tolerated in many cases. Therefore, the ability to predict failures prior to treatment could save a great deal of pain and expenses, helping make better clinical decisions.

Diverse predictor markers have been reported to influence the outcome of anti-HCV treatment such as virus genotype, viral load, complexity of viral population and viral genome sequence (1, 5-6, 10, 16). Recently, several genome-wide association studies (GWAS) have reported associations between different single nucleotide polymorphisms (SNP), located near the IL28B gene, and antiviral treatment, spontaneous viral clearance and progression to chronicity (8, 14, 18-19, 21). These findings suggest that these polymorphisms could be used as predictor factors to personalize antiviral therapy.

The goal of this work was to develop a rapid, highly specific and sensitive assay suitable for the identification of two IL28B SNPs (rs12979860 and rs8099917) strongly associated with therapy outcome. For this, 20 HCV chronic cases, aged 52–65 years, and 30
healthy donors, age matched, were enrolled in this study. All patients had completed the corresponding antiviral treatment and were being seen as part of the follow-up standard protocol after completion of therapy.

The genomic regions including SNPs rs12979860 and rs8099917 were used to design two different primer sets capable of differentiating between the two alleles for each polymorphism, based on their respective nucleotide pattern (table 1). For this, the 3’ ends in both forward primers were designed to carry the complementary nucleotide to either allele. Additionally, one mismatch, located at the penultimate nucleotide position, was also incorporated to the allele-specific primer increasing the primer specificity and enhancing the discrimination power between the two alleles (Figure 1). The incorporation of a GC clamp (17-base pair GC tail) at the 5’ end in one of the allele-specific primers was used to increase the Tm (~2.5°C) of that particular PCR fragment, thus facilitating visual identification (Figure 2). Primers including one mismatch at the antepenultimate and two mismatches, both the penultimate and antepenultimate positions, were also tested to determine the optimal experimental mismatching combination.

Four different constructions bearing the corresponding nucleotide substitutions for each target SNP were generated. All plasmid constructions were subjected to MELT MAMA PCR using the corresponding primers. MC analysis showed SNP rs12979860-C as a melting peak at 87.5 °C while rs12979860-T exhibited a peak at 88.5 °C. For SNP rs8099917, the G-allele showed a melting peak at 79.3 °C and the T-allele displayed a melting peak at 80.5 °C (Figure 3). Primers including only one mismatch at the penultimate nucleotide position exhibited good discrimination between both alleles (Figure 3). On the contrary, both primer sets containing a unique mismatch at the antepenultimate position did not achieve the desire
degree of discrimination for either SNP. PCR efficiency was significantly reduced when primers including two mismatches were used, resulting in poor amplification.

Blood samples from thirty healthy donors were simultaneously genotyped by MELT-MAMA PCR and Sanger sequencing. Concordance between the two methods was 100%. For SNP rs12979860, twenty five subjects were homozygous (3 T- and 22 C-allele) and five were heterozygous. For SNP rs8099917, fifteen individuals exhibited a homozygous genotype (5 G- and 10 T-allele) and 15 individuals were heterozygous (table 2).

Twenty HCV cases with known therapy outcome were genotyped. The most common genotype for SNP rs12979860 (16 patients) among the HCV cases was homozygous for the T-allele. Four patients were homozygous for the C-allele and no heterozygous individuals for this particular SNP were identified in this cohort. All four C-allele carriers (100%) successfully achieved SVR, while only 2 individuals (12.5%) out 16 carrying the T-allele attained SVR. In general, patients carrying the T-allele (87.5%) were prone to fail the antiviral treatment (table 2).

For SNP rs8099917, fifteen cases showed a homozygous genotype (5 G- and 12 T-allele, respectively), and three heterozygous cases were also identified (table 2). Seven individuals (87.5%) carrying the G-allele did not achieve SVR and only 1 subject (12.5%) successfully eliminated the virus. Homozygous subjects for the T allele achieved SVR in 41.6% of the cases while 58.3% showed null viral response.

Until very recently, there were no reliable baseline markers that could predict the outcome of anti-HCV therapy. The findings of associations between several SNPs located in the proximity of the IL28B gene and viral response have shown promising results suggesting that they can be used for decision making before start of treatment, preventing patients from...
undergoing a great deal of unnecessary distress. Thus, this might potentially represent a major step towards customization of medical care for HCV patients. Remarkable attention has been given to IL28B SNPs rs12979860 and rs8099917 which have shown strong association with therapy outcome, and therefore are leading candidates as predictor markers.

The predictive value of the SNPs located near the IL28B gene needs to be compared, and possibly complemented, with other predictors factors such as the infecting genotype, viral load, viral population complexity, nucleotide substitutions, etc, in order to improve their capacity to predict therapy outcome. The importance of IL28B SNPs as predictor markers is crucial even in the case of future therapies based on viral enzyme inhibitors since IFN-α will remain as the backbone of the anti-HCV treatment.

Despite the capacity of both IL28B SNPs to predict SVR among HCV cases, these markers seem to lack the ability to forecast when a patient could relapse, and therefore IL28B genotype should not be used exclusively to determine treatment outcome. Moreover, IL28B genotyping does not provide any guidance regarding treatment duration and should always be supplemented with a response-guided, personalized approach based on viral titer monitoring during the patient’s follow-up.

Development of reliable methods for the correct identification of IL28B SNPs is of high importance for the management of HCV cases. MAMA PCR has been largely used for punctual nucleotide substitution identification in diverse settings (3, 11, 15). The combination of MC analysis resolution and the simplicity of MAMA-PCR based assays provides a powerful tool for the accurate identification of SNPs without the need of extra steps after DNA amplification (13).
In summary, we present a reproducible, inexpensive and accurate method that allows rapid genotyping of IL28B polymorphisms in either clinical or research laboratories with various throughputs. This methodological approach is especially convenient in clinical scenarios where rather quick decisions should be made regarding medical attention of HCV chronic cases.
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**Figure Captions**

**Figure 1.** MELT-MAMA PCR principle. Two allele-specific primers were designed in such a way that the last position at the 3’ end matches the SNP of interest, and therefore if the SNP is not complementary the DNA polymerase fails to extend the primers preventing the synthesis of the nascent DNA strand. Mismatches purposely incorporated on the 3’end of the MAMA primer improve allele discrimination by further destabilizing the nucleotide bonds and avoiding amplification of the undesired allele. One of the allele-specific primers also differs at the 5’ end where a GC tail was incorporated in order to increase the melting temperature of the corresponding amplicon to facilitate allele identification. Measurement of fluorescence (SYBR Green) allows amplicon identification and allele discrimination by MC analysis.

**Figure 2.** Primer annealing sites. Sequences of both plasmids rs12979860 (A) and rs8099917 (B) along with the allele-specific primers are depicted. The conserved nucleotides positions throughout the alignment are indicated by ‘ ⋄ ’. The target SNP position is designated by “ * ”. Solid line arrows represent the position for the MELT-MAMA (forward) primer and the dashed line arrows depict the location of the corresponding reverse primers.

**Figure 3.** IL28B genotyping by MELT-MAMA PCR. A) Primer performance for both SNPs was assessed by MELT-MAMA PCR. Primers bearing one single mismatch exhibited good discrimination between alleles. The Tm for SNP rs12979860 corresponding to the C-allele amplicon was 87.5 °C while the T-allele exhibited a Tm of 88.5 °C. For SNP rs8099917, the experimental Tm observed for the G- and T-allele were 79.3 and 80.5°C, respectively.
MATCHED PRIMER-TEMPLATE

MISMATCHED PRIMER-TEMPLATE

HOMOLOGOUS (ALLELE A)

HOMOLOGOUS (ALLELE B)

HETEROLOGOUS (ALLELE A or B)
Figure 3

Melting Peaks

- rs8099917 G-allele
- rs8099917 T-allele
- rs12979860 C-allele
- rs12979860 T-allele
**Table 1. Primer sequences**

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<th>Reverse Primer</th>
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<tr>
<td>(allele C)</td>
<td>CCGAAGGCTC</td>
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| rs12979890 | CGCGCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCC GCC polymorphic sites are shown in bold
Mismatching nucleotides are in italics
GC tail is underlined
* Mismatches according to Li et al., 2004.
Table 2. IL28B genotyping and patients’ demographic characteristics

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