Hepatitis C Infection, Genotypes and Frequency of Interleukin-28B Polymorphisms

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Introduction: Hepatitis C is an infectious disease caused by the hepatitis C virus (HCV), with a worldwide prevalence between 123 to 170 million people infected. This virus has high heterogeneity being classified in six different genotypes: 1a, 1b, 2, 3, 4, 5 and 6. Therapeutic response is associated with HCV genotype and nowadays to the sequence of nucleotides from IL-28B gene. IL-28B gene different polymorphisms were identified. These polymorphisms seem to contribute for therapeutic efficiency. Individuals with the polymorphism rs12979860 (C/C) and rs8099917 (T/T) seem to have higher likelihood of treatment success.

Aim: The aim of this study is to evaluate the association between different HCV genotypes and the presence of rs12979860 IL-28B polymorphism.

Material and methods: The cohort study consisted in 59 HCV positive samples from the Centre Hospital and University of Coimbra. The HCV genotyping was made with the VERSANT® HCV Genotype 2.0 (LiPA). The evaluation of IL-28B polymorphisms was made using the Fast Set IL-28B from Arrow Diagnostics. Statistical analysis was performed in SPSS®.

Results and discussion: It was observed that 44 individuals were HCV genotype 1, of which 77.8% (14) were CC, 70.4% (19) were CT and 78.6% (11) were TT. The others IL-28B genotypes were equally distributed between genotypes 3 and 4 from HCV.

Conclusion: In this study it was not possible to observe an association between IL-28B genotype and HCV genotypes, however, it was observed that individuals with HCV genotype who have a poor response to therapy, are also IL-28B genotype that do not respond effectively to therapy.

Keywords: hepatitis C; genotype; polymorphisms; Interleukin-28B

Introduction

Hepatitis C is an infectious disease caused by hepatitis C virus (HCV) with tropism for liver cells, causing severe inflammation with many long-term complications. In most cases an asymptomatic chronic infection progresses, leading to cirrhosis 10 to 20 years after infection, and about 10% to 20% of cases evolve into hepatocellular carcinoma after 30 years of infection (1, 2).

The average incubation period of this disease is 15 to 90 days knowing that the asymptomatic individual can transmit the virus before the symptomatic phase, with the acute phase of infection by asymptomatic standard (2). Estimates indicate an overall prevalence of HCV range from 2% to 3%, i.e. a value between 123 million and 170 million people infected worldwide, which is considered a worldwide epidemic (3–6). In Portugal epidemiological data indicate that about 15% (100000–140000) of the population are infected with HCV (7).

HCV belongs to the gender Hepacivirus in the Flaviviridae family, about 60 nm in diameter, consisting of RNA and capsid surrounded by an envelope (8, 9). Its genome consists of about 10,000 nucleotides encoding 3,000 amino acids, which originates different proteins, contributing to the heterogeneity of this virus. These proteins can be classified into structural (and the capsule constitute the viral envelope) and nonstructural (NS2, NS3, NS4A, NS4B, NS5A and NS5B) (9,10).

Since there is a hypervariable region (HVR1) in the gene encoding the envelope (E1) and a zone at the amino-terminus of the genome (5’end) of 342 nucleotides end, this is the most conservative region of the genotype having a crucial role in viral replication (1, 8, 9, 11–13).

The high heterogeneity of the nucleotide sequences of the virus allows the appearance of different genotypes of HCV. Being divided into 6 types and 11 subtypes (1a, 1b, 1c, 2a, 2b, 2c, 3a, 3b, 4a, 5a and 6a). The genotypes differ in a percentage that can vary between 30% and 35% in its nucleotide sequence (2, 12).

Genotypes 1, 2 and 3 are distributed worldwide, and the number infected is higher in Western countries. In Egypt and Central Africa genotype 4 is the most common. Genotype 5 is most prevalent in South Africa. Macau, Hong Kong and Vietnam are the places where genotype 6 is prevalent (6.14).

Patients carrying genotypes 2 and 3 by standard have a better prognosis because they usually respond better to treatment. The use of pegylated interferon (IFN-PEG) plus ribavirin (RIB) is the most commonly used treatment in the clinic. RIB acts directly on viral DNA, inhibiting DNA polymerase. It is known that this drug alone has no effect on HCV. The INF binds to specific receptors on the surface of cells infected by this virus, initiating an intracellular signaling process and rapid activation of transcription of different genes. These genes encode viral proteins which, after
binding to non-infected cells, promote inhibition of viral replication and inhibition of cell proliferation. PEG-INF acting together with theRib discloses a more effective therapy (15–17).

Genotype 1 is more common among patients with hepatitis C in Europe, genotype 1b is the most prevalent and therefore more resistant to therapy. The remaining genotypes are less prevalent in Europe (14, 18).

The clinical advantage in determining the genotype concerned is to predict disease progression and better adaptation of treatment (11). The treatment for this infection may act both in the acute phase or chronic phase of infection, with different objectives: In the acute phase, the goal is to reduce the risk of progression to chronicity; in the chronic phase, the objective is to control the progression of liver disease by inhibiting viral replication in order to reduce the inflammatory activity and subsequent progression to cirrhosis and eventual liver cancer (1, 19).

The phenomenon of viral persistence occurs due to genetic mutations of the virus, which limit the action of the immune defenses, occurring immunosuppression and protection of infected cells (20).

Recently began therapy with a new group of drugs (Boceprevir and Telaprevir), whose main target is the enzyme HCV NS3 serine protease, this portion of the HCV genome is nonstructural and allows the production of new virus, the inhibition promotes reduction of viral replication (12, 21, 22).

The pathogenicity of this infection occurs mainly due to induced hepatocyte injury due to persistent viral replication. The presence of the virus in hepatocytes promotes an immune response, leading to a constant activation of T helper cells, which act on target hepatocytes causing their destruction and elimination of virus, the greater the intensity of the immune response, the higher the lesions in these cells (1, 2).

As mentioned previously the T-cells have an important role in infection, because they produce cytokines, which are produced during the activation and effector phase in order to mediate and regulate immune and inflammatory response. They also are responsible for activating B lymphocytes, which subsequently produce specific antibodies. Interleukins (IL) are family of cytokines, which are responsible for the activation of lymphocytes. Each IL acts on a limited and specific group of cells expressing specific receptors for this (23, 24).

IL-28B is from the III interferon family, which plays a crucial role in the immune defense against viruses, including HCV (24, 25).

Recent studies have suggested that the efficacy of therapeutic response is dependent on the nucleotide sequence of the IL-28B gene (26, 27). The polymorphism of a single nucleotide will be sufficient to help in predicting the effectiveness of therapy, and is described in the following literature polymorphisms: rs8099917 polymorphism and rs12979860 polymorphism (4, 5, 25, 28).

The first is associated with sustained viral response, with which individuals with genotype C/C have a higher probability of response to treatment compared to individuals with genotype C/T or T/T (wild type) (24, 26, 29, 30). Individuals with the rs8099917 polymorphism of the IL-28B genotype and T/T are also associated with a greater likelihood of treatment success (29, 31).

Thus, this study aimed to evaluate the association between rs12979860 genotype of the IL-28B and the presence of different HCV genotypes in the population.

Material and methods

The study population consists of 59 individuals with proved HCV infection. Data collection was performed at the Department of Blood Service and Transfusion Medicine (SSMT) in the Centre Hospital and University of Coimbra (CHUC), between September 2013 and April 2014. 74.6% being males (44) and 25.40% (15) females. The median age of patients studied was 46 years, with a minimum of 28 years and maximum of 68 years.

The samples used in this study came from different services, and the service with the highest number was Infectious Diseases – Consultations, followed by the Service of Internal Medicine (Figure 1).

The samples for determination of HCV genotype were collected in a tube without preparation, and centrifuged at 1350 g for 10 minutes, separating the serum at a maximum within 4 hours after harvest and freezing it at –20 °C until processing.

The Samples that were designed to determine the polymorphism of IL-28B were collected in a tube containing ethylenediaminetetraacetic acid tripotassium anticoagulant (EDTA K3). Were
then stored at –20 °C, with a maximum time space 4 hours after harvest.

The determination of HCV genotype and IL-28B was carried out using molecular biology techniques. After a positive test for HCV screening the next step was to perform confirmatory of infection. After this there was the confirmation of HCV genotyping test.

The IL-28B genotyping test is not part of the standard procedure of the SSMT, being made after viral genotyping.

The determination of the HCV viral load is performed using the COBAS® AmpliPrep/COBAS® TaqMan® HCV Quantitative Test (Roche Diagnostics, Mannheim, Germany).

**HCV genotyping**

Genotyping was performed in three steps. The first step consisted in the extraction of RNA; using the NucliSens® EasyMAGTM equipment (Frimley Camberley, United Kingdom) for automatic isolation (purification and concentration) of total nucleic acid (RNA) in biological samples. The second step of this RNA amplification was performed using the Versant HCV Amplification 2.0 (LIPA) (Frimley Camberley, United Kingdom). This is a test that uses sensors online, using reverse hybridization, for use in vitro diagnostics, allowing the identification of the six genotypes and their respective subtypes.

The third step corresponds to amplification with the reverse transcriptase (RT) and polymerase chain reaction (PCR), being performed sequentially in a single tube. The RNA extracted in the previous step is added to a reaction tube containing enzymes and other reagents necessary for the amplification steps.

At the beginning of the reaction, the HCV specific primers for reverse transcription yields complementary DNA (cDNA). Then the reaction is heated to activate the DNA polymerase for the PCR amplification step and simultaneously inactivate the reverse transcriptase. The 5’UTR region and the core region of the HCV genome are co-amplified from cDNA using two pairs of biotinylated primers, for producing two separate fragments of biotinylated DNA, which will represent the 5’UTR region and the core region of HCV, using the Versant HCV Amplification 2.0 (LIPA) (Frimley, Camberley, United Kingdom) for genotyping.

The identification of genotypes was performed using the VERSANT® HCV Genotype assay 2.0 (LIPA) (Frimley, Camberley, United Kingdom). This assay uses probes aligned to identifying genotypes 1 to 6 and b referring to the subtypes of genotype 1 HCV.

In this assay strips, there are three control bands 22 and parallel bands containing DNA sequences specific for HCV genotypes. The band conjugate control monitors the color development reaction. In the band 2, the control amplification, the band 23 is contained in the universal probes which hybridize with the PCR product of the core region. The HCV genotypes are determined by aligning the strips of the test to the standards of interpretation presented in this essay table.

The equipment used for automation of the processing steps of the strips is the Auto-LiPA (Frimley, Camberley, United Kingdom).

**IL-28B genotyping**

The extraction of human DNA was performed according to the manufacturer’s instructions using NucliSENS® easyMAG® (bioMérieux, Marcy l’Etoile, France).

Genotyping IL-28B was performed using the Fast Set IL28B (Arrow Diagnostics, California, USA), which is a diagnostic device based on the in vitro amplification of nucleic acids by real-time PCR (Real Time PCR).

This allows the detection of polymorphism rs12979860 in the human IL28B gene and differentiation of CC, CT and TT genotypes. The test is based on the continuous reading of fluorescence that occurs during the amplification reaction. This feature allows to detect simultaneously the signal produced by two or more different fluorochromes.

It is possible to monitor in real time during their course of the amplification reaction, detecting the increase in fluorescence intensity. The mixture of oligonucleotides provided by the test, contains a specific probe for the mutation that affects sensitivity to anti-HCV drugs, marked “FAM”, and a probe specific for the sequence “wild type” labeled “HEX”. Amplification occurs in both channels in the case of heterozygous samples.

**Statistic analysis**

The statistical treatment of the data was conducted using the Statistical Package for Social Sciences (SPSS Inc., Chicago, USA) version 21.0 for Win-
Results

From all genotypes of HCV, genotype 1a was predominant with a larger number of individuals, 47.5% (28); followed by genotype 1b with 27.1% (16) (Table I).

Genotype 2 was represented with 1.7% (1) as genotype 4e and 4, each with only one observation. The genotype 4a/4c/4d had an occurrence of 8.5% (5), followed by genotype 3a with 11.9% (7) (Table I).

The genotype 1, subtypes 1a and 1b HCV represents a group of 44 subjects, the genotype with the highest number of cases, as expected (Table I).

The IL-28B genotypes present in the study group were analyzed: 30.51% (18) of the subjects showed the CC polymorphism, 45.76% (27) the genotype CT and 23.73% (14) the TT genotype.

In the HCV genotype 1 the most prevalent IL-28B polymorphism was CT (43.20%, 19), followed by the CC genotype with 31.80% (14) and the TT genotype with 25.00% (11). With regard to the one HCV genotype 2 identified it was IL-28B CC genotype. The HCV genotype 3 had a higher frequency of IL-28B CT (42.90%, 3). The CC genotype of IL-28B was represented with 28.60% (2) and the TT genotype with 28.60% (2).

HCV genotype 4 showed a frequency of 71.40% (5) in the CT genotype of IL-28B. CC and TT genotypes occurred in 14.30% (1), which can be seen in Figure 2.

The viral load values were evaluated and the median viral load obtained was 1,745,194.00 IU/mL. The minimal viral load observed was 0 IU/mL and the maximum 23,059,696 IU/mL.

In respect to the viral load values for each IL-28B genotype no statistically significant differences were found. Subsequently, we analyzed the possible association between the most prevalent HCV genotypes (1a and 1b), with the IL-28B genotypes. It was observed that from the 28 individuals of HCV genotype 1a, 32.14% (9) were CC genotype of IL-28B; 39.29% (11) had CT genotype of IL-28B and 28.57% (8) were TT genotype of IL-28B. The evaluation of the 16 individuals of HCV genotype 1b, showed that 31.25% (5) were IL-28B CC genotype; 50% (8) were IL-28B CT genotype and 18.75% (3) were IL-28B TT genotype (Figure 3). No statistically significant differences were observed between genotypes.

Discussion

In the study population it was observed that the IL-28B TT and CC genotypes have a lower prevalence in individuals who are HCV genotype 1. CT genotype of the IL-28B in turn has a higher prevalence in HCV genotype 1 subjects, corresponding to 43.20% of cases. The frequencies of HCV genotypes 3 and 4 were equivalent, with a higher frequency (71.40%) of IL-28 CT polymorphism in the HCV genotype 4 (Figure 2). The results observed in our study may be due to the fact that HCV genotype 1 and CT polymorphism of IL-28B is of poor response to treatment, thus giving a greater volume of these requests by the clinical team. This is supported by the percentage of HCV genotype 1 individuals, which is much higher than the percentage of the other HCV genotypes in tested samples. The discrepancy between the number of samples per HCV genotype may interfere with the statistical results, increasing the standard deviation. Aalaei-Andabili and colleagues concluded that the HCV genotypes 2 and 3 (normally more favorable response to therapy) are strongly associated with the presence of IL-28B genotypes, contributing to a favorable response to therapy (rs12979860 and rs8099917 CC TT). The probability of finding the rs12979860 polymorphism in individuals CC genotype 1 or 4 of HCV is high, so that therapy in these patients reveals to be more effective than in individuals rs12979860 Non-CC (32). Alajos Pár et al also concluded that the rs12979860 polymorphism CC has a high probability of increasing the effectiveness of therapy, and rs80999917 TT individuals require more aggressive therapies for the same effect on rs12979860 CC individuals. The same was observed for the rs12979860 CC individuals who were mostly HCV genotype 1 or 4 (24). E. Riva et al obtained similar conclusions about the effectiveness in determining the response to therapy by the HCV genotypes and IL-28B polymorphisms, adding further that the rs12979860 CC polymorphism predicts treatment efficacy, even in the absence of HCV genotype information (27).
In the HCV genotypes 1 and 3, the IL-28B CT polymorphism frequency was similar, according to the previously published studies demonstrating that the CT individuals have poor response/efficacy regarding the treatment used, which are followed up by the hospital, thus increasing the number of individuals of this group over the others (24, 27, 32).

The results obtained from the analysis of the association between HCV genotypes 1a and 1b and IL-28B polymorphisms determine that there is not a statistical association, although it is possible to verify that the CT genotype of the IL-28B has a larger number of individuals, either in the genotype 1a and 1b, as was observed for HCV genotypes 3 and 4. However, in the HCV genotype 1a, the difference is lower than the percentage obtained from individuals of genotype 1b in CT genotype of IL-28B.

These results are consistent with published studies (24, 27, 32).

**Conclusion**

Our results suggest that there is no statistical association between HCV genotypes with worse prognosis and IL-28B genotypes of poor response to therapy (CT). To improve the results, we should increase the number of samples analyzed, as well as balancing the different study groups (HCV genotype 1/2/3/4).

However, a trend was observed. The CT genotype of the IL-28B seems to us to have a higher prevalence in genotype 1 HCV. To correct this bias some adjustments will be necessary, such as to balance the number of samples from each study group for each genotype, in order to be able to determine if the trend is proven and statistically significant.

One of the difficulties experienced in conducting this study was that we did not have access to all patient data, including viral load, only the values obtained on the day of genotyping HCV or completion of the polymorphism of the IL-28B, no information about the possible ongoing treatment and baseline viral load.

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**Scientific disclosures**

This work was presented in the form of oral communication in the 2nd Congress of Croatian Chamber of Health Professions – Department for Professional Medical Laboratory Activities, between May 29 and June 1, 2014, in Zagreb, Croatia.

This work was also accepted for communication as poster at 31st World Congress of Biomedical Laboratory Science in Taipei, Taiwan, hold from October 3–7, 2014.

**References**


**Figure 3 – Frequency of distribution of 1a and 1b HCV genotypes by frequency of IL-28B genotypes.**


