

RESEARCH ARTICLE

Evaluation of the Frequency of the IL-28 Polymorphism (rs8099917) in Patients with Chronic Hepatitis C Using Zip Nucleic Acid Probes, Kerman, Southeast of Iran

Zahra Iranmanesh¹, Hamid Reza Mollaie^{1*}, Seyed Alimohammad Arabzadeh¹, Mohammad Javad Zahedi², Mehdi Fazlalipour³, Saeede Ebrahimi¹

Abstract

Polymorphisms in the region of the interleukin IL-28 gene on chromosome 19 have been related with clearance of hepatitis C virus (HCV), a major human pathogen responsible for chronic hepatitis, cirrhosis and hepatocellular carcinoma. About 3% of the world's population is infected with HCV. The long-term response to therapy is influenced by many host and viral factors, and recent evidence has indicated that some host genetic polymorphisms related to IL-28 are the most powerful predictors of virological response in patients with HCV. This study assessed frequency of the IL-28 polymorphism (rs8099917) in 50 patients (39 men and 11 women) with chronic hepatitis C using ZNA probe real time PCR new method. All patients were tested for genotype of HCV and the HCV viral load. In parallel, the levels of SGOT, SGPT and ALK enzymes were assessed. Treatment using Peg-interferon alpha with ribavirin was conducted for patients and subsequently samples were collected to detect any change in viral load or liver enzyme rates. The overall frequency of the TT allele is 74%, TG allele 20% and GG allele 6% and the percent of patients who had T allele was 84%. Clear reduction in viral load and liver enzymes was reported in patients with the T allele. Especially for genotype 1 which is relatively resistant to treatment, these alleles may have a role in this decline. In conclusion, we showed that IL-28 polymorphism rs8099917 strongly predicts virological response in HCV infection and that real-time PCR with Zip nucleic acid probes is a sensitive, specific and rapid detection method for detection of SNPs which will be essential for monitoring patients undergoing antiviral therapy.

Keywords: Chronic hepatitis C - interleukin 28 polymorphism - zip nucleic acids - real time PCR

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Introduction

Global prevalence of hepatitis C is about 3% and approximately 170 million people in worldwide are infected with the virus. Hepatitis C virus has a positive linear RNA and this virus classified in the family of Flaviviridae and the genus of Hepacivirus. HCV virion is about 50 to 60 nm and has 9.4 Kb length which is responsible to code about 3010 amino acids (Keyvani et al., 2012). The prevalence of HCV in America, Canada and Northern Europe is less than 1% and in Thailand, Malaysia and India is from 2.1 to 1.4%. This prevalence in Ukraine is 9.2% and in North and sub-Saharan Africa and Arabic countries including Egypt is 14.5% (Zekri et al., 2012). HCV infection can be seen all over the world, but in the Eastern Mediterranean Region and Africa have the highest rates of incidence.

HCV is divided into six major genotypes and more

than 80 subtypes. HCV genotypes 1 and 4 are common in Western countries and type 1 is dominant in North America, West Europe and Japan (Al-Kubaisy et al., 2014). Genotype 1 has the minimum response to treatment but genotype 2 has the best action in treatment and type 3 has the highest rate of spontaneous resolution. While getting chronic HCV is common in genotype 4. Generally, types 1 and 3 are in Europe and America and type 3 is prevalent in Africa (Petruzzello et al., 2014). In Iran, the highest prevalence 1.5% and the lowest 0.2% has been reported but still now HCV is the most important reason of hemophilia, thalassemia and renal failure and hemodialysis (Jamalidoust et al., 2014).

Sustained virological response (SVR) is known as a follow up of HCV 6 months after treatment. This response is varied between patients. Some factors such as genotype 2 and 3, low-level in viral load, absence of cirrhosis, female sex, and the age less than 40 years increase the

¹Department of Medical Microbiology, ²Department of Medical Virology, Kerman University of Medical Sciences, Kerman, ³Department of Gastroenterology, Iran University of Medical Sciences, Tehran, Iran *For correspondence: hamid2008kmu@gmail.com

response to treatment. In addition, the study of the human genome has shown that the IL28B polymorphism is involved on SVR response (Lazarus et al., 2014; Yoshida et al., 2014a). The IL28B gene is encoding interferon $\lambda 3$. This IFN with interferon $\lambda 1$ (coded by IL29) and interferon λ (coded by IL28A) make interferon λ family.

Genes of IL28A, B and IL29 are located on chromosome 19 and in gene of IFN $\lambda 3$ some polymorphisms such as rs8099917, rs12979860, rs4803219, rs10853727, rs8109886, rs4803223, rs12980602 and rs12980275 have been reported. The most important polymorphisms in IL28B are rs12979860 and rs8099917 (Riva et al., 2014; Rosso et al., 2014). Single nucleotide polymorphisms (SNP) rs12979860 is 3 kb upstream of the IL28B gene and SNP rs8099917 is located 8 kb upstream of the IL28B gene (Lapie and Gilgenkrantz, 2010) (Figure 1). For treatment of hepatitis C, newly peg-interferon plus Ribavirin is an antiviral drug for the individual based on genotypes being used. Although, these materials have side effects such as: flu-like symptoms, fever and muscle aches for interferon alpha and destruction of red blood cells for Ribavirin have been reported (Callau Monje et al., 2014; Riva et al., 2014).

Many methods were developed to detect SNPs, such as direct polymerase chain reaction (PCR) sequencing, restriction fragment length polymorphism (PCR-RFLP), High resolution melting curve Analyses (HRM), matrix-assisted laser desorption/ionization time of flight-mass spectrometry (MALDI TOF MS) with their respective advantages and disadvantages (Moosavy et al., 2011). Sequencing remains the best approach to the identification of new mutations. However, it cannot detect total mutations in less than 25% of total Gene population and is not appropriate for large-scale use in large cohort studies or clinical laboratories because of its labor intensive and time consuming (Aghasadeghi et al., 2011). MALDI TOF MS are capable to detect variants, but more strict experiment conditions and equipment's are required. PCR-RFLP could only detect mutations in a high proportion and is also labor intensive and time consuming (Aberle et al., 2001). Monitoring of gene mutations and SNPs will play an important role in assessing treatment. Prolonged treatment with antiviral agents can lead to the emergence of drug-resistant virus (Chen et al., 2010).

Zip nucleic acids (ZNAs) are oligonucleotides conjugated with cationic spermine units which increase affinity for their target by decreasing electrostatic repulsion between negatively charged anionic single strand Nucleic Acids to improve hybridization, thus enhancing and accelerating target recognition (Noir et al., 2008). The possibility of modulating the global charge of the ZNA oligonucleotide-oligocation conjugates by the number of cationic spermine moieties attached to the Nucleic Acid oligomer, is a key to predict melting temperature of ZNA-DNA/ZNA-RNA hybrids easily. T_m increases linearly with the length of the oligocation (Afshar and Mollaie, 2012; Alvandi and Koohdani, 2014). ZNAs were shown to enable specific and sensitive reactions when used as primers for PCR and Reverse Transcription. Moreover ZNA probes provide broad flexibility in assay design and represent an effective alternative to Minor Groove Binder

(MGB) and Locked Nucleic Acid (LNA) containing oligonucleotides (Voirin et al., 2007).

With the study of the human genome, some genes and polymorphisms have been found which are important in HCV treatment. Scientists ensure that polymorphisms in IL28B gene are directly linked with spontaneous clearance of HCV and the result of treatments (Bibert et al., 2013). Thus, detection of mutants or SNPs to treatment is necessary to prevent disease progression and malignancy and recommended for rapid identification and sensitive treatment monitoring (De et al., 2014). In this study described a sensitive method for detection of IL-28 polymorphism (rs8099917) by Real-time fluorescent quantitative PCR using ZNA-probes in Chronic HCV patients who had been long-term Antiviral therapy in Kerman, southeast of Iran. So far no study has been done in this area with same method. This method can be used to detect rs8099917 and other SNPs in IL-28 in a single multiplex reaction simultaneously.

Materials and Methods

Patients

In retrospective study, fifty patients (39 cases were male (78%) and 11 cases were female (22%) and 23 of males (58.9%) and 3 of females (27.2%) had genotype 1. On the other hand, 16 of men (41.1%) and 8 of women (72.8%) had HCV genotype 3. mean age=42 years, range 38-55 years) who were diagnosed as Chronic Hepatitis C infection from Jan 2012 to Sep 2014 in our Laboratory (Virology Laboratory of the Besat Specialist Clinic, Kerman, IRAN) following the guidelines of prevention and treatment of chronic hepatitis C were enrolled in this study. The decision to treat is primarily based on the combination of three criteria: a) serum HCV RNA levels, b) serum alanine amino transferase (ALT) levels and c) histological grade and stage of the underlying liver disease. Exclusion criteria included a coexisting severe illness, organ or bone marrow transplantation, recent treatment with systemic corticosteroids, immunosuppressant's or chemotherapeutic agents, liver disease not due to hepatitis C, and seropositivity for human immunodeficiency virus (HIV) or hepatitis B (HBV) or hepatitis D virus.

Serological tests

The blood from the patients with chronic hepatitis C was assayed for alanine transaminase at our clinical laboratory using an automated analyzer. HBsAg and HBeAg as well as Anti-HCV and Anti-HIV were determined using commercial radioimmunoassay kits (Abbott Laboratories, Chicago, IL). Hepatitis D antigen was detected using enzyme immunoassay kits (Abbott Laboratories, Chicago, IL).

HCV RNA level and Genotyping

Five ml of peripheral blood were collected from each patient into EDTA-containing vacutainer tubes. Plasma was separated and stored at -70°C . HCV RNA was extracted from 200 μL of plasma with High Pure Viral Nucleic Acid kit (Roche Diagnostics GmbH, Mannheim, Germany). Plasma HCV-RNA levels were

Table 1. Sequence Primer and Probe for Detection rs 8099917 Polymorphism

| Name | Sequence | Tm |
|----------------|------------------------------------|------|
| Forward primer | GTTCCCTCCTTTTGTGTTTCC | 49.6 |
| Reverse Primer | GTCATTTGCCTTTACTATCC | 49.7 |
| T allele Probe | FAM-TCTGTGAGCAATTTACCCAAATT- BHQ1 | 58.6 |
| G allele Probe | HEX-TCTGTGAGCAATGTCACCCAAATT- BHQ1 | 60.2 |

quantified via quantitative Real-time polymerase chain reaction with a commercial detection kit (artus HCV kit, Qiagen, Germany). The Real-time PCR had a lower limit of 10 copies/mL. HCV genotyping was performed with a commercial HCV genotyping detection kit (Inter Lab Service, Russia).

Detection IL28 polymorphism rs 8099917

Detection of rs 8099917 was done with specific primers and probes from position 301 of the IL-28B region that were design by Dr H.R.Mollaie Using Beacon designer software that were shown in Table 1 (Version 8 Primer, Biosoft, USA). Primers and probes synthesized by Metabion company (metabion international AG, Germany). five μ l of DNA sample that was extracted from PBMNs, combined with 15 μ l reaction mixture of fermentas TaqMan Master mix (Thermo fisher Scientific, USA) contain primers and probe were subjected to Real-time PCR. The condition for the ZNA mediated probe assay was initially 15 min with hot start Taq DNA polymerase at 95°C followed by 40 cycles at 95°C for 5 sec and 60°C for 20 sec. Of each cycle at the extension step, the fluorescent signals of Probe rs 8099917 were measured at Green channel for T allele, Yellow for G allele. Quantitative determination of the amplified products was done with the Rotor Gene 6000 (Corbett Research, Australia).

Statistical analyses

Chi square and Fisher's exact Tests were used to analyze the data obtained by SPSS 11.5 software (SPSS Inc, Chicago; USA). The differences or association with $p < 0.05$ were considered statistically significant.

Results

In our research, 50 patients with chronic hepatitis C were studied before and after treatment by Peg-interferon alpha with Ribavirin and the frequency of allele polymorphism rs8099917 for interleukin 28 were investigated. The result revealed that the allele frequency of rs8099917 polymorphism is 74% for TT genotype (37 patients), 20% for TG allele (10 patients) and 6% for GG allele (3 patients). Out of the 50 patients, 39 cases were male (78%) and 11 cases were female (22%) and 23 of males (58.9%) and 3 of females (27.2%) had genotype 1. On the other hand, 16 of men (41.1%) and 8 of women (72.8%) had HCV genotype 3. Our result shows that, from the total 37 patients with alleles TT, 16 cases (43.3%) had genotype 1 and 21 samples (56.7%) were HCV genotype 3. From total 10 patients with allele TG, 8 samples (80%) had genotype 1 and 2 cases (20%) were HCV genotype 3. From 3 persons with allele GG,

2 patients (66.6%) with genotype 1 and 1 case (33.3%) was HCV genotype 3. The viral load, AST, ALT and ALK levels in three alleles (GG, TT, and GT) were studied before and after treatment. The mean of viral load level in TT and GT is decreasing from $(6.39 \pm 0.4) \times 10^6$ copies/ml to $(44.17 \pm 0.2) \times 10^2$ copies/ml, $(7.49 \pm 0.7) \times 10^7$ copies/ml to 102.74 ± 0.363 copies/ml respectively. The rate of liver enzymes has a similar reduction (Figure 2). As it is reported, for patients with TT allele, the mean of AST enzyme was 323 ± 58.9 before treatment and after treatment became 70.87 ± 7.6 . For patients with TT allele, the mean of ALT enzyme was 335.68 ± 65.18 before and 73.7 ± 7.6 after treatment. For ALK enzyme in TT allele, the mean of 668 ± 20 before and 153.31 ± 78.83 after treatment were reported. As it is shown, the rate of AST, ALT and ALK enzymes is dropping after treatment in HCV genotype 1 patients with GT allele. Viral load levels and liver enzymes were significantly reduced in TT and GT allele and since genotype 1 is relatively resistant to treatment, these alleles may have a role in this decline (Figure 3). The mean of viral load level in TT and GT is decreasing. The rate of liver enzymes has a similar reduction. As it is reported, for patients with TT allele, the mean of AST enzyme was 325 ± 44 before treatment and after treatment became 63 ± 5 . For patients with TT allele, the mean of ALT enzyme was 248 ± 35 before and 61 ± 30 after treatment. For ALK enzyme in TT allele, the mean of 680 ± 54 before and 136 ± 13 after treatment were reported. As it is shown, the rate of AST, ALT and ALK enzymes is dropping after treatment in HCV genotype 3 patients with GT allele. Viral load and liver enzymes were significantly decreased in patients with TT and GT allele but because there is only one sample with GG allele, statistical comparison is not available. In our survey, the viral load of HCV genotype 3 was studied some weeks before and after treatment as. In Figure 4, the frequency mean of HCV viral load in different weeks during drug therapy were shown. The viral load in patients with HCV genotype 1 was evaluated to compare changes in weeks before and during treatment. In 11 patients the mean of AST level 12 weeks before drug therapy was 197 ± 58 , and after treatment became 62 ± 8 and also 7 samples were 275 ± 99 and 60 ± 15 , 13 weeks before and after treatment. Other changes in AST enzyme were clarified for 14, 15 and 21 weeks before and after treatment. The rate of ALT enzyme in patients with HCV genotype 3 studied some weeks during treatment. In 11 patients the mean of ALT rate 12 weeks after drug therapy was 209 ± 65 and also 7 samples were shown 298 ± 55 . Other changes in ALT enzyme were clarified for 14, 15 and 21 weeks after treatment. In our survey, the ALK enzyme in patients with HCV genotype 3 was studied some weeks during treatment. In 11 patients the mean of ALK 12 weeks after drug therapy was 718 ± 13 , and 21 weeks after

treatment became 122 ± 13 and also 7 samples were 580 ± 12 and 130 ± 10 , 13 weeks after treatment. Other changes in ALK rates were clarified for 14, 15 and 21 weeks before and after treatment. The AST enzyme rate in patients was

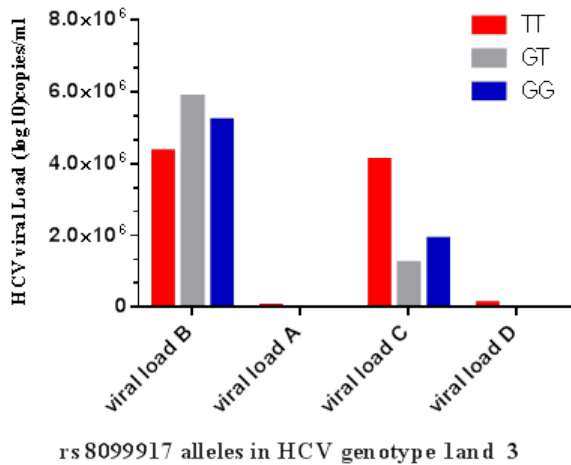


Figure 2. Relative Differentiation between HCV Viral Load before and after Treatment (Mean±SD). (Viral Load B) Viral Load before treatment in HCV genotype 1, Viral load A) Viral Load after treatment in HCV genotype 1, Viral Load C) Viral Load before treatment in HCV genotype 3, viral Load D) Viral Load after treatment in HCV genotype 3)

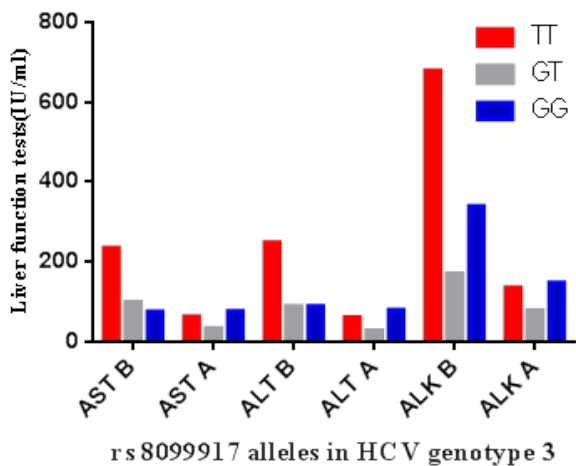


Figure 3. Differentiation between Liver Function Tests (Mean±SD) before and After Treatment in Patients with HCV Genotype3 with Different Allel for rs 8099917 (A) After treatment; B) Before treatment)

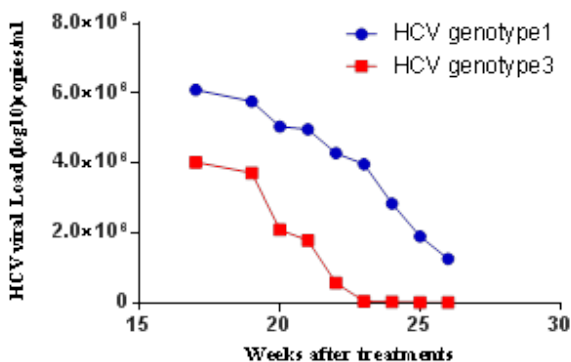


Figure 4. Reduce HCV Viral Load in Weeks During Treatment

analyzed to compare changes in weeks during treatment. In 6 cases the mean of AST was reported 22 weeks after treatment was 294 ± 83 and after drug therapy was 73 ± 12 . Other patients who had HCV Genotype 1 were studied in 12-26 weeks after treatment and strong reduction in their AST enzymes were published. The ALT enzyme of patients with HCV genotype 1 was shown some weeks after treatment. In 6 patients the mean of ALT rate 22 weeks after drug therapy was 301 ± 45 and 26 weeks after treatment became 74 ± 6 . In 12-26 weeks after treatment in patients who had HCV Genotype 1, sharp reductions in their ALT enzymes were published.

Discussion

To date, many assays have been used for detection of IL-28 mutants in patients with hepatitis C. Differences in sensitivity, specificity, cost, and time required, exist in these methods. Real-time PCR is able to quantitatively detect a small portion of mutants in populations and ZNA probe detection method is a newly developed method for detection of low abundant mutants in the background of wild-types. So we used these methods and we could detect single mutation about IL-28. In our study most of drug resistance cases were in men. Sequence analysis, on the other hand, has already proven to be a useful technique for mutation detection, however, it takes more time and cannot be used everywhere and also cannot detect any mixtures of variants, but sequencing results of this study were same to our method which used in this study. Using ZNA probe method is quick and inexpensive comparing to other probe methods for detection of SNP mutation; and ZNA probe method is more trustable than other methods. The results obtained with both methods were completely concordant in all samples. ZNA was able to detect as low as 10 copies/mL of SNP, while other method only detected 1000 copies/mL of Mutants. In addition, the cost of ZNA is slightly lower and is much more rapid so it requires less manual work than PCR-RFLP. The total assay time for ZNA real-time PCR was 1.5 hours, respectively. Another advantage of the real-time PCR method is it is able to calculate the ratio of mutants to total virus in samples. This will be useful in clinical studies on the dynamics of resistant mutants during lamivudine therapy (Li et al., 2012). The presence of IL-28 polymorphisms like rs12980275, rs8099917 and rs12979860 can make big effects on instant treatment responses against HCV infection. According to the given role for interferon lambda, it is mentioned that the polymorphisms of this cytokine family can be associated with a variety of physiological conditions and diseases (Melis et al., 2011). Several investigators on polymorphisms and their importance in hepatitis C and response to treatment of this disease have examined and the association between the SNP of IL28B and the response of virus in HCV positive patients have noted. Unfortunately, the treatment of this group of patients is very costly and time consuming with a lot of side effects. So this project was done on HCV positive patients in Kerman, southeast of IRAN, to get a valuable resource for further study and having got a good plan for host based genetic basis. According to the role

of IL28B polymorphisms in hepatitis C treatment, we examined the frequency of genotypes 1 and 3 for rs8099917 gene in HCV patients who had received PEG-IFN- α and Ribavirin therapy. It has been proved that variant IL28 is important in HCV infection and T allele is called responder allele and G allele is known no responder allele. These can be used as prognostic factors in patients with hepatitis C virus (Fischer et al., 2013; Harada et al., 2014). In a study to examine allele polymorphism rs8099917 for interleukin 28 and showed that the allele TT frequency has a significant positive relationship with SVR and suggests that host genetics may be useful for prediction of viral response (Aparicio et al., 2010). In 2009 in Japan, 314 patients evaluated and TT allele frequency 64% reported and showed that TT has more role than GG in viral response in patients. In a same research done by Ravesh in Switzerland in 2010 on whites 68% for TT allele have mentioned and said that GG allele has a role for chronic HCV. Our data revealed that the frequency of TT allele is 74%, TG allele 20% and GG allele is 6% and also in our study the percent of patients who had T allele is 84% of cases and G allele is 16% of cases (Seaberg et al., 2014; Tanaka et al., 2014). Most of the cited studies are explaining that the T allele is dominant in their studies which are similar to our data that shows 74% in T allele frequency (Yoshida et al., 2014b; Zhang et al., 2014). Different variants of IL-28 are strongly predictive factors for sustained viral response, particularly in patients with genotype 1 virus and T allele for rs12979860 is a good reason for viral load reduction after treatment (Bibert et al., 2013). In Iran a study was done on 48 patients with HCV type 1 in Tehran that CC genotype is more relevant than TT in rs12979860 (IL28) for making better SVR which is different comparing with our result. Another survey was done in Iran and 118 patients were studied for IL28 polymorphism rs8099917 and revealed that TT allele is dominant in Iranian patients. The analysis of our data shows similar result (Hashemi et al., 2012). In our survey the viral loads and the rate of liver enzymes evaluated before and after treatment based on different alleles of rs8099917 in patients with genotype 1. The result shows a significant decrease in viral loads and the rate of liver enzymes in patients with TT allele. Since it can be difficult to treat HCV genotype 1, TT allele probability can reduce the viral load and the enzymes. In summary, our findings suggest that SNP rs8099917 of IL28B are useful baseline predictors for virological response in patients infected with genotype 1 and 3 HCV when treated with Peg-IFN plus ribavirin.

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