Original Article

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Effect of Interleukin 28B SNP rs12979860 Genotype on Viral Load in Hepatitis C Virus in Kolar Population, Karnataka, India

Microbiology Section

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ABSTRACT

Introduction: The natural course of Hepatitis C Virus (HCV) infection is influenced by a number of host and viral variables. Interleukin 28B (IL28B) is a kind of interleukin. A Single Nucleotide Polymorphism (SNP) designated as rs12979860 was reported to predict viral clearance with and without treatment. Subjects with the GG (favourable) IL28B rs12979860 genotype were more likely to clear the infection spontaneously and respond well to therapy. These findings imply that people who have the "favourable" GG genotype have a lower viral burden than those who have the "unfavourable" AA genotype.

Aim: To determine the effect of IL28B SNP rs12979860 genotype on HCV viral load in Kolar population, Karnataka, India.

Materials and Methods: The present study was a case-control study which was carried out in Department of Microbiology, Sri Devaraj Urs Medical College, Kolar, Karnataka, India. Subjects were enrolled from Department of Medicine of R.L. Jalapa Hospital and Research Centre, teaching hospital of Sri Devaraj Urs Medical College between November 2020 to March 2021. A total of 248 patients were taken of which 124 were HCV antibody-positive and 124 were controls. The effect of IL28B rs12979860 SNP on HCV viral load and clearance among HCV-infected patients were examined. Detection and quantification of HCV-RNA was determined by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). IL28B rs12979860 genotyping was performed using PCR and Restriction Fragment Length Polymorphism (RFLP) technique and specific primers. Statistical analysis was done by using open Epi tool. The frequency, percentage and Chi-square test were used to analyse categorical variable. A p-value <0.05 was considered as significant.

Results: In the present study, in group 1, the frequency of G/G genotype was considerably high 83 (67%) compared to G/A 17 (13.7%) and A/A 24 (19.3%) and in group 2, the frequency of G/G genotype 84 (67.7%), G/A genotype 34 (27.4%) and A/A genotype 6 (4.9%). There was a statistically significant difference in both the HCV infected and healthy controls groups (p=0.002). The average (±SEM) HCV viral load was $4.6\pm3.6\times10^7$, $9.4\pm7.7\times10^7$ and $5.5\pm5.2\times10^7$ IU/mL in patients with the IL28B rs12979860 GG, GA and AA respectively. Also there was a significance between the viral load and IL28B rs12979860 (p-value <0.05).

Conclusion: Thus, present study results indicate that the IL28B rs12979860 genotype has an effect on viral load in untreated HCV patients. These findings highlight the importance of viral-host interactions in influencing the outcome of HCV infection.

Keywords: Infection, Polymorphism, Ribonucleic acid levels

INTRODUCTION

The HCV spreads by blood-to-blood contact and is a leading cause of chronic liver disease, with an estimated 170 million people infected worldwide [1]. Approximately 18 million individuals in India are assessed to be contaminated with HCV. In 75-85% of infected people, HCV infection is persistent [2]. However, 20-40% of people with acute HCV infection obtain spontaneous clearance, the time course and factors that influence clearance remain unclear [3]. HCV is a positive-sense single-stranded RNA virus having a place with the family *Flaviviridae*. Also, SNP at the rs12979860 site of IL28B, also known as interferon λ3, has been used as a predictor of viral clearance with and without therapy [4-6].

Interferon λ3, coded by the IL28B gene located on Chromosome 19, induces antiproliferative and antiviral activity and also up regulates interferon-stimulated genes, via the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway [7]. The IL28B binds to IL28B receptors on hepatocytes that further activate the JAK-STAT pathway. Then STAT activated by JAK through phosphorylation and up regulation of the Interferon Regulatory Genes (ISGs) that leads to spontaneous clearance of the virus. Several Genome Wide Association Studies (GWAS) have shown that response to Pegylated Interferon/Ribavirin (PEG IFN/RBV) therapy in genotype 1 infections are strongly associated with SNPs within or adjacent to the IL28B gene (rs12979860 and rs8099917) [4,6,8]. Besides, rs12979860 have genotypes CC, CT and TT, while rs8099917 have genotypes TT, TG and GG. A better treatment response in Chronic Hepatitis C Infection (CHC) has been shown to be associated with C/C genotype at rs12979860 and T/T genotype at rs8099917. In different ethnic groups, better outcomes in the patients who have protective genes have been observed, this is because the efficacy of standard anti-HCV treatment is affected by the SNPs of IL-28 [4,5]. Besides hepatitis C, the role of these polymorphisms and their impact on response has been studied, in a large number of other infectious and non infectious diseases, i.e., human T lymphotropic virus, herpes simplex virus, Human Immunodeficiency Virus (HIV), and cytomegalovirus to name a few [9]. In India, IL28B and HCV RNA levels studies are limited [10]. Moreover, In Kolar region this was the first study giving evidence about the IL28B and HCV viral loads levels. The link between IL28B SNPs and HCVRNA levels is still challenged. Later IL28B can also compare with HCV genotypes. Therefore, in order to predict the treatment therapy of interferon and clearance rate of HCV infection, understanding IL-28B gene polymorphism is important. The aim of the is to determine the effect of interleukin 28B SNP rs12979860 genotype on HCV viral load in Kolar population, Karnataka.

MATERIALS AND METHODS

The present study was a case-control study which was carried out in Department of Microbiology, Sri Devaraj Urs Medical College, Kolar, Karnataka, India. The study was approved by Institutional Ethics Committee (IEC) of Sri Devaraj Urs Medical College (DMC/KLR/IEC/467/2020-21). Subjects were enrolled from Department of

Medicine between November 2020 to March 2021. Written informed consent was obtained prior to the recruitment of the subjects.

Inclusion criteria: Symptomatic and asymptomatic Hepatitis C infected patients.

Exclusion criteria: HBsAg positive patients, HIV infected patients and pregnant women.

A total number of 248 subjects were enrolled which were further categorised as group 1 and group 2 as follows:

Group 1: (n=124) HCV infected patients were diagnosed by Chemiluminesense Immunoassays (CLIA).

Group 2: (n=124) Healthy controls.

Study Procedure

Sample collection: A whole 5 mL of peripheral blood sample was collected from all the HCV positive patients and 3 mL from healthy controls and sample transferred into the sterile tubes containing Chemiluminesense. After centrifugation the plasma was separated and was stored in aliquots at -80°C for further analysis.

RNA extraction: Viral RNA was extracted using QIAamp Viral RNA Mini Kit according to the manufacturer's instructions (#catalog number 52904 QIAamp Viral RNA mini kit, Qiagen, USA).

DNA extraction for IL28B genotyping: The blood sample was further processed for Deoxyribonucleic Acid (DNA) extraction by using manual method i.e. salting out method [11]. Whole blood was treated with Erythrocyte Lysate Buffer (ELB) and Red Blood Cells (RBCs) were lysed. Total isolated White Blood Cells (WBCs) pellets were exposed to proteinase K for protein digestion and further DNA was extracted. The genomic DNA was used to check the IL28B polymorphism (rs12979860).

qPCR viral load detection: quantitative PCR was performed for the detection of HCV RNA using HCV-K-004 kit (CoSara diagnostics. Pvt. Ltd) by quantitative qPCR by using Magnetic Induction Cycler-qPCR (Mic-qPCR, biomolecular systems). The HCV viral load kit included reagents and a readymade master mix with internal positive control along with five standards. The negative control was included in parallel for each batch of analysis. The single-step reverse transcription real-time PCR transcribes and amplifies conserved sequence of RNA from the 5' untranslated region of HCV genome was detected by using Quasar 670 dye (Q670). A human RNase P gene marker was used as an internal positive control to check the reliability of each reaction, and the CAL Fluor Red 610 dye was being used to detect inadequate samples (CF610). The qPCR reaction was performed by using the total reaction volume of 10 µL and the HCV viral load thermal cycling conditions were as follows: activation at 42°C for 2 minutes hold, 70°C for 1 minute hold, 95°C for 20 seconds and 45 cycles of 95°C 15 seconds and 55°C for 60 seconds. The PCR product was detected by using Quasar 670 dye (Q670).

Genotyping of IL28B Polymorphism (rs12979860): PCR was used to amplify the reverse complementary region by using a set of specific Forward Primers: (5'-CTCAGGGTCAATCACAGAAG-3') and Reverse primers (5'-GAGGATGCAGAGAAGCTG-3'). Using online software, the primers flanking the polymorphic region were designed. (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). (Primer3 plus bioinformatics) The PCR product was amplified by using total volume of 25 µL comprising 7 µL of distilled water, 1 µL of each forward and reverse primers, a significant amount of 2.5 mm $MgCl_2$ 1.7 µL 2.5% of DMSO, 10 µL of Master mix (Mapleton Taq DNA polymerase 2x master mix catalog number A120301) and 4 µL of template DNA. The initial denaturation was allowed at 95°C for 5 minutes; then 35 cycles were performed with denaturation of 95°C for 30 seconds, annealing at 64.7°C for 30 seconds and extension at 72°C for 1.15 minutes. The final extension was carried out at 72°C for 5 minutes. Then further 10 µL amplified product was processed for RFLP at 37°C for 2 hours. By using restriction ensyme HPY1II6. A 10 µL amplified product was subjected to 2% electrophoresis to get the bands GG 467 bp, 132 bp, AA 467 bp, 102 bp and GA 467 bp,132 bp and 102 bp fragments.

STATISTICAL ANALYSIS

Statistical analysis was carried out by using done by using open Epi web tool, Microsoft excel and statistical analysis were done by using Statistical Package for the Social Sciences (SPSS) 25.0 version. The mean and standard deviation was used for age, aspartate transferase, Alanine transferase and their p-value was calculated by using t-test. The gender was calculated by using Chi-square test. Categorical variable were analysed by using in frequency, percentage and Chi-square test. Difference in IL28B genotype and allele between the cases and controls were compared by calculating p-value from Chi-square test. A p-value <0.05 was considered as significant.

RESULTS

In the present study, all 248 participants were genotype for SNP rs12979860 of IL28B gene, includes two groups, in cases 124 HCV positive patients and in controls, 124 controls. The mean age and standard deviation of cases 51.33±16.58 years and in control 50.61±16.21 years. In cases, males were 90 (72.6%) and females were 34 (27.4%) whereas in controls males were 83 (66.9%) and females were 41 (33.1%) and AST and ALT level described in [Table/Fig-1]. The distribution of genotype and allele frequency of SNP rs12979860 is described in [Table/Fig-2]. In cases, the frequency of G/G genotype was considerably high 83 (67%) compared to G/A 17 (13.7%) and A/A 24 (19.3%) and in controls the frequency of G/G genotype 84 (67.7%), G/A genotype 34 (27.4%) and A/A genotype 6 (4.9%). The frequency of allele "G" was found to be considerably higher in both the cases and controls. It would be designated as major allele in the study group while "A" as minor allele. There was a statistically difference in both cases and controls (p=0.002 value). The genotype frequencies of the IL28 B (rs12979860) variants were also analysed by adopting different models. The results are given in [Table/Fig-3]. Both the variants showed the highest association with the multiplicative model. The genotype frequencies in the control group were in conformity with the Hardy-Weinberg equilibrium $\chi^2=(2.6\times10^{-14})$.

Parameters	Cases n=124 Mean±SD	Control n=124 Mean±SD	p-value
Age (years)	51.33±16.58	50.61±16.21	0.80
*Gender M/F	90 (72.6%)/34 (27.4%)	83 (66.9%)/41 (33.1%)	0.35
AST (U/L)	77.67±69.75	28.12±9.58	<0.0001
ALT (U/L)	74.87±66.72	24.04±6.98	<0.0001

[Table/Fig-1]: Demographic presentation of hepatitis C infected patients and healthy controls.
AST: Asparatate transferase; ALT: Alanine transferase; Age- t test; Gender- Chi-square test; p-value significant <0.05, <0.0001 highly significant

Gene	SNP	Genotype/ Allele	Cases n=124	Controls n=124	p-value*	Odd ratio CI#
IL28 B	rs12979860	GG	83	84	0.001	NA
		GA	17	34		
		AA	24	6		
		G	183	202	0.0002	1.56 (1.0-2.3)
		A	65	46		

[Table/Fig-2]: Distribution of allele and the genotype with cases and control group.
Hardy Weinberg Equilibrium: $\chi^2=2.6\times10^{-14}$; Chi-square test (two-tailed)
*Significant (p-value <0.05); #OR: Odds ratio; CI: Confidence interval; NA: Not applicable

To assess the effect of the IL28B rs12979860 SNP on Hepatitis C infected patients (n=124) viral load, we quantified the viral load in the patients infected with HCV with different IL28B rs12979860 genotypes as shown in [Table/Fig-4]. The average (±SEM) HCV viral load was $4.6\pm3.6\times10^7$, $9.4\pm7.7\times10^7$ and $5.5\pm5.2\times10^7$ IU/mL

Model	Genotype	p-value*	Odds ratio
Dominant	GG vs GA+AA	0.5	1.037 (0.6-1.764)
Recessive	GG+GA vs AA	0.0003	4.68 (1.84-11.9)
Additive	GG>GA>AA	-	1>0.5>4.0
Multiplicative	G vs A	0.0002	1.56 (1.0-2.3)

[Table/Fig-3]: Genotype model of evaluate the association of SNP rs12979860 with cases and control.
*Chi-square, one-tail (Fisher's-exact test); Mantel Haenssel Chi-square for linear trend
*p-value <0.05 is statistical significant

in patients with the IL28B rs12979860 GG, GA and AA respectively. Moreover, the median and Interquartile range of viral load was 1675200 (43410-16928250), 203925 (35100-873150), 11736900 (4927500-30956775). Also there was a significance between the viral load and IL28B rs12979860 (p-value <0.05).

	Viral load			
ILR28B	<800000 IU/ML	>800000 IU/ML	Total	p-value*
AA	21 (16.94%)	3 (2.42%)	24	<0.05
GA	10 (8.06%)	7 (5.65%)	17	
GG	58 (46.77%)	25 (20.16%)	83	
Total	71 (57.26%)	53 (42.74%)	124	

[Table/Fig-4]: Comparison between viral load and IL 28B (SNP rs12979860) in cases.
*Chi-square test showed that there is statistically significant association between viral load and IL 28B

DISCUSSION

In patients infected with HCV, the connection between HCV RNA viral load and IL28B genotype studies are still limited. In the present study, the effect of the IL28B rs12979860 SNP on HCV viral load was investigated. The IL28B genotype was discovered to be able to predict the outcome of HCV infection but not the HCV viral load. The most recent genome-wide association studies found a link between multiple SNPs in the IL28B gene (IFN-gene domain) and antiviral drug efficacy, with the SNP rs12979860 (C/T) of the IL28B gene was particularly strong [12,13]. The ability to determine Sustained Virologic Response (SVR) in patients with HCV infection, before beginning antiviral therapy is critical in determining the probability of therapeutic success.

The present study was an attempt to detect the viral load and their association with IL28B in hepatitis C infected patients. In the present study, the reverse complementary polymorphic region of SNP 12979860(G/A) were studied. The frequency of allele G is significantly higher which act as a major allele but as compared to other studies they are considered C as a major allele. In present study the results showed that GG genotype 83 (67%) as compared to GA 17 (13.7%) and AA 24 (19.3%) and in control the frequency of GG genotype 84 (67.7%), GA genotype 34 (27.4%) and AA genotype 6 (4.9%) concordance with the previous study from Andhra Pradesh in the year 2012 [11].

In the present study the frequency of G/G genotype was the highest 83 (67%) and there was a significant correlation found between the viral load and IL28B rs12979860. Studies conducted in Indian subcontinent results were also correlated that IL28B is significant predictors of SVR with HCV genotype 1 and 3 [14]. The CC genotype was discovered in 54.4% of patients, whereas the variant genotype (CT+TT) was detected in 45.6% of patients who had their rs12979860 IL28B genotypes determined. IL28B polymorphisms could be a key factor in modifying antiviral therapy for CHC patients by Khan MS et al., [15]. These results were in coherence with the ones obtained by Sarrazin C et al., [16]. They found a significant correlation between SVR and IL28B rs12979860. The genotypes 2 and 3 were taken in their study. Also in another study it was also found that the patients having the HCV patients with the CC or TT genotypes are found to be having more viral load than the patient with CT/TT or TG/GG [17]. In a study by Firdaus R et al., was also in favour that the genotype 3 infected patients; the rs12979860 CC genotype was markedly related with prolonged virological response. In patients with high viral load, CC genotypes at rs12979860 were observed to be linked with persistent virological response (OR=6.75, p-value <0.05) [18]. A cohort study from India, also suggested that patients infected with genotype 3 and IL28B SNP is linked to both early viral decrease and long-term viral response [19]. In the present study, although there was such association observed apart from the finding that the highest percentage of patients consisted of GG genotype. The study finding the coherence with the present study was the one conducted by Abdelwahab SF et al., [20]. They examined 375 Egyptians with genotype 4 and found out that there was no significant association between viral load and IL28B rs12979860. When considering the SVR a study by Ge D et al., concluded that rate of SVR becomes two fold greater for the rs12979860 CC genotype when compared with the TT genotype [4]. In another study by Rauch A et al., a considerable number of infected patients having genotype 2/3 also showed no correlation between the rs8099917 genotype and virological response to the compounded therapy of paginated interferon/ribavirin [21]. This points towards the least correlation between the viral load and the IL28B rs12979860. In another study in spain Labarga P et al., it was found that CC genotype was associated with the higher HCV viral load than all other genotypes [22]. It can suggested that IL28B rs12979860 plays a crucial role as determinant of baseline virological load and further there should be more studies on the polymorphism of IL28B to access the clinical outcomes and treatment response.

Limitation(s)

To better confirm the results of polymorphism study, more number of samples are required. Further polymorphism study of IL28B with HCV genotypes and also follow-up study can also be helpful for future vaccine development.

CONCLUSION(S)

The present study concluded that, the IL28B rs12979860 SNP was found to be help in spontaneous clearance of infection in untreated HCV patients, it had effect on the viral load in infected patients. Since the GG genotype has been shown to be a strong predictor of HCV treatment clearance, extending the current findings to a larger study cohort should provide information for better HCV-mediated illness management in Indian participants. However, these findings highlight the importance of viral-host interactions in defining the course of HCV infection.

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