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Original Article

GENETIC SUSCEPTIBILITY OF HCV RNA IN VARIOUS ISONYM GROUPS OF PUNJAB, PAKISTAN

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ABSTRACT

Objective: To uncover the ecology and genetic factors affecting the susceptibility of infectious diseases of HCV RNA and to understand the role of genetic characters in the outcome and pathogenesis of hepatitis C virus (HCV) infection.

Methods: A total of 349 patients with chronic hepatitis have been included in this genetic susceptibility study. The infected individuals were diagnosed anti-HCV positive and then confirmed by HCV Polymerized Chain Reaction (PCR) qualitative test. All these selected patients had been diagnosed for HCV and taken the standard treatment of interferon and ribavirin from 20 w to 36 w. The end product of PCR was confirmed by gel electrophoresis. The following restriction enzymes Ear I, Rsa 1 and Mae III were used to digest the PCR product. This study focused only on patients who were non-responsive and had relapsed from conventional therapy.

Results: Our study proposes that both the interleukin genes IL-10 and IL-28B are found common in two major castes of the Punjab. We determined that HCV genotype 3a is very common (84.0%) among the group of responders whereas genotype 1a is more familiar in relapse (66.2%) as well as non-responders (54.0%). Excluding Genotype 4, the rest five main genotypes, namely 1a (61.40%), 2a (0.50%), 2b (20.00%), 3a (13.70%) and an untraceable (4.40%) were found among the 12 different castes/tribes ethnic groups I Pakistan.

Conclusion: To better manage and improve the fact behind understanding human susceptibility to HCV infection, a cohort study is beneficial.

Keywords: HCV, Genotypes, Genetic, Polymorphism.

INTRODUCTION

Hepatitis C virus (HCV) infection is a major public health problem faced by more than 130 million individuals globally. Hepatitis C is an infectious disease that mainly targets the liver in human's and chimpanzees. This infectious virus is made up of 9,600 nucleotides, which is fully sequenced. They were originally named as non-A, non-B hepatitis after its discovery in 1989[1]. HCV is an envelope, small size (55-65 nm) and single-stranded positive sense RNA virus (fig. 1). Its genus is hepatitis C virus and it belongs to Flaviviridae family [2].

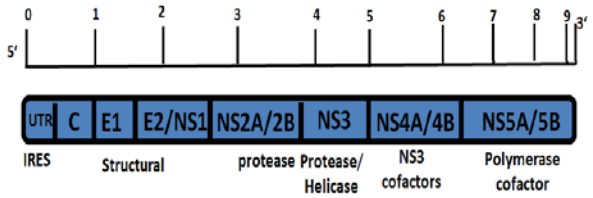

Fig. 1: Viral hepatitis C

Meanwhile, HCV infection being a chronic blood-borne disease, it causes a broad route of diseases in patients from subclinical infection to acute and chronic infection[3]. About 60 to 80% of infected individuals have chronic hepatitis from HCV which further involves liver cirrhosis in 20% of the infected patients and thereby leading to liver cancer [4].

With the development and use of molecular tools over the last 5 y, involving both genotyping methods and quantification of HCV RNA has paved a way to understand better the HCV-related liver disease [5, 6]. Currently, the quantification of HCV RNA has been mainly used in patients with chronic active hepatitis (CAH) to study their response to interferon (IFN) alpha therapy or to monitor replication of HCV in the infected patients during the treatment [7-9]. However, studying the genetic susceptibility may also provide a deeper understanding into the pathogenesis of HCV-related disease. This pathogen has been notoriously difficult to move from the infected individuals into the (laboratory environment) experimentation. Firstly, to identify it, to grow its culture and then study it, is still quite challenging. For more than 30 y, since the discovery of the virus in aHCV RNA vaccine has not been developed. It has been estimated that in Pakistan the prevalence of HCV is 8% of the total population [10]. Acute infection of HCV is often symptomless and mild, while chronic infection shows a lethargic course, which may lead to cirrhosis and hepatocellular carcinoma (HCC) [11]. This study was planned to screen the genetic susceptibility of HCV RNA with respect to ecology in different isonym groups in Punjab.

MATERIALS AND METHODS

Sample collection

A total of 349 patients with chronic hepatitis has been included in this genetic susceptibility study. Documents of statement for the consent and ethical approval for the collection of the samples are submitted as a supplementary file. Infected individuals were diagnosed anti-HCV positive and then confirmed by HCV Polymerized Chain Reaction (PCR) qualitative test. All these selected patients had been diagnosed for HCV and taken the standard treatment of interferon and ribavirin from 20 w to 36 w. The patients, who after the treatment of interferon therapy respond successfully to it and their HCV PCR test result became negative labeled as "cured", called as responders. Whereas the patients, who got the standard therapy of interferon and responded/recovered very well but turned up in HCV clinics after 6 mo to 24 mo showing the symptoms of HCV and their PCR test also indicated positive-were relapsed cases. While the other group of patients did not respond to the therapy even after 16 to 20 w therapy, called as non-responsive. After the formation of three groups of patients, the following criteria were considered for the recruitment of the patients. Both males and females, who were 18 to 56 y old, belonging to different districts of Punjab, were included in the study. The patients with mixed infection of hepatitis B and HIV, or receiving interferon treatment, or diagnosed as having HCV infection but not having started any treatment (Naive patients), were used as control samples.

Data collection

The data for each patient were recorded on a questionnaire. DNA extraction and molecular analysis were performed in Human

Genetics and Molecular Biology Department at The University of Health Sciences, Lahore.

DNA extraction

Genomic DNAs were extracted from all collected blood

Selection of susceptible genes to study

This study focused only on patients who were non-responsive and had relapsed from conventional therapy. The objective of the study was to find a gene which is susceptible in HCV RNA virus, so, that it became obvious to study the gene polymorphism which was selected in the previous studies. Hence, the gene polymorphism of different genetic variants as IL10 and IL28B was the most important genetic variants. The picture of genetic polymorphism would enable us to do the genetic variability testing for accurate and right time treatment of the patients resulting in a decrease of anxiety and economic burden, as HCV is a major health problem.

Polymerase chain reaction (PCR) for polymorphism of IL-10

To amplify the region of IL-10 gene as IL-10_1082, IL-10_819, and IL-10_592, required to detect three Singles nucleotide polymorphism (SNPs), the following primers described in the table were used. Solutions of these primers (shown in table 1), along with PCR master mix by Fermentas was used. The thermal cycling conditions set for IL-10 PCR are denoted in table 2. Table 3 represents primer sequence to detect the IL-28B gene polymorphism. Previously extracted sample DNA, which was to be amplified, a total of 25µl reaction mixture was prepared in a 0.2 ml PCR tube.

The end product of PCR contains amplified IL-10 gene in the region of 1082bp and its presence was confirmed by gel electrophoresis. In normal (i.e.) wild-type gene, product size is between 327-330bp and is more than 330bp. It varies between 12 and 204bp over and above the normal length. The following restriction enzymes Ear I, Rsa 1 and Mae III were used to digest the PCR product.

Table 1: Primer sequence for IL-10 gene polymorphism detection

S. No.	Gene	Position of polymorphism	Allele position	Phenotype frequency	Primer sequences 5-3	Annealing temperature TA[a]C
1.	IL-10	-1082	G	High	F→CCAGGTAGAGCAACACTCCT	53 ˚C
			A	Low	R→CTCTTACCTATCCCTACTCCC	
2.	IL-10	-819	C	High	F→TCCAGCCACAGAAGCTTACA	60 ˚C
			T	Low	R→GGCACATGTTTCCACCACCTCTT	
3.	IL-10	-592	C	High	F→GTGGAAACATGTGCCTGAGA	58 ˚C
			A	Low	R→ATGAGGGGGTGGGCTAAATA	

Table 2: Shows thermal cycling conditions for IL-10 PCR

Step	Time	Temperature	Number ofcycles
Denaturation of template DNA	5 min	95 °C	Initial cycle
Denaturation of DNA into single strands	1 minute	95 °C for	35 cycles
Annealing of primers to their complementary sequences	30 seconds	57 °C	
Extension of DNA strands	1 min	72 °C	

Table 3: Primer sequence for IL-28B Gene polymorphism detection

S. No.	Gene	Allele position	Phenotype frequency	Primer sequences 5/-3/	Annealing temp TA ˚C
1.	IL-28B	G	High	F→5' GGACGAGAGGGCGTTAGAG	53 ˚C
		A	Low	5'→AGGGACCGCTACGTAAGTCA	
2.	IL-28B	C	High	F→CCTATGTCAGCGCCCACAATTCC	60 ˚C
		T	Low	R→GGAGCTCCCCGAAGGCCC	
3.	IL-28B	C	High	F→ CGTTATGCATCCTTTCTGTGAGCATGG	57˚C
		A	Low	R→CCCAGGAGCTTGCACTAGCTCTTG CCAGGGCCCCTAACCTCTGCA GGGAGCGCGGAGTGCAATTCA	

Genotyping of IL10

Three different genotypes of IL10 were studied.

a) 1080 G/A b) 819 A/C c) 592 C/T

SNP were genotyped by Restriction Fragment Length Polymorphism (RFLP). For 1st genotype which was transcribed in 15 ml reaction tube, that contains PCR product and 'the specific restriction enzymes (Ear I). The digested products were visualized at 4% DNA gel electrophoresis.

Biological characteristics of interleukin 10

Moreover, IL-10 has dual biological roles in inhibiting. Both IL-10 deficiency and excess synthesis may be responsible for some lesions. Too low levels of the cytokines may result in the lack of pro-inflammatory cytokines suppression, an increase in inflammatory reactions, which results in some autoimmune disorders [12]. On the other side, excess synthesis may result in augmented susceptibility to viral infections or cancer. Various studies have established that IL-10 contributes in the growth of human infectious diseases [13].

The analysis of 349 patients has revealed five of the six subtypes namely 1a, 2a, 2b, 3a, of HCV as well as one unclassifiable genotype described in fig. 2. The frequency distribution of the genotype is provided table 4 along with their percentages. The data with respect to the frequency of six subgenotypes (namely 1a, 1b, 2a, 2b, 3a, and 3b) of HCV as well as one unclassifiable (UNTP) in assorted12 primary castes and their sub-caste is enumerated below.

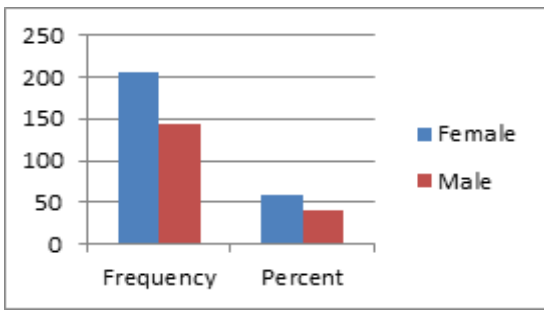

Fig. 2: Shows male and female distribution from different ethnic groups of punjab with chronic hepatitis C infection

Table 4: Shows the frequency and percentage distribution of six different genotypes of HCV in different caste/isonym groups of the Punjab

Caste	Sub-caste	Genotype 1a	Genotype 1b	Genotype 2a	Genotype 2b	Genotype 3a	Genotype 3b	Untypeable	Frequency
Arian	Chaudhry, Mian,	12	0	0	2	18	0	0	32
Gujjar	Chaudhry	15	0	0	4	16	0	1	36
Jutt	Bajwa, Dhiloo, Gondal, Sulehri	35	0	0	12	18	0	4	69
Kashmiri	Butt, Dar, Khwaja, Mir	17	0	0	7	8	0	3	35
Kumhar		2	0	0	1	2	0	0	5
Mughal	Rehmani	5	0	0	3	3	0	0	11
Malik	Baig, Lohaar, Mirza	13	0	0	5	6	0	0	24
Pathan	Khan, Pashtoon, Kundi, Tareen, Yousufzai	28	0	0	11	18	0	4	61
Rajput	Janjua, Rana, Khokhar, Sulehri	14	0	1	5	19	0	1	40
Shaikh		8	0	0	2	2	0	0	12
Syed	Alvi, Naqvi, Rizvi, Zaidi,	10	0	0	1	6	0	0	17
Unknown	No cast known	4	0	0	0	2	0	1	7
Total		163	0	1	53	118	0	14	349

RESULTS

This study is done to uncover the ecology and genetic factors affecting the susceptibility of infectious diseases of HCV RNA. The response of this pathogen is different in patients belonging to different caste/isonym groups. During this study period, we encountered a total of 402 patients out of which 53 (13.18%) refused to take part in the study. In the end, we were left with 349 persons consisting of 205 females and 144 males suffering from a chronic infection of HCV RNA (fig. 3). These 349 patients belonged to 12 different caste/isonym communities of the Punjab. To date no work seems to have been done by considering vis a vis chronic infection of HCV and different isonym communities in the sub-Indian subcontinent, where caste system is in vogue. The ecology and genetics of our caste system, because of their isolation, present a different genome structure to the invading HCV/RNA for interaction with the host cells. The genetic analyses of our data of HCV, mode of transmission, and the seroconversion of HCV in different isonym communities of the Punjab gave us different results. According to the progression of HCV infection and patient's status, after the standard therapy (according to the hospital protocol), these patients were divided into three groups:

Responders

Patients who responded to the standard therapy of interferon were well and declared cured. This group consisted of 100 blood samples 84% of this group belonged to genotype 3a, 10% to 1a, 3% to 2b and 3% and UNTP.

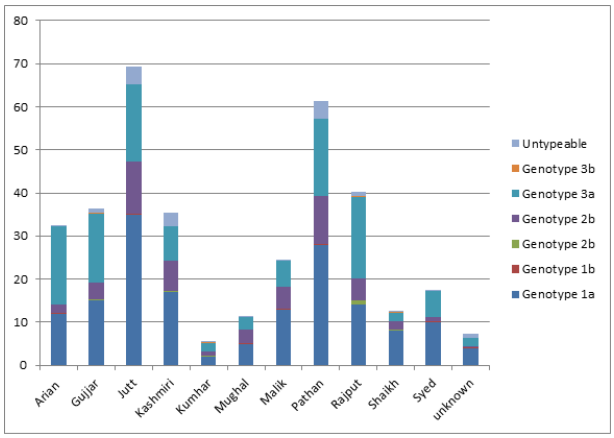

Fig. 3: Shows the frequency distribution of six different genotypes of HCV in different caste/isonym groups of the Punjab

Non-responder

Those who had not responded to therapy even after 16 to 24 w periods of treatment. Their HCV RNA remained active and replicating throughout the period of treatment. This group consisted of 92 blood samples, of which 53.73% belong to genotype 1a, 0.95% of 2a, 28.42% to 2b, and 13.68% to 3a and3.22% to UNTP.

Relapser

This group consisted of patients who responded to the therapy very well and were declared cured after HCV PCR, but after 6 to 18 mo again found positive by PCR testing showing active replicating viremia. This group, also called as recurrence of HCV, consisted of 154 blood samples, out of which 66.24% belonged to genotype 1a, 14.93% to 2b, 13.64% to 3a and 5.19% to UNTP.

DISCUSSION

This study was undertaken on the ecology and susceptibility of HCV RNA in different isonym communities inhabiting the Punjab, where the caste system is deeply rooted due to cultural and economic reasons [14, 15]. The philosophy of various caste systems was explained by Ibbetson *et al.* [16], who stated that it was impossible to make any statement regarding anyone caste. Punjab is the biggest province of Pakistan, containing about 60 percent population of the country, which is composed of thousands of major and minor castes/isonym communities. In a study conducted by Awan *et al.* [17], a sample of 250 IDPs of South Waziristan (SWA) aged between 1–60 y was collected and found that 28.8% of the sample size had HCV positive, of these 21.6% had the HCV RNA. HCV was found to be more in males and old age people. Similarly, illiteracy was found to be a main cause of the disease. The other causes found in the study were Dental Surgery, Tattooing, Reused Blade, Blood transfusion and drug addiction.

CONCLUSION

We determined that HCV genotype 3a is very common (84.0%) among the group of responders whereas genotype 1a is more familiar in relapser (66.2%) as well as non-responders (54.0%). Excluding Genotype 4, the rest five main genotypes, namely 1a (61.40%), 2a (0.50%), 2b (20.00%), 3a (13.70%) and an untypeable (4.40%) were found among the 12 different castes/tribes ethnic groups I Pakistan. The HCV frequency in 12 isonym groups is as follows: Jutt (18.91%), Pathan (17.19%), Arain (15.26%), Rajput (11.46%), Malik (10.44%), Gujjar (10.02%), Kashmiri (10.02%), Sayyed (4.87%). Sheikh (3.43%) and Mughal (3.21%). The highest number of HCV infections was found in the Jutt caste, whereas the percentage of infection was less in Mughals caste. Rajput caste had most common strain genotype 3a was present among the responder.

Table 5 and Fig.4 shows the gene polymorphism of IL 10 for three different positions. In SNP IL-10at 1082 position, GA (80.30%),GG (5.20%) and AA (14.5%); and SNP at 819, AC (84.7%) CC (12.0%)

and AA (3.2%), and SNP at 592 position, CC (24.1%). AA (6.0%), and CA (69.9%). The frequency of gene polymorphism CA was high than CC and AA homologous. In IL-28B SNP at location a, TG (40.6%), TT (54.6%), GG (4.8%), TT (54.6%); SNP at location b, TT (6.8%), CT (58.2%), CC (34.9%), TT (16.1%) CT (43.8%) andCC (40.2%) was found (Shown in table 6). Fig.5shows the three gene polymorphisms of IL-28B with the relapsed and non-responder group occurrence of TT homologous high at one position. CT heterozygous polymorphism was frequently at the second and third position. Major caste found was of Jutt (Jatt), with genotyped 1a and 2b, while other main caste representing chronic infection of HCV was of Pathans, who had major relapses. The most important genotype uncommon in this region was found in Rajput group that is 2a, only one sample of 1a was found in Rajput tribe, these caste do not marry out of caste and restrict to their own gene pool and after a certain period of time become mutated and cause infectious diseases as well as abnormalities in the next generations

Both HCV genotypes and genetic susceptibility seem to be of significance in attaining the HCV infection. In conclusion, this present study proposes that both IL-28B and IL-10 interleukin genes are familiar with two main castes in Punjab. Therefore a detailed research is must to improve the better understanding of human genetic susceptibility with various strains of HCV infection and its management.

Table 5: Frequencies of IL-10 SNP's polymorphism

Gene	SNPs	Polymorphism	Frequency	Percentage
IL-10	IL-10_1082	AA	36	14.5
		GA	200	80.3
		GG	13	5.2
		AA	8	3.2
	IL-10_819	AC	211	84.7
		CC	30	12.0
		AA	15	6.0
	IL-10_592	CA	174	69.9
		CC	60	24.1

Table 6: Frequencies of IL-28B SNP's polymorphism

Gene	SNPs	Polymorphism	Frequency	Percentage
IL-28	IL-28B_a	GG	12	4.8
		TG	101	40.6
		TT	136	54.6
	IL-28B_b	CC	87	34.9
		CT	145	58.2
		TT	17	6.8
	IL-28B_c	CC	100	40.2
		CT	109	43.8
		TT	40	16.1

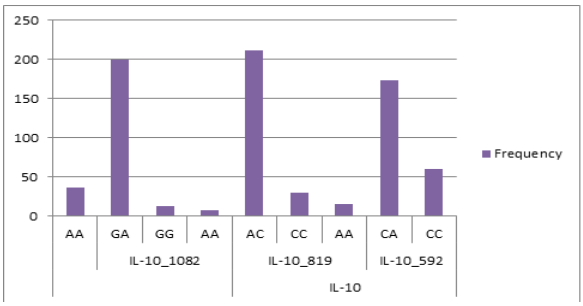

Fig. 4: Shows the frequency of 3 polymorphisms of IL-10 single nucleotide polymorphism

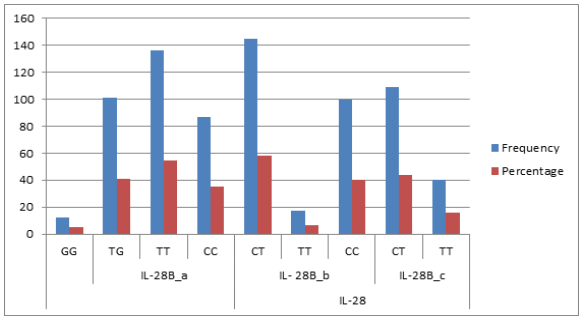

Fig. 5: Shows 3 Gene polymorphisms of IL-28B with the relapsed and Non-responder group

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ABBREVIATION

Hepatitis C virus (HCV); Chronic active hepatitis (CAH); Hepatocellular carcinoma (HCC); Polymerized Chain Reaction (PCR); Single nucleotide polymorphism (SNPs).

CONFLICT OF INTERESTS

All authors disclose no conflict of interest.

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