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Distribution of Hepatitis C Virus Genotypes Amongst the Beta-thalassemia Patients in North of Iran

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Abstract: Beta-thalassemia patients have high prevalence for HCV infection. In developing countries, HCV antibody is reported to be high in this group of patients. This study carried out to determine the distribution of HCV genotypes amongst the beta-thalassemia patients in North of Iran. The present study has been carried out between February and March 2010 amongst a group of 245 beta-thalassemia patients (125 male and 120 female) referred to the hospitals Mazandaran and Guilan provinces for a blood transfusion. Qualitative analysis of these samples using ELISA and PCR. The PCR positive samples were subjected to genotyping by RFLP method. Of total 245 beta-thalassemia patients who were the subjects of this study, 28 of these patients were diagnosed through PCR test to have RNA virus. For this reason, the prevalence of this illness in this study group was estimated as 11.42%. By using the RFLP technique, the above genotyping were identified and the prevalence of three genotypes, including 3a, 1a and 1b were proved. The genotype 3a was most prevalent. Out of 28 positive samples, 18 (64.3%) samples had this genotype. After that, genotype 1a with 9 positive occurrences (32.1%) and genotype 1b with only 1 positive occurrence (3.6%) were most prevalent. This study demonstrated that the main reason the beta-thalassemia patients became infected with the genotype of the virus was due to receiving infected blood that entered into Iran during the past two decades.

Key words: Hepatitis C virus, genotypes, beta-thalassemia patients

INTRODUCTION

The hepatitis C virus is genus of the Flaviviridae family, identified for the first time in 1989. Afterwards, the whole genome has been identified and sequenced (Afridi *et al*., 2009; Amadi *et al*., 2009). According to the World Health Organization to report more than 3% of the world population is infection with this virus and 170 million of these have chronic infection (Kandil *et al*., 2007; Amini *et al*., 2009; Lawrence, 2009; Abro *et al*., 2010). Moreover, approximately 85% of the patients after getting infected to acute HCV will progress to chronic hepatitis, of which 10 to 20% will develop liver cirrhosis (Alkahtani, 2009) and 1 to 5% hepatocellular carcinoma (Davis *et al*., 2003; Strader *et al*., 2004; Abo Elmagd *et al*., 2011).

Hepatitis C virus has a great number of genetic diversity classified to many genotypes (Zein and Persing, 1996). There are also many other genotypes which have been formed within the studied virus, which consequently resulted in the identification of many different subtypes (Kazemi *et al*., 2005; Garcia-Montalvo and Galguera-Colorado, 2008). The prevalence of these genotypes is different geographic areas. For example, while genotypes 1, 2 and 3 are spread around the world, their prevalence is different from one region to another. Genotype 4 appears to be limited to central Africa and Middle East, while genotype 5 is more common in South Africa and genotype 6 has been observed mostly in Hong Kong, Vietnam and other South East Asian countries (Zein, 2000; Gish and Lua, 1997). Having some information on the prevalence of various genotypes can show how the virus got in. Moreover, since some of the genotypes are antiviral drug resistance, identifying the genotype can help patients' recovery (Izopet *et al*., 1998; Zein, 2000; Hosogaya *et al*., 2006). Hepatitis C can be transmitted by transfusion and percutaneous routes (Khedmat *et al*., 2007). This virus is prevalent in IV-drug abusers, multiple sex partners, hemophilia and Beta-thalassemia Patients (Talaie *et al*., 2007).

Beta-thalassemia Patients are considered as major factor for prevalence of HCV infection (Hejazi *et al*., 2007; Shahraki *et al*., 2010). In developing countries, HCV antibody is reported to be high in this group of patients

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which endanger the health of these patients and increase their treatment costs. The main reason for liver damage from HCV infection is the high level of iron in the liver (Shahraki *et al*., 2010). In recent times, due to pay special attention to the better screening of donated blood, the risk of transmitting the virus blood through transfusion has decrease to 1 to 2 million units (Stramer *et al*., 2004).

Because of having not sufficient information about the genotypes of HCV amongst the beta-thalassemia patients in North of IRAN, the present study attempts to identify the genotypes in this area and their relation with the age, gender and other epidemiological factors.

MATERIALS AND METHODS

The present study has been carried out between February and March 2010 amongst a group of 245 beta-thalassemia patients (125 male and 120 female) referred to the hospitals Mazandaran and Guilan provinces for a blood transfusion. The average age of the patients were 18.38±4.5 ranging between 10 to 55 years old. Upon filling an application form contained demographic, laboratory and epidemiological related information, 10 mL of blood was obtained from which patients. Using the third generation enzyme Immunoassay kit (Acon Laboratories, Inc, Sandiego, USA), the blood was tested for the presence of antibodies against HCV in the plasma.

In the next phase, by an extraction kit (High Pure Viral Nucleic Acid, Roche Applied Science, Penzberg, Germany) the RNA virus was extracted from samples. Then, RNA was used to the synthesis of cDNA. The reaction for synthesis of cDNA, including, 7 μL of extracted RNA, 200 U of AMV reverse transcriptase (promega, USA), 40 U random hexamer (promega, USA), 20 U of RNase inhibitor (promega, USA) and 10 mM of dNTPs (Promega, Madison, USA) (Amini *et al*., 2009).

Were used two specific primers for 5'UTR hepatitis C virus (Table 1) Nested PCR test was carried out. First reaction was performed in a total volume of 50 μL contained 35.2 μL of molecular biology-grade water (Sigma Aldrich, California, USA), 5 μL of 10×PCR buffer, 0.15 mmol of $MgCl_2$, 100 μM of dNTPs, 2.5 U of *Taq* DNA polymerase (Promega, Madison, USA), 20 pmol of each outer primer and 5 μL of cDNA. PCR amplification conditions on the thermocycler (Eppendorf, Hamburg, Germany) were as follows: 94°C for 4 min, followed by 30 cycles of 94°C for 30 sec, 58°C for 35 sec and 72°C for 40 sec, with a final extension at 72°C for 4 min.

Table 1: Sequence of primers used for HCV RNA Nested PCR assay

Function	Locus	Sequence sense	Nucleotide Sequence 5' to 3'
Upstream primer	-4 to -22	Antisense	GCA CGG TCT ACG AGA CCT
Downstream primer	-268 to -251	Sense	AGC GTC TAG CCA TGG CGT
Nested primer 1	-26 to -43	Antisense	GGG CAC TCG CAA GCA CCC
Nested primer 2	-199 to -183	Sense	GTG GTC TGC GGA ACC GGG

After the execution of the primary cycle, the elements of PCR with the same composition of the first round and inner primer were transferred to another micro tube with the 5 μL from the first round PCR product was added. After the execution of 30 secondary cycles, 10 μL from the second round PCR was electrophoresis on a 2% agarose and visualized under UV-transilluminator. The presence of 174 bp band signified PCR's positive reaction.

Then, to identify genotypes of the virus, the extract of the second round PCR was used to perform RFLP test (Pohjanpelto *et al*., 1996). Accordingly, for every sample, three micro tubes were considered. The final capacity of each micro tube was 20 μL. the composition of three micro tubes was respectfully:

- Two μL of buffer, 11.6 μL of distilled water; 0.2 μL from each restriction enzyme (ScrFI and HinFI)
- Two μL of buffer, 11.6 μL of distilled water; 0.2 μL from each restriction enzyme (MvaI and HinFI)
- Two μL of buffer, 11.8 μL of distilled water; 0.2 μL from each restriction enzyme (BstUI)

Finally, 6 μL from the second round PCR was added to each Micro tube and left over night in 37°C. Upon enzyme digestion, the end product in the vicinity of a size marker 50 bp (fermentas, Germany) was electrophoresis on a 2.5% agarose gel. With this method, the following genotypes are identifiable: 1a, 1b, 2a, 2b, 3a, 3b, 4, 5 and 6 (Pohjanpelto *et al*., 1996).

In order to determine the relationship between the degree of prevalence and the HCV genotypes with the information collected from beta-thalassemia patients, the results obtained were entered into SPSS. V, 16 software and t-tests were conducted.

RESULTS

In the total 245 beta-thalassemia patients, 36 patients were identified through ELISA test to have HCV antibody. Twenty eight of these patients were diagnosed through PCR test to have RNA virus. For this reason, the prevalence of this illness in the studied group was estimated as 11.42%. By RFLP technique, the above genotyping were identified and the prevalence of three genotypes, including 3a, 1a and 1b were proven (Fig. 1).

The genotype 3a was the most prevalent. Out of 28 positive samples, 18 samples have been infected to.

As such, its prevalence is reported to be 64.3%. Afterward, genotype 1a with 9 positive occurrences (32.1%) and genotype 1b with only one positive occurrence (3.6%) were the most prevalent.

The demographic information indicated that none of the known genotypes were found in the 20 sec age group ($p = 0.000$). Genotype 3a the most prevalent in both men and women was 66.7 and 61.5% in men and women population respectively. The genotype 1a was also equally prevalent in both men and women and no significant differences were observed ($p = 0.735$).

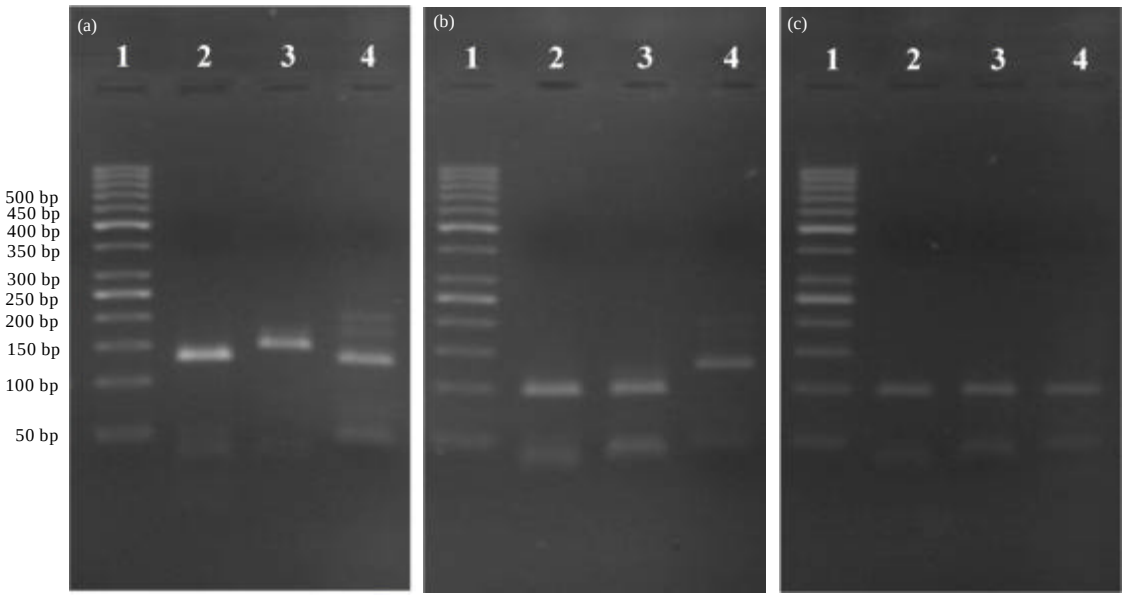

Fig. 1(a-c): 2.5% agarose gel electrophoresis of the digestion products of the amplified DNA from different genotypes, 1: Marker 50 bp (fermentas, Germany), (a) 3a (2: 120, 3: 150, 4: 120 bp), (b) 1a (2: 100, 3: 100, 4: 120 bp) and (c) 1b (2: 100, 3: 100, 4: 100 bp)

Table 2: Distribution of HCV genotypes by demographic variables in beta-thalassemia patients in North of Iran

			Genotype						
			1a		1b		3a		
Demographic variable	No. tested	No. HCV RNA positive	No.	%	No.	%	No.	%	p-value
Age (year)									
<20	96	0	0	0.0	0	0.0	0	0.0	0.000
20-29	86	20	6	30.0	1	5.0	13	65.0	
>30	63	8	3	37.5	0	0.0	5	62.5	
Sex									
Male	125	15	5	33.3	0	0.0	10	66.7	0.735
Female	120	13	4	30.8	1	7.7	8	61.5	
Place of residence									
City	137	18	5	27.8	1	5/6.0	12	66.7	0.629
Village	108	10	4	40.0	0	0.0	6	60.0	
Level of education									
Analphabet	107	9	2	22.2	0	0.0	7	77.8	0.258
Diploma	78	10	1	10.0	1	10.0	8	80.0	
Master	60	9	6	66.7	0	0.0	3	33.3	
Social status									
affluent	22	0	0	0.0	0	0.0	0	0.0	0.000
Good	116	7	4	57.1	0	0.0	3	42.9	
Mediate	83	9	1	11.1	1	11.1	7	77.8	
Poor	24	12	4	33.3	0	0.0	8	66.7	
Total		245	28	9	32.1	1	3.6	18	64.3

Table 3: Distribution of HCV genotypes by risk factors in beta-thalassemia patients in North of Iran

				Genotype						
				1a		1b		3a		
Demographic variable	Status	No. tested	No. HCV RNA positive	No.	%	No.	%	No.	%	p-value
History of hospitalization	Yes	152	15	3	20.0	1	6.7	11	73.3	0.275
	No	93	13	6	46.2	0	0.0	7	53.8	
History of transplantation	Yes	12	0	0	0.0	0	0.0	0	0.0	0.653
	No	233	28	9	32.1	1	3.6	18	64.3	
History of surgery	Yes	131	18	5	27.7	1	5.6	12	66.7	0.500
	No	114	10	4	40.0	0	0.0	6	60.0	
History of accident	Yes	51	10	3	30.0	0	0.0	7	70.0	0.160
	No	194	18	6	33.3	1	5.6	11	61.1	
History of dentistry	Yes	108	21	7	33.3	1	4.8	13	61.9	0.006
	No	137	7	2	28.6	0	0.0	5	71.4	
History of risk behavior	Yes	15	0	0	0.0	0	0.0	0	0.0	0.560
	No	230	28	9	32.1	1	3.6	18	64.3	
Smoking	Yes	15	3	1	33.3	0	0.0	2	66.6	0.713
	No	230	25	8	32.0	1	4.0	16	64.0	
Total		245	28	9	32.1	1	3.6	18	64.3	

As for the distribution of this genotype with place of residence, the majority of the patients lived in cities, with genotype 3a (66.7%), moreover, the patients had been the most prevalent that were among (p = 0.629) (Table 2).

Also, there weren't any significant statistical differences in the prevalence of genotypes and the level of education. The Infection to the hepatitis C virus was mostly observed among those who had graded diploma and below level. There wasn't any patient with a master's degree and higher, while, we observed these patients were infected to the virus (p = 0.258).

The majority of infected patients were from an unstable (poor) social background. In this group also the genotype 3a was the most prevalent (66.7%). Conversely, patients with an affluent social background were not infected. Considering the number obtained for (p = 0.000), there seems to be a correlation between the degree of prevalence of genotypes and the social status of the patients.

As for the level of prevalence of the HCV genotypes in relation with risk factors studied (Table 3) including history of hospitalization, transplantation, surgery, accidents, background illnesses, foreign travel's smoking and addiction, in related to, we didn't observe any significant statistical differences, However, there seems to be a correlation with using dentistry related services.

DISCUSSION

Currently, it is possible to improve the lives of beta-thalassemia patients through proper screening of donated blood. Nevertheless, considering the fact that this virus is not detectable during the so called window period, beta-thalassemia patients are still in danger of getting infected with hepatitis C virus through their monthly blood transfusion (Raghraman *et al*., 2003; Pour *et al*., 2006; Garcia-Montalvo and Galguera-Colorado, 2008). The prevalence of hepatitis C virus in the study group is reported to be 11.42%, which in comparison to its prevalence in society as a whole, 0.5%, is quite high (Kabir *et al*., 2006). For this reason, utilizing a more proper screening system is essential.

At present, the hepatitis C virus seems to have six genotypes that are localized in different geographical areas (Kazemi *et al*., 2005; Garcia-Montalvo and Galguera-Colorado, 2008). There are many documents indicating that patients infected with the hepatitis C virus demonstrate different degrees of infections and different responses to treatment by interferon alpha (Kazemi *et al*., 2005; Amini *et al*., 2009). As a result, to undertake epidemiological studies, a reliable genotyping system is essentially needed. Utilization of these tools will help identify the sources of infection and the paths through which the virus has been entering among the studied patients (Garcia-Montalvo and Galguera-Colorado, 2008; Lawrence, 2009) In this study, a genotyping based on Restriction enzyme was used.

The most prevalent genotype in this study is reported to be genotype 3a with 64.3%. After that, there are genotype 1a with 32.1% and genotype 1b with 3.6%. According to studies carried out to identify genotypes of hepatitis C virus among Iranians infected to beta-thalassemia, the genotype 3a is the most dominant.

While 3a was the dominant genotype among the patients of this study group, studies done on the prevalence of genotypes of this virus among none beta-thalassemia population in North of Iran identify 1a as the dominant genotype (Hejazi *et al*., 2007; Amini *et al*., 2009). In this group of patients infected with hepatitis, the prevalence of genotype 1a was reported to be 64%. After

that, there are genotype 3a (20%) and 1b (16%). The fact that beta-thalassemia patients and non beta-thalassemia patients were found to have two different dominant genotypes could be associated with the difference in the way the virus enters one's body (Alavian *et al*., 2002, 2005).

It is worth to mention that in the study group, the under 20 year old beta-thalassemia patients were not infected with the virus. However, infection with the virus was more prevalent among those over 20 years old. The main reason for the higher rate of infection in this age group and the dominance of the genotype 3a among Iranian patients could be related to the importing of infected blood and its transfusion to the beta-thalassemia patients during the past decade. Overall, this study demonstrated that there is a significant relationship between age, social background and dental work history with the type of genotype. However, to understand the exact relationship requires further study.

CONCLUSIONS

The predominant HCV genotype in beta-thalassemia patients in Mazandaran and Gilan provinces is type 3a followed by 1a and 1b. The frequency of genotype 3 was observed to be increasing in this patient's whereas the prevalence of genotypes of this virus among none beta-thalassemia population in these area identify 1 as the dominant genotype.

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