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ORIGINAL ARTICLE

Molecular epidemiology of hepatitis C virus and its relation with persistence or clearance of infection in Hamadan, West-Iran

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ABSTRACT

Background and Objectives: Hepatitis C Virus genotyping appears to be vital for predicting the response to antiviral therapy. The present study aimed to analyze the *HCV genotypes* in relation to persistence or clearance of the virus in residents of Hamadan Province, West-Iran.

Material and Methods: A total of 1159 recorded questionnaires of HCV infected people were evaluated in this prospective study. Several parameters including HCV genotypes, *anti-HCV antibodies, viral load, drug treatment,* response to therapy and amount of *ALT* and *AST* were analyzed.

Results: HCV genotyping in 637 samples revealed a predominance of type 1a (52.1%) followed by 3a (42.4%), type 1b (2.7%) and type 2 (0.2%) respectively. Mixed genotypes (3a-1a) were detected in 0.9%, and 1.7% had untypable genotype. High frequency of genotypes 1a and 3a were observed in drug-resistant (group-a) and drug-sensitive (group-b) patients respectively (P<0.0001). Additionally, duration of drug treatment was significantly higher in group-a than group-b (P<0.0001). During follow-up period, 92 cases showed spontaneous clearance of HCV infection and more importantly 86 of 92 cases were positive for anti-HCV antibodies compared with 59 of 455 antibody positive cases with treatment-induced clearance of HCV infection (P<0.0001).

Conclusion: HCV genotyping and also antibody screening could be useful for proper therapeutic intervention in HCV infected subjects.

Keywords: Epidemiology, Genotype, HCV, Antibody.

INTRODUCTION

Hepatitis C virus (HCV) is a member of the Flaviviridae family. HCV has been classified into 1-7 major genotypes and each genotype is further divided into subtypes. HCV genotypes present diverse clinical outcome, biological properties, and reactions to antiviral treatment which play essential roles for studying the pathogenesis and epidemiology of HCV infectious disease (1, 2). Data now supports a key role for different genotypes in developing specific mechanisms that lead to diverse pathological signs such as insulin resistance,steatosis and progression toward cirrhosis, fibrosis and hepatocellular carcinoma. Also, HCV genotype can affect pharmacological treatment in terms of duration and dose of therapy (3). It has been reported that genotype 1 is more likely in relation to higher

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incidence of destructive disease with increased insulin resistance, higher threat of cirrhosis, progress of hepatocellular carcinoma and also resistance to therapy. In compare with genotype 1, genotype 3 is correlated to enhanced liver steatosis and fibrosis (3). In this context, Rolfe *et al*. showed a higher frequency of spontaneous clearance of HCV-RNA in younger infected patients with genotype 1 in compare with genotype 3 (4). Likewise, a relationship has been observed between HCV genotype 2 and a more active liver disease (5).

To predict the response to interferon therapy in patients with chronic hepatitis C infection, HCV-genotyping seems to be vital (6). Although the role of HCV genotypes has been reported to be important in pathogenesis and epidemiology of HCV-related disease but, so far little is known of this connection in western provinces of Iran.

The present study aimed to analyze the prevalence of HCV genotypes, anti-HCV antibodies, and evaluate some clinical features in relation to para-clinical data such as serum viral load, liver function tests and drug consumption in HCV infected people in Hamadan, a western province of Iran. The findings in turn can lead to better monitoring and therapeutic intervention in HCV infected patients. To our knowledge, the current study is one of the few researches which surveyed the epidemiological data of the frequency distribution of HCV genotypes in this part of Iran.

PATIENTS AND METHODS

A total of 1159 recorded questionnaires of HCV infected people in Hamadan province, West of Iran, who referred to Shahid-Beheshti University Hospital, Hamadan University of Medical Sciences, between January 2006 and December 2011 were surveyed in this prospective study approved by institutional research ethics committee.

RNA extraction and Real-time PCR quantification. HCV-RNA was extracted from 100 mirolitters of sera samples from all referred patients by using commercial DNA/RNA extraction kit (K2.9.Et.50.CE. RIBO-PREP, InterLabService, Russia) based on manufactures protocol. Then, the number of HCV-RNA copies per milliliter of serum was quantified using quantitative real-time PCR kit (AmpliSens® HCV Monitor-FRT, InterLabService, Russia) according to the manufactures' instructions.

HCV genotyping. Genotypes of HCV-RNA positive samples were determined by conventional PCR kit from the above mentioned company.

Anti-HCV antibody detection. Anti-HCV antibodies were determined using commercial ELISA kit (DIA.PRO, Milano - Italy).

Some data in the questionnaires were missed and therefore we analyzed the existing available data. The evaluated parameters in this study were HCV genotypes, viral load in sera samples, anti-HCV antibodies, monitoring of therapy (Recurrent infection or successful treatments leading to clearance of infection), duration of drug treatment (Peg interferon alfa-2a (PEGASYS) and Ribavirin (COPEGUS®) combination ther-apy) and the level of ALT and AST as the liver function tests. However, efficacy of treatment was monitored by the periodic HCV-RNA testing and the evaluation of liver enzymes.

Statistical analysis. The results were analyzed using Prism 5.01 (Graphpad Software, San Diego, CA, USA), EPI info 6.04 (CDC, Atlanta, Georgia, USA), and SPSS version 16 software (Texas, USA). Baseline characteristics were summarized as means and proportions of selected variables. The distribution of quantitative variables was determined using the Kolmogorov–Smirnov test. Mean values of quantitative variables among groups were compared using an unpaired t-test for data distributed normally and a Mann–Whitney test for non-normal data. The Kruskal–Wallis test or ANOVA with Bonferoni were used to compare means among two or more groups, as measured by interval variables. The data were considered significant if P values were less than 0.05.

RESULTS

Demographic data of the study population are summarized in Table 1. Of 1159 patients, 904 (77.9%) were male and 255 (22.1%) were female. Among the 1159 HCV-RNA positive patients included in the current study, HCV genotype was determined in 637 cases. Five hundred and two cases of 1159 were categorized as new referrals to the laboratory. The results of genotyping in 637

samples revealed a predominance of type 1a (52.1%) followed by 3a (42.4%), type 1b (2.7%) and type 2 (0.2%) respectively. Mixed genotypes (3a, 1a) were also detected in (0.9%) of the samples. Also, 1.7% of the cases had a non-typeable genotype (Fig. 1).

In view of patients' medical conditions, 1056 (91.1%) cases had only HCV infection without any risk factor, 49 (4.2%) of patients belonged to the groups with thalassemia, hemophilia, multi transfusion and patients under hemodialysis. Drug abusers consisted of 25 (2.2%) of infected patients. Liver disease, co-infection (HCV infection along with HBV or HIV infections), and other clinical history (unknown source of infection) were 10 (0.9%), 11 (0.9%) and 8 (0.7%) cases respectively (Table 1). Comparison of the different groups of patients regarding to the medical conditions depicted a significant difference for the pattern of antibody response, drug consumption status and finally post treatment evaluation (Table 2).

Post-treatment appearance of HCV infection (HCV-RNA in serum) during three years follow up was evaluated in 110 of 565 cases who received antiviral therapy. Among this group, 42 cases showed recurrent infection (group a) in compare with 68 cases who had treatment-induced clearance of infection within 6-12 months of antiviral therapy (group b). Notably, higher significant frequency of genotype 1a and 3a were observed in group a and b respectively ($P<0.0001$ and $P<0.0001$, Table 3). As expected, duration of drug treatment was significantly higher in group (a) compared to group (b) ($P<0.0001$, Table 3).

Table 1. Demographic characteristics of the study subjects

Parameters	Patients (n=1159)
Age in year (Mean±SD)	37.6±18.23
Gender (Female/ Male)	255 (22.1%) / 904 (77.9%)
Medical conditions (Risk factors or background disease)	
Liver diseases	10(0.9%)
Only HCV infection	1056(91.1%)
Co-infection	11 (0.9%)
Tx, Tf, Dial, Thal, Hemophilias	49 (4.2%)
Drug abusers	25(2.2%)
Others	8 (0.7%)
Antiviral treatment for HCV (%)	
Interferon and Ribaverin	565 (48.8%)
No treated	92 (7.9%)
New referrals to laboratory	502 (43.3%)
Post-Treatment HCV infection	
positives /negatives	110 (19.5%) / 455 (80.5%)
Reappearance of HCV post therapy [a]	42/110
Initial stages of treatment[b]	68/110
Anti-HCV antibodies testing	
Positives / Negatives	529 (45.6%) / 5 (0.40%)
unknown	625 (54.0%)
HCV Genotyping	
Done / Not tested	637 (54.9%) / 522 (45.1%)
Pre-treatment SGOT (IU/ml) (Mean±SEM)	40.01±1.54
Pre-treatment SGPT (IU/ml) (Mean±SEM)	48.95±2.61
Pre-treatment Viral load (Mean±SEM)	191780±10493

a:HCV-RNA positives for three times during meanly three years of follow up, b: Patients underwent antiviral thearpy within first year of diagnosis, Tx: Transplant patients, Tf: Transfusion, Dial: Dialysis, Thal: Thalassemia.

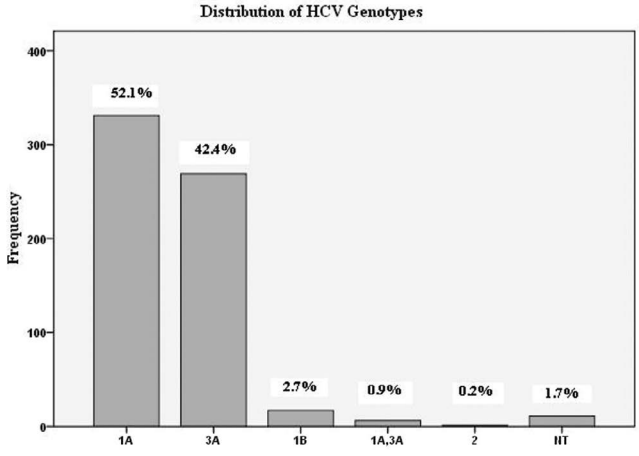

Fig 1. Distribution of HCV genotypes among 637 cases of study population (n=1159)

Table 2. Comparison of different groups of the study subjects with regard to the HCV genotypes, viral load and antibody response and antiviral therapy

Variables	HCV Infection N=1056	Tx-Tf- Dia- Thal N= 49	Liver Diseases N=10	Co-infection N=11	Drug abusers N=25	Others N=8	P Values[a]
Gender (F/M)	237/819	8/41	3/7	2/9	1/24	4/4	0.25
HCV genotypes							
1a	296	21	2	4	7	2	0.15
3a	250	5	4	1	8	2	
1a, 3a	5	0	0	0	1	0	
1b	17	0	0	0	0	0	
UT	9	9	2	0	0	0	
X	461	21	4	7	9	4	
Antibody status							
Positives (%)	479 (45.3%)	11 (22.4%)	5 (50.0%)	9 (81.8%)	20 (80.0%)	5 (62.5%)	<0.0001
Unknown	574	35	5	2	5	2	
Viral Load (copies/ml)*	192030±11287	241021±66351	138818±66450	144158±30517	207596±46424	140468±58746	0.79[b]
Post-treatment HCV infection Pos /Neg	101 / 414	2 / 33	2 / 2	1/ 3	4 / 1	1 / 2	<0.0001
Antiviral treatment (%)	522 (49.4%)	35 (71.4%)	3 (30%)	2 (18.2%)	2 (8.0%)	1 (12.5%)	<0.0001

Significant differences were shown for seropositivity, persistence of HCV infection after therapy and frequency of cases who received antiviral therapy. a: Yates corrected P values by Chi-square test, b: One-way ANOVA test. UT: untypable, X: Not tested.* HCV-RNA copies per milliliter of serum (Mean ± SD). Tx: Transplant patients, Tf: Transfusion, Dial: Dialysis, Thal: Thalassemia.

Table 3. Comparing the HCV genotypes, antibodies, viral load and liver enzymes between drug-sensitive and drug-resistant patients (recurrence of infection)

Parameters	Recurrent HCV infection (n=42)	Responsive to drug-treatment (N=68)	P values
HCV genotypes			
1a	27	3	<0.0001[a]
3a	1	10	<0.0001 [a]
Anti-HCV antibodies			
Pos / Neg	14 / 1	6 / 0	0.62 [a]
Viral load (Copies/ml)			
Mean±SEM	219582±93659	122861±35134	0.93 [b]
Duration of drug treatment (Months)			
(Mean±SD)	17.94±12.25	6.28±2.7	<0.0001 [c]
SGOT (IU/ml) (Mean±SEM)	49.67±4.0	46.67±5.9	0.23 [b]
SGOT (IU/ml) (Mean±SEM)	52.08±4.17	45.97±5.91	0.06 [b]

a: Yates corrected P values by Chi-square test, b: Two-tailed P values by Mann-Whitney U test, c: Unpaired T-test.

During follow-up period, 92 cases showed spontaneous clearance of HCV infection and more importantly 86 of 92 cases were positive for anti-HCV antibodies in compare with 59 antibody positives among 455 cases with treatment-induced clearance of HCV infection (P<0.0001). There was not statistically difference with respect to the frequency of antibody positives between post-treatment HCV-RNA positives (17 of 110) and HCV-RNA negatives (59 of 455) (P=0.59).

Among a total of 600 cases who were diagnosed as HCV genotypes 1a (N=331) and 3a (N=269), 222 known cases were located in category of post-treatment evaluation of HCV-RNA and the remaining 378 cases were categorized as new referrals (Table 4). Out of 222 cases, 45 patients were HCV-RNA positives after initiation of antiviral therapy (30 cases with genotype 1a vs. 15 cases with 3a, P=0.06, Table 4). However, 4 of 15 cases with genotype 3a had not received any treatment. In spite of antiviral therapy, 27 of 30 cases showed recurrence of infection compared to one case in 3a genotype patients (P<0.0001, Table 4). Additionally, patients with 1a genotypes showed higher content of viral load in serum compared to the patients with type 3a although it was not statistically significant (P=0.77, Table 4).

Comparison of viral load among the patients who received drug treatment and in those without therapy revealed a significant decreased level of serum viral load in the presence of drug treatment (145530±19647 vs. 196910±11427, P=0.02). Moreover, the patients with antiviral therapy showed significant decreased levels of liver enzymes (SGOT and SGPT) in comparison to the patients without treatment (30.89±1.69 vs. 50.91±2.50, P<0.0001 and 31.60±2.14 vs. 69.68±4.70, P<0.0001 respectively).

Table 4. Differences between two main HCV genotypes in the study subjects

Parameters	HCV genotypes		
	1a (n=331)	3a (n=269)	P values
Post-treatment HCV infection (N=222)			
Positives /negatives	30 / 143	15/ 34	0.06[a]
New referrals to laboratory	158	220	<0.0001 [a]
Post-treatment HCV-RNA positives (N=45)			
With / Without treatment	30 / 0	11 / 4	0.009[b]
Recurrence of HCV infection	27	1	0.0001 [b]
Post-treatment HCV-RNA negatives (N=177)			
With / Without treatment	142 /1	33 / 1	0.34 [b]
Viral load (copies/ml)	199200±19040	195634±16531	0.77

a: Yates corrected P values by chi-square test, b: Two-tailed P values by Fisher exact test.

DISCUSSION

In the sense of HCV treatment, genotype 1 appeared to be related with a poor response, while type 2 and 3 infections have promising reactions to interferon therapy (7). Thus, determining the HCV genotypes may help the clinicians to have an appropriate scope in HCV therapy. To achieve this goal, HCV genotypes of positive patients were evaluated in the current study in Hamadan Province, West of Iran. Our study revealed that genotype 1a was the most common genotype with frequency of 52.1%, followed by genotype 3a, 1b and mixed genotype with frequencies of 42.4%, 1.1%, and 2.7%, respectively. Our findings appear to be consistent with the previous reports from Iran such as Keyvani *et al*. and Kabir *et al*. studies that showed the highest frequency for genotype 1a, followed by genotype 3a and 1b in Tehran, the capital of Iran (8, 9). Similarly, Jahanbakhsh Sefidi *et al*., found the highest rate for subtype 1a (44.9%) followed by subtype 3a (39.6%), and 1b (11.3%) (10). Moreover, a study which performed in Bushehr Province, South-West of Iran, indicated that most frequent genotypes of HCV were 1a, 3a and 1b respectively (11). Among samples in the current study, 11 (1.7%) had a non-typeable genotypes due to using commercial genotyping kit that determined only type 1, 2 and 3 as well as their subtypes based on the higher frequencies of these genotypes in Iran. In our study mixed genotypes (3a, 1a) were also detected in 0.9% of the samples which is in agreement with the study performed in Bushehr province that indicated the presence of mixed genotype (1a, 3a) in 1% of patients (11). Of note, our results are different from Keyvani *et al*. study that showed mixed infection in 1.6% of samples (8) and also with Jahanbakhsh *et al*. report that indicated mixed genotypes in 2.5% total cases (10). Observation of higher frequency for the mixed genotypes may be due to the larger sample size in above studies.

Notably, we found that only 1 (0.2%) patient with HCV genotype 2 infection. Similarly, other investigations in Iran have confirmed the absence of genotype 2 (8). However, in another study in Shiraz, South of Iran, distribution of HCV genotypes among

kidney, liver and bone marrow recipient candidates were 50% for genotype 1, 35.3% genotype 3, 2.9% genotype 2 and 2.9% genotype 4 (12). Another investigation in Tehran, also confirmed that 50% of patients were infected with HCV genotype 1a, 30.3% with 3a, 14.1% with 1b, 2.1% with 4d, 1.4% with 4a, 0.7% with 2b, and 0.7% with genotype 6a (13).

From reports made in Iran's neighboring countries, it can conclude that type 4 is the most frequent genotype in Kuwait, Iraq, Saudi Arabia and Yemen (8). Moreover, subtypes 3a and 1b are dominant in Pakistan as the eastern border country of Iran and in Turkey as western border country of Iran respectively (1, 14). Frequency of genotype 4 is rarely reported in Iran and attributed to different routes of contamination such as piercing, minor surgery, hemodialysis, and not to transfusion, sexual contacts, or intravenous drug abuse (IVDA). Nevertheless, one study showed over-representation of genotype 4 among hemodialysis patients in Tehran, Iran (15). Comparatively, genotype 4 is reported in Central and North Africa, particularly in Egypt and Middle East and western countries (16). Data from other studies demonstrate the prevalence of genotype 3a in Southeast Asian countries (7), type 1 in Brazilian patients (17) and type 1 and 3 among Belgian patients (18). In the majority parts of USA and Europe, infection with genotype 3 is found mostly in younger patients, especially in intravenous drug abusers (19). Similarly, we observed higher prevalence of type 3 among IVDA group in the current study.

It has been reported that different types and/or subtypes of HCV infection are related with geographic distributions, different transmission mode, and responses to interferon treatment (7). In our study, most of patients did not mention or aware of the probable acquisition mode. Nevertheless, some of the most high risk groups were thalassemics, hemophiliacs, multi-transfusion, and patients under hemodialysis (Tables 1 and 2). In addition, we found that 25 (2.2%) patients were intravenous drug abusers (IVDA) which seems notable for sanitation authorities in this part of country.

Totally, 92 cases showed spontaneous clearance of HCV infection and more importantly 86 of 92 cases were positive for anti-HCV antibodies compared to 59 antibody positives among 455 cases with treatment-induced clearance of HCV infection ($P<0.0001$). Survey of 144 antibody positives among 547 HCV-RNA negative cases during three years follow-up revealed that 86 of 92 cases had spontaneous clearance of HCV infection without receiving antiviral therapy. This was confirmed by serial testing during 3 years follow up of those cases. This may be attributable to differences in specific MHC class II alleles which may influence the susceptibility or resistance to HCV infection and even the strength of anti viral immunity (20-22). It has been reported that the human leukocytes antigen (HLA) molecule is implicated in the control of inhibition or development of viral diseases (23). Also, some studies have shown that the presence of some alleles such as HLA-DRB1*11 to be correlated with less severe liver disease and also virus elimination (20, 24). This issue must not be overlooked but remains to be investigated by future research in this region of Iran.

It has demonstrated that a pretreatment of HCV RNA level is helpful in the planning for proper durations of treatment and also administration of interferon dosages (7). A comparable geographical distribution of the titers of HCV RNA has not been recorded in the societies such as Iran. We found the lower but insignificant quantities of viral load in patients infected with genotypes 3a than patients with type 1a (Table 4). This is partially in line with a study that reports significant lower RNA levels in type 3 infected patients compared with infected patients with type 1. Also, this factor had been considered as an explanation for the better response to therapy (7). However, socioeconomic status of some patients and also the prisoners in the current study did not provide the opportunity for them to perform the viral load tests. This accounts as a limitation for our research. Remarkably, we found a significant decreased level of serum viral load in patients with drug consumption versus those without drug admission which seems reasonable ($P=0.02$).

With regard to the reappearance of HCV infection, 42 patients showed recurrent infection (group a) mostly belong to genotype 1a in compare with 68 cases who responded to drug treatment (group b) (Table 3). Of note, observation of non-responsiveness or responding but with recurrence of HCV infection among patients with genotype 1a in our study is in line with the reports that indicate the treatment of type 1 as a challenge which necessitates novel strategies for treatment (19, 25). Notably, higher frequency of genotype 1a and 3a were observed in group a and b respectively ($P=0.0000001$, Table 3). Expectedly,

duration of drug treatment was significantly higher in group (a) compared to the group (b) (P<0.0001, Table 3).

In conclusion, since monitoring the response to HCV treatment is critical, our findings in this study could be supportive for the view of defining proper therapeutic intervention in HCV infected subjects. As would be expected, this will help the sanitation authorities to design the novel planning in HCV therapy. Also, determining HLA alleles and other genetic variation (e.g. IL-28 gene polymorphism) seem to be vital for promising therapy. It has to be emphasized that further work is required to determine the prevalence of HCV genotypes in relation to outcome of HCV infection in other parts of Iran.

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