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Original Article

Determination of HCV Genotypes among Chronic Hepatic Patients in Ahvaz

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Abstract

Background and Aims: Chronic hepatitis C is one of the most important etiologies of cirrhosis and hepatocellular carcinoma worldwide. Clinical condition of hepatic patients and the outcome of chemotherapy are under impact of Hepatitis C virus (HCV) genotypes. Therefore, HCV genotyping is important for prediction of success of chemotherapy and progression of liver diseases.

Methods: In this article the distribution of HCV genotypes detected in a population in Ahvaz, southwest of Iran is reported. This was a study on 80 patients suffering from chronic hepatitis C. Following extraction of the viral ribonucleic acid (RNA) patient's sera, from it was used as the template for the synthesis of complementary deoxyribonucleic acid (cDNA). The amplified fragments of HCV cDNAs by nested PCR were subjected to restriction enzyme analysis. Restriction Fragment Length Polymorphism (RLFP) was performed on all HCV isolates to determine the subgenotype.

Results: Out of 80 HCV positive samples 43 (53.8%) were genotype 1a and 37 (46.2%) were genotype 3a. The dominant genotype of HCV isolated from patients suffering from chronic hepatitis C in Ahvaz was genotype 1a. Therefore, interferon therapy may not help some of these patients against HCV infection.

Conclusion: It is suggested to perform HCV genotyping prior to interferon therapy. Besides, the result suggests that the major route of HCV infection in Ahvaz population is the use of contaminated syringe.

Keywords: HCV genotype; hepatitis; RFLP

Introduction

Hepatitis C virus (HCV) is a significant etiology of chronic hepatitis and the leading cause of hepatic transplantation in the world (1). In more than 70% of the cases, infection with hepatitis C virus becomes chronic and 40% of chronic cases lead to cirrhosis or hepatocellular carcinoma (2). Liver biopsy histological assessment delivers the most reliable information concerning the degree of liver pathology (3). According to world health organization (WHO) reports, more than 3% of world population is infected with HCV (4). Prevalence of HCV in Iran is estimated about 1% which is far lower than prevalence of infection with this virus in population of neighboring countries (5).

HCV genome consists of a single-stranded RNA about 9.5 kilo base (kb). This virus was discovered in 1989 and it belongs to the family of Flaviviridae. Several genotype variations of

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the virus have been isolated. Less than 80% of the genotypes' sequences are identical (6). Sequence variation is not distributed evenly through the genome of HCV. The most conserved part of the genome is 5'-UTR. E1 and E2 regions display the most variable part of its genomic RNA (7, 8). The long single Open Reading Frame (ORF) of HCV genome is placed between 5' and 3'-UTR. The single ORF of HCV encodes a primary polyprotein. Following digestion of polyprotein by viral and cellular proteases, several structural and non-structural proteins are produced (9). Researchers have classified HCV into 6 genotypes and several subtypes (10). HCV genotyping is performed by several molecular techniques, such as sequencing of cloned genome, hybridization, Restriction Fragment Length Polymorphism (RFLP) and genotype-specific primer PCR. Since 5'-UTR of the HCV genome is highly conserved, PCR primers are designed for this region. The amplified fragment is subjected to restriction enzyme digestion for RFLP determination. In addition to 5'-UTR, core, E1 and NS5B regions of the genome have been used by researchers for genotyping of this virus (9).

The gold standard method for HCV genotyping is sequencing but this technique is expensive and requires many equipments and facilities, therefore not formidable in most regional laboratories. Contrary, RFLP is a sensitive and cost-effective method especially when a numerous amount of samples need to be genotyped. In RFLP, part of 5'-UTR is amplified by PCR and the amplicon is digested by restriction enzymes. The genotype of the HCV is determined based on the pattern of the fragments following digestion (11). In this paper, genotyping of HCV isolated from hepatic patients was performed by PCR-RFLP (12).

Methods

This was a cross-sectional study including 80 cases, 65 males and 15 females suffering from chronic hepatitis C infection. The patients were attended at Imam Khomeini Hospital. This hospital is a referral hospital in Ahvaz city for hepatic patients. The collected bloods from the patients were transferred to the virology department of our university, centrifuged and the sera were kept at -70 ºC till RNA extraction.

HCV RNA extraction

HCV RNA was extracted from 250 µl of each serum by guanidinium isothiocyanate method as described (13). Briefly, the RNA is precipitated by isopropanol and following a wash with 70% ethanol, the pellet of RNA is dissolved in 30 µl diethyl pyrocarbonate (DEPC) water.

cDNA synthesis

Five microliter of the extracted RNA was used as the template for synthesis of cDNA by QIAGEN Sensiscript RT kit. In a microtube, 2 µl 10x RT buffer, 2 µl 5mM dNTPs, 2 µl 10mM random primer, 1 µl 10U/µl RNase inhibitor were added. The final volume of RT reaction was adjusted to 20 µl by DEPC water. The reaction tube was incubated at 37 ºC for 1 hour and the product was kept at -20 ºC.

Nested-PCR

The sequences of external and internal PCR primers specific for 5'-UTR region of HCV genome have already been published by researchers (12). The sequence were follow:

ExF; 5'-AGCGTCTAGCCATGGCGT-3'
ExR; 5'-GCACGGTCTACGAGACCT-3'
InF; 5'-GTGTCTGCGGAACCGG-3'
InR; 5'-GGGCACTCGCAAGCACCC-3'.

The first round of PCR reaction mixture contained 5 µl cDNA as template, 5 µl 10x PCR buffer (Roche, Germany), 1 µl 10mM dNTPs, 1 µl of each external primers and 0.3 µl *Taq* DNA polymerase. The final volume was adjusted to 50 µl with DEPC water. Thermocycler machine (Techne, TC-512 USA) was programmed as: 94 ºC, 5 minutes followed by 30 cycles of denaturation temperature 94 ºC, 35 seconds, annealing temperature 58 ºC, 40 second and extension Tm 72 ºC, 45 second and 5 minutes at 72 ºC for final extension.

For nested PCR, 3 µl of the first round PCR product was used as template and 1 µl of each internal primer was added to the reaction. Other PCR components were similar to the first round. The volume was adjusted to 50 µl by DEPC water. Apart from annealing Tm

which was 64 °C for 40 seconds, other steps of nested-PCR program were similar to the first round. Finally 8 µl of PCR product was loaded onto 2% agarose gel, electrophoresed, stained with ethidium bromide and visualized under UV transilluminator (VILBERT LAOURMAT France). The product size was 174 bp.

HCV genotyping

To do RLFP of HCV, 2 pairs of restriction enzymes, Hinf I/ScrF I, Hinf I/MvaI and Bsh1236 I, were used. Each nested-PCR products were divided in 3 1.5 ml microtubes and 4 units of the restriction enzymes were added to the assigned tube (12). Following incubation at 37 °C for 2.5 hours, digested products were loaded onto 3% agarose gel. Ethidium bromide staining visualized the pattern under UV.

Results

Nested-PCR

Patients were referred to Imam Khomeini hospital in Ahvaz city by specialists and were suspected of chronic hepatitis C. Sixty five (81.25%) subjects were male and 15 (18.75%) were female. The mean age of patients was 40 years (age range of 18-66 years old).

Primarily, they were screened for anti-HCV antibody. Sera samples from 80 patients were positive for anti-HCV antibody and all of them also were showed positive by nested-PCR. The product size of 174 bp was detected in the positive cases for nested-PCR.

Table 1: RFLP pattern of HCV genotypes after digestion of PCR products with restriction enzymes.

HCV genotypes	Sizes of Fragments		
	Tube A (ScrF I and Hinf I)	**Tube B** (Mva I and Hinf I)	**Tube C** (Bsh1236 I)
1a	97 bp	97 bp	129 bp
1b	97 bp	97 bp	99 bp
2a	97 bp	174 bp	174 bp
2b	174 bp	174 bp	174 bp
3a	129 bp	145 bp	99 bp
3b	97 bp	145 bp	99 bp
4	97 bp	145 bp	129 bp
5	97 bp	174 bp	99 bp
6	97 bp	97 bp	174 bp

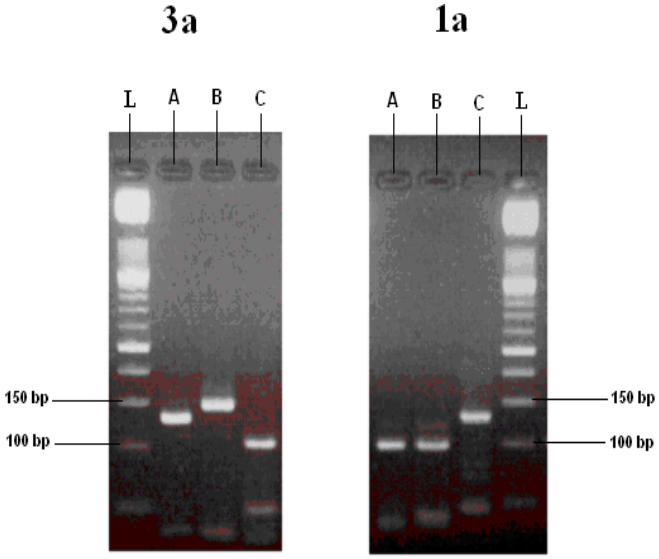

Fig.1: RLFP patterns of genotypes of 1a and 3a.
1a → A=97 bp, B=97 bp, C=129 bp.
3a → A=129 bp, B=145 bp, C=99 bp.
(L) Roche (Germany) DNA Molecular Weight Marker XIII (50 bp ladder).

HCV genotyping

The HCV genotypes were determined in all 80 patients based on PCR-RFLP method.

The genotypes of 43 (53.8%) cases were 1a. While the rest 37 specimens (46.2%), were genotype 3a of HCV based on their RFLP pattern.

In the case of genotype 1a, the sizes of fragments were 97 bp, 97 bp and 129 bp, and the sizes of fragments for the case of genotype 3a were 129 bp, 145 bp and 99 bp. Table 1 shows the sizes of each fragment following restriction enzyme digestion of HCV nested-PCR product (12).

Thirty four (52.3%) patients with genotype 1a and 31 (47.7%) patients with genotype 3a were

Table 2: Distribution of HCV genotypes among male and female.

Sex	No	Genotype 1a	Genotype 3a
Male	65	34 (52.3 %)	31 (47.7 %)
Female	15	9 (60 %)	6 (40 %)

Table 3: Distribution of HCV genotypes in relation to age.

Age	No	Genotype 1a	Genotype 3a
<40	20	11 (55%)	9 (45%)
40-60	53	28 (52.84%)	25 (47.16%)
>60	7	4 (57.14%)	3 (42.86%)

male while 9 (60%) patients with genotype 1a and 6 (40%) patients with genotype 3a were female. Table 2 shows the distribution of HCV genotypes among males and females.

Twenty (25%), 53 (66.25%) and 7 (8.25%) patients were <40, 40-60 and >60 years old, respectively. Table 3 compares the distribution of HCV genotypes in relation to age.

The dominant genotype of HCV in three ranges of age, male and female was similarly 1a.

Figure 1 shows RFLP of the digested products on agarose gel. The pattern of genotypes of 1a and 3a are visible.

Discussion

Different genotypes of HCV demonstrate different geographical distribution, besides, the severity of HCV hepatitis and the outcome of chemotherapy against HCV genotypes is not similar. For these reasons, determination of HCV genotypes is of great importance. HCV genotyping can be performed with several molecular techniques. According to Furione *et al* the sensitivity of RFLP for genotyping of HCV is 96.2 % (11). This level of sensitivity of RFLP is very close to sequencing technique for determination of HCV genotypes (12). Therefore we decided to use RLFP for genotyping of HCV in chronic hepatic patients in Ahvaz, southwest of Iran.

The results of our study showed that the dominant genotype of HCV in Ahvaz was 1a. This result is consistent with the results of other researchers reported from other parts of our country. Ahmadi-Pour, Samimi-Rad and Zali determined the genotypes of HCV in Iran by RFLP (14-16). Despite of the HCV genotype 1a prevalence in Iran, the prevalent HCV from Iran neighboring countries like Iraq, Kuwait, Saudi Arabia and Yemen has been reported genotype 4 (17). HCV genotype 1 is the most resistant genotype to interferon (1b is the most resistant then 1a) (18, 19). Therefore, interferon therapy will not help some of these patients against HCV infection and HCV genotyping is recommended prior to commencing interferon therapy.

The route of infection with genotypes of HCV varies slightly, e.g. genotypes 1b and 2a spread through contaminated blood and blood products. Our blood transfusion centers screen the donated bloods for HCV. We believe that genotypes 1b and 2a are not rampant in our area and in this study we could not found either of these genotypes. The major route of infection with genotypes 1a and 3a is application of shared contaminated syringe of HCV by addicts. Unfortunately the frequency of these genotypes is elevating in population in recent years (9, 20). According to the results of this report, possibly the major route of distribution of HCV in Ahvaz population is contaminated syringe.

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References

1. Arens M. Clinically relevant sequence-based genotyping of HBV, HCV, CMV, and HIV. J Clin Virol. 2001;22(1):11-29.

2. [International consensus conference on hepatitis C. Conclusions. The European Association for the Study of the Liver (EASL) Paris, February 26-28, 1999]. Gastroenterol Clin Biol. 1999;23(6-7):730-5.

3. H LA, Simon K, Gladysz A. Clinical and epidemiological assessment of hepatitis C virus

infection among voluntary blood donors. Med Sci Monit. 2002 Aug;8(8):CR591-6.
4. Hepatitis C--global prevalence (update). Wkly Epidemiol Rec. 1999;74(49):425-7.
5. Alavian SM, Gholami B, Masarrat S. Hepatitis C risk factors in Iranian volunteer blood donors: a case-control study. J Gastroenterol Hepatol. 2002;17(10):1092-7.
6. Simmonds P. Variability of hepatitis C virus. Hepatology. 1995;21(2):570-83.
7. Bukh J, Purcell RH, Miller RH. At least 12 genotypes of hepatitis C virus predicted by sequence analysis of the putative E1 gene of isolates collected worldwide. Proc Natl Acad Sci U S A. 1993;90(17):8234-8.
8. Chan SW, McOmish F, Holmes EC, Dow B, Peutherer JF, Follett E, et al. Analysis of a new hepatitis C virus type and its phylogenetic relationship to existing variants. J Gen Virol. 1992;73 (Pt 5):1131-41.
9. Zein NN. Clinical significance of hepatitis C virus genotypes. Clin Microbiol Rev. 2000;13(2):223-35.
10. Tokita H, Okamoto H, Luengrojanakul P, Vareesangthip K, Chainuvati T, Iizuka H, et al. Hepatitis C virus variants from Thailand classifiable into five novel genotypes in the sixth (6b), seventh (7c, 7d) and ninth (9b, 9c) major genetic groups. J Gen Virol. 1995;76 (Pt 9):2329-35.
11. Furione M, Simoncini L, Gatti M, Baldanti F, Grazia Revello M, Gerna G. HCV genotyping by three methods: analysis of discordant results based on sequencing. J Clin Virol. 1999;13(3):121-30.
12. Pohjanpelto P, Lappalainen M, Widell A, Asikainen K, Paunio M. Hepatitis C genotypes in Finland determined by RFLP. Clin Diagn Virol. 1996;7(1):7-16.
13. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem. 1987;162(1):156-9.
14. Ahmadipour MH, Keivani H, Sabahi F, Alavian SM. Determination of HCV Genotypes,in Iran by PCR-RFLP. Iranian J Publ Health. 2006;35:54-61.
15. Samimi-Rad K, Nategh R, Malekzadeh R, Norder H, Magnius L. Molecular epidemiology of hepatitis C virus in Iran as reflected by phylogenetic analysis of the NS5B region. J Med Virol. 2004;74(2):246-52.
16. Zali MR, Mayumi M, Raoufi M, Nowroozi A. Hepatitis C virus genotypes in the Islamic Republic of Iran: a preliminary study. East Mediterr Health J. 2000 Mar-;6(2-3):372-7.
17. Simmonds P, Holmes EC, Cha TA, Chan SW, McOmish F, Irvine B, et al. Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. J Gen Virol. 1993;74 (Pt 11):2391-9.
18. Poynard T, Klepping C, Audigier JC, Barthelemy C, Bel A, Delmont J, et al. Randomized double-blind clinical trial of aluminum phosphate versus ranitidine in the acute treatment of duodenal ulcer. Digestion. 1990;47(2):105-10.
19. Martinot-Peignoux M, Marcellin P, Pouteau M, Castelnau C, Boyer N, Poliquin M, et al. Pretreatment serum hepatitis C virus RNA levels and hepatitis C virus genotype are the main and independent prognostic factors of sustained response to interferon alfa therapy in chronic hepatitis C. Hepatology. 1995;22(4 Pt 1):1050-6.
20. Pybus OG, Charleston MA, Gupta S, Rambaut A, Holmes EC, Harvey PH. The epidemic behavior of the hepatitis C virus. Science. 2001;292(5525):2323-5.