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Article *in* Archives of Virology · June 2012

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Molecular epidemiology of hepatitis C virus among injection drug users in Iran: a slight change in prevalence of HCV genotypes over time

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Received: 13 February 2012 / Accepted: 24 April 2012

Abstract Injecting drug users (IDUs) are the main at-risk population for hepatitis C virus (HCV) transmission. We studied HCV infection, risk factors, and genotype distribution in relation to the year of first injection among Iranian IDUs. Of a total of 126 specimens positive for HCV antibody, 93 (74 %) had detectible HCV RNA, and the NS5B gene was sequenced for 83, with genotype 3a (n = 48, 58 %) being predominant, followed by 1a (n = 35, 42 %). Tattooing was an independent predictor for HCV infection. No significant difference was found between HCV genotypes and IDU characteristics. Although there was no change in the distribution of prevalent genotypes before and after 1997, a slight variation in the prevalence was observed ($p = 0.71$). The difference in the prevalence of subtypes 1a and 3a (9.1 % in the period 1984-1996 and 18.2 % in the period 1997-2009) during 25 years was 9.1 %. These findings indicate a high prevalence of HCV infection among Iranian IDUs and highlights HCV-3a as the most prevalent subtype for the past 25 years. Harm-reduction strategies appear to be the most important measures to reduce the transmission of HCV in Iran.

Blood transfusion safety and better health care conditions have changed the HCV epidemiology in Iran. Systematic screening of blood samples for HCV since 1996 has led to a significant decrease in HCV transmission through blood transfusion [1]. Similar to other developed countries, injection drug use has become the major route of new HCV infection in Iran. Recent studies on the Iranian injecting drug users (IDUs) report a high prevalence of HCV

GenBank accession numbers: Partial sequences of the NS5B gene of hepatitis C virus obtained in this study were deposited in GenBank under the accession numbers JQ433573 to JQ433655.
* The release date of these sequence data to the public database is on 01 March 2012.

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Published online: 13 June 2012

infection, ranging from 50 to 75 % depending on the area of the study, and also a significant increase in HCV infection over the last decade [2, 3]. Reports from different regions in Iran show an HCV antibody prevalence rate ranging from 31.5 to 88.9 %, with a median of 60 % [2–10] among IDUs.

There is typically an association between the genotype and HCV transmission route. The predominant HCV subtype among IDUs in Europe is 3a, whereas 1b is common in patients who have acquired the virus through blood transfusion [11, 12]. In Iran, subtype 1a is the most prevalent in transfusion-related risk groups, such as hemodialysis, thalassemia, and hemophilia patients [13–15]. The data available on the molecular epidemiology of HCV among Iranian IDUs is scarce [16]. Additional studies in this area could help in finding the right treatment for the IDU population as well as predicting changes of genotype frequency and distribution in the general Iranian population mediated through IDUs.

In this cross-sectional study, we describe the HCV genotype distribution, genotype variation in relation to the time when injection started, and the risk factors behind the HCV epidemic among IDUs in Iran. Moreover, phylogenetic relationships between detected HCV sequences were analyzed.

A total of 126 serum samples were collected from anti-HCV-positive IDUs registered at the hepatitis clinic of the Imam Khomeini Hospital and a methadone clinic at West Health Center in the capital city of Tehran between October 2008 and June 2009.

The sample size calculation was based on the estimated prevalence of HCV RNA of 80 % (16) among IDUs and an acceptable error, d = 7 %. Thus, a sample size of 125 IDUs was calculated to be appropriate for this study.

The study subjects were divided as either short- (ST) or long-term IDUs (LT). ST (n = 68) and LT (n = 58) were defined as those who have injected drugs for $\leq$5 and >5 years, respectively, with a mean length of injection of 3.3 ± 1.3 vs. 11.34 ± 5.4 years.

Among the participants, 61(48 %) were ex-IDUs (former drug users) and the remaining 65 (52 %) were current IDUs. Current IDUs were defined as individuals who injected drugs at the time of interview, and ex-IDUs were defined as those who had injected drugs for at least two years, but not in the last one year. The research protocol was approved by TUMS Ethics Committee as well as the Committee for Research on Infectious Diseases at West Health Center. Participation in this study was voluntary, and a signed informed consent was obtained from each participant.

All of the subjects were interviewed, using a questionnaire on demographic information, at-risk behaviors, and legal problems. HCV RNA was extracted from 140 µl of each serum sample using a QIAamp Viral RNA Min Kit (QIAGEN, Valencia, CA) following the manufacturer's instructions. HCV RNA sequences were amplified targeting 377 bases of the NS5B region using a QIAGEN One Step RT-PCR Kit. After purification of the PCR products with a PCR Purification Kit (QIAGEN), NS5B amplicons were sequenced directly (Eurofins MWG Operon, Germany). Nucleotide sequences were aligned by using the Muscle program. Mega software (version 5; available at http://www.megasoftware.net) was used to construct the phylogenetic trees by the UPGMA method based on Kimura 2-parameter distances. Average evolutionary distances were estimated by the Kimura 2-parameter method [17] and used to analyze the relationships both between the two subtypes 1a and 3a and within each subtype of the strains isolated from the participating IDUs. Associations between possible risk factors and HCV infection and genotypes were assessed by either χ^2 or Fisher's exact test where appropriate. Statistical analysis was performed using the SPSS software (version 11.5). A p-value lower than 0.05 was considered to be significant.

The mean age of the 126 participants was 34.4 ± 10.1 years, ranging from 19 to 65 years old. One third of the patients were under the age of 30, and the highest rate of IDUs (44 %) was seen in the 30- to 40-year age group. They included 124 males and 2 females. Among the participants, 37 % had been married, 69 % reported a history of syringe sharing, 60 % had been tattooed (43 % of them in prison), and 79 % had a history of incarceration (26.2 %, one or two times; and 52.4 %, three times or more). About 77 % of the subjects had started injection drug use in the last 10 years (1997-2007) compared to only 23 % of those in the period before 1997. The difference was statistically significant ($p = 0.001$). The vast majority (93 %) of the study subjects were non-injecting drug users during their first years of addiction. They resorted to injection 6.1 ± 4.7 years after starting drug use. The mean age at first drug use and at first injection of the IDUs was 18 ± 5.1 and 24.03 ± 6.1, respectively.

HCV RNA was detected in 74 % (93/126) of the samples that were positive for HCV antibody. Eighty-three of the 85 samples that were positive by PCR for NS5B were sequenced in this region. The remaining eight samples were positive in the 5′-UTR. Subtype 3a was predominant (n = 48, 58 %), followed by subtype 1a (n = 35, 42 %). Of the injectors who were RNA positive, 33.3 % (31/93) had no history of sharing syringes and 75.8 % (25/33) of subjects who were RNA negative, had a history of sharing syringe. About one third (33/99) of the IDUs with a history of incarceration had injected drugs while in prison.

The HCV RNA status was significantly associated with tattooing ($p = 0.04$). No significant relationship was found between demographic and other drug-use-associated risk factors with HCV infection (Table 1).

Table 1 Characteristics of the IDUs and HCV prevalence

	N (%)	HCV RNA positive (%)	*p*-value
Study participants	126 (100)	93 (73.8)	
Sex			
Male	124 (98.4)	91 (97.8)	0.39
Female	2 (1.6)	2 (2.2)	
Age			
<30 years	39 (31)	27 (29)	0.55
30-40 years	56 (44.4)	41 (44.1)	
>40 years	31 (24.6)	25 (26.9)	
Marital status			
Single	58 (46)	43 (46.2)	0.96
Married	47 (37.3)	35 (37.6)	
Divorced	21 (16.7)	15 (16.1)	
Mean age (years)	34.4 ± 10	34.59 ± 10	0.86
Needle-sharing			
Yes	87 (69)	62 (66.7)	0.38
No	39 (31)	31 (33.3)	
History of incarceration			
Never	27 (21.4)	18 (19.4)	0.56
One or two times	33 (26.2)	24 (25.8)	
Three times or more	66 (52.4)	51 (54.8)	
Injection drug use in prison			
Yes	33 (33.3)	24 (25.8)	0.61
No	66 (66.7)	51 (54.8)	
History of tattooing			
Yes	76 (60.3)	61 (65.6)	0.04*
No	50 (39.7)	32 (34.4)	
Duration of injection drug use			
≤5 years	68 (54)	50 (53.8)	0.93
>5 years	58 (46)	43(46.2)	
Age at first injection			
≤25 years	65 (51.6)	50 (53.8)	0.41
>25 years	61 (48.4)	43 (46.2)	
Main mode drug use			
Injecting	9 (7.1)	6 (6.5)	0.61
Injecting & non-injecting	117 (92.9)	87 (93.5)	
Mean duration of drug injection	8.1 ± 5.8	8.4 ± 5.4	0.88
Mean age at first injection	24.03 ± 6.1	24.2 ± 8.2	0.46

Note that after the first row, the percentages shown in columns 2 and 3 are calculated based on the total for the same column. For example: (91/93) × 100 = 97.8 %

* Statistically significant

Table 2 Characteristics of HCV-RNA-positive IDUs according to genotype (n = 83)

Variables	HCV genotype		*p*-value
	1a (n = 35) N (%)	3a (n = 48) N (%)	
Age			
≤40 years	24 (38.7)	38 (61.2)	0.386
>40 years	11 (52.3)	10 (47.6)	
Year injection started			
<1997[a]	10 (45.4)	12 (54.5)	0.71
>1997[b]	25 (40.9)	36 (59.1)	
History of incarceration			
Never	6 (40)	9 (60)	0.864
One or two times	8 (38.0)	13 (61.9)	
Three times or more	21 (44.6)	26 (55.3)	
Study group			
Ex-IDUs	20 (46.5)	23 (53.5)	0.309
Current IDUs	15 (37.5)	25 (62.5)	
Short-term IDUs	17 (39.5)	26 (60.4)	0.805
Long-term IDUs	18 (45.0)	22 (55.0)	
Mean age	35.2 ± 10.5	34.3 ± 9.6	0.369
Mean age at first injection	24.6 ± 8.7	23.9 ± 7.6	0.545

Note that the percentages for each subtype are calculated based on the sum of both subtypes for each row. For example: [24/(24 + 38)] × 100 = 38.7 %

[a] The period 1984-1996

[b] The period 1997-2009

The association of the genotype and characteristics related to HCV-RNA-positive IDUs is shown in Table 2. In contrast to subtype 1a (≤40 years, 38.7 %; >40 years, 52.3 %), the prevalence of subtype 3a was higher among IDUs under 40 years old (61.2 %) compared to those older than 40 (47.6 %). There was no significant relationship between the prevalence of subtype 1a and 3a and the risk factors related to the HCV-RNA positive IDUs ($p > 0.05$) (Table 2).

The difference in the prevalence of subtypes 1a and 3a among ex-IDUs (1a, 46.5 %; 3a, 53.5 %), LT- IDUs (1a, 45 %; 3a, 55 %), and those who started injection before 1997 (1a, 45.4 %; 3a, 54.5 %) was 7 %, 10 %, and 9.1 %, respectively. This difference was higher among current IDUs (25 %; 1a, 37.5 %; 3a, 62.5 %), ST-IDUs (20.9 %; 1a, 39.5 %; 3a, 60.4 %), and those who started injection after 1997 (18.2 %; 1a, 40.9 %; 3a, 59.1 %) (Table 2). Phylogenetic trees of NS5B sequences obtained in this study with reference sequences from GenBank and sequences from other Iranian risk groups are shown in Fig. 1a, b. Phylogenetic analysis indicates a similar clustering pattern for the strains within subtypes 1a and 3a. Three small clusters were formed by six subtype 1a sequences isolated from the participants (Fig. 1a) Seven strains generated from IDUs in this study formed seven clusters with strains from IDUs and non-IDUs published in a previous work [16]. Overall, 15 of the subtype 1a strains were not closely related to the other strains. Twenty-seven

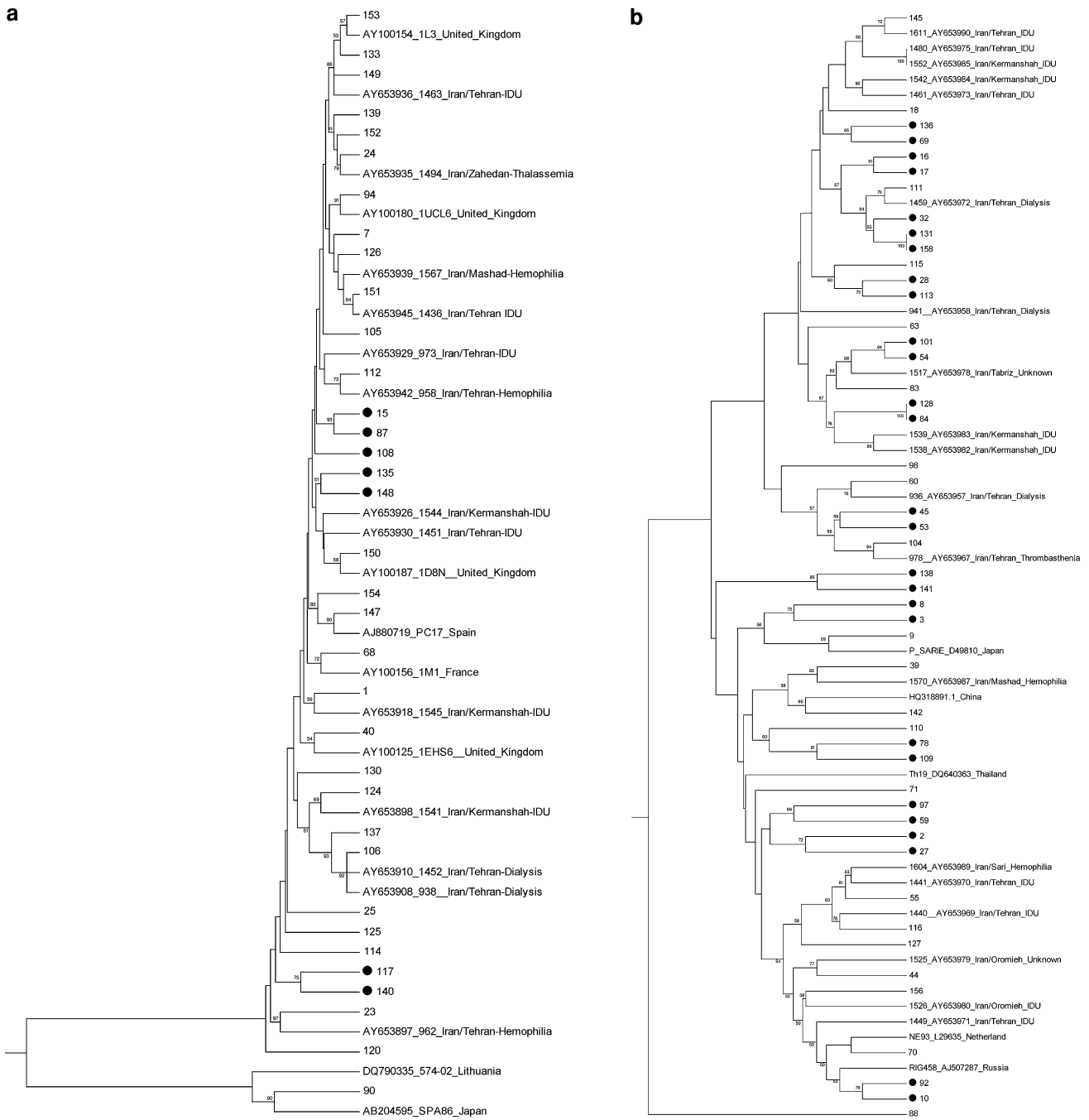

Fig. 1 Phylogenetic trees of HCV strains obtained by UPGMA based on 377 nucleotides within the NS5B region of 128 sequences representing (**a**) the sequences of subtype 1a strains and (**b**) the sequences of subtype 3a strains. The designation and origin of the strains obtained from GenBank are given at the nodes. Bootstrap values (1000 replicates) are given at branch points. The six subtype 1a and 27 subtype 3a sequences (isolated from IDUs in this study), which formed 3 and 13 clusters, respectively, are indicated by black circles

subtype 3a sequences isolated from IDUs in this study formed 13 small clusters, while eight strains formed eight clusters with previously published Iranian sequences from IDUs and non-IDUs (Fig. 1b) [16]. Two patients (131 and 158) who had identical strains were brothers and had shared needles. The two other identical strains, 84 and 128, were from IDUs who knew each other. Ten HCV-3a strains were distantly related to other strains. The remaining subtype 1a and 3a strains were intermixed with sequences of European and Asian origin. The average evolutionary distances obtained from Mega 5.0 with subtype 1a and 3a sequences were 0.064 and 0.074, respectively.

The present study provides important findings about the major HCV genotypes circulating among IDUs, disease frequency, and the potential route of HCV transmission in Iran. The predominant subtype, in terms of genotype, was 3a (58 %), followed by 1a (42 %). This is in agreement with the reports from Western European studies [11, 18], and it is also in line with previous work on a limited number (n = 39) of IDUs from several regions of Iran, with subtype 3a being the most prevalent type (54 %), followed by 1a (38 %) and 1b (8 %) [16]. However, the distribution and prevalence of subtypes among IDUs are different from those in Iranian non-IDUs, in which subtype 1a (50 %) is the most prevalent, followed by subtypes 3a (30.4 %), 1b (8.5 %), 2b (1.2 %) and 4 (9.7 %) [16, 19]. Considering the fact that injection of drugs is the only difference between IDUs and non-IDUs, these findings emphasize the importance of injection drug use in the spread of subtype 3a among the Iranian IDUs.

The finding that 74 % of the participants were positive for HCV RNA ranks as one of the highest HCV prevalences observed among IDUs worldwide [20, 21]. Taken together with the high prevalence of HCV antibody and the significant increase in the rate of injection drug use in the last decade ($p = 0.001$) [2, 3], this is predictive of an accelerating high rate of HCV infection among Iranian IDUs.

The high rate of HCV RNA among IDUs in this study (74 %) is similar to the rates observed in patients infected due to receiving contaminated blood or blood products, as in the case of hemophilia and thalassemia (82 %) and dialysis patients (71 %) [16].

However, the higher prevalence of HCV antibody among IDUs (median 60 %) in comparison with the transfusion-related risk groups such as in hemophilia (42.5 %), thalassemia (16 %), and dialysis patients (5 %) [1, 22] shows that the rate of HCV infection is higher among IDUs.

In this study, we found tattooing to be an independent variable in HCV infection. Although this is the first study from Iran that reports a significant association between tattooing and HCV RNA positivity, there are several studies from different regions of Iran [3, 5, 8, 10, 23] that indicate that tattooing is an independent risk factor for HCV antibody positivity among IDUs. Tattooing is common among Iranian IDUs because most of them are unaware that it is an important route of HCV transmission. Thus, without any preventive measures, they get tattooed by their IDU friends or prisoners while they are in prison. The higher risk of HCV transmission when tattooing is practiced inside prison has been reported in an earlier study from Iran [5]. Our data indicate that the rate of IDUs has significantly increased since 1997 compared to the period before. This trend has led to an increase of HCV infection among Iranian drug addicts. A recent study has demonstrated a highly significant increase in anti-HCV antibody prevalence from 47 % to 80 % over the last decade among Iranian IDUs [3]. The finding that 33 % of the injectors who were HCV RNA positive had no history of syringe sharing indicates that other risk factors are also involved in infection of IDUs with HCV. Possibly, a percentage of these injectors hid the fact or did not remember that they had shared syringes. Furthermore, we observed that 75.6 % of the subjects who were HCV RNA negative had a history of syringe sharing. The IDUs in this group might either have had shared syringes only a few times or knew that those with whom they shared syringes were HCV negative. Demetriou et al. also found no significant differences between HCV- positive and HCV-negative groups with regard to having shared syringes [21].

We found no significant association between the history of imprisonment and HCV infection. This differs from that observed by Hickman et al. [24]. One of the possible explanations for failure to find such association could be the low rate (33 %) of injection drug use in prison by IDUs. There are two possible reasons for this low rate: (1) there is a lack of disposable syringes in prison, and most IDUs are aware of the possibility of being infected with HCV by syringe sharing; and (2) 93 % of the study IDUs has used both modes of IDU and non-IDU during their drug addiction. Therefore, they could remain drug addicts through the mode of non-IDU while they were in prison.

The higher prevalence of subtype 3a among IDUs under 40 years old (61.2 %) compared to those older than 40 years (47.6 %) suggests that, in comparison with the past, subtype 3a has been spread at a higher rate in recent years among younger IDUs. The slight variation in the frequency of subtypes 1a and 3a both prior to and after 1997, between ex-IDUs and current IDUs, and also LT vs. ST groups shows a gradual decrease in the prevalence of subtype 1a and an increase in that of 3a over time. This differs from reports indicating a significant variation in the frequency of HCV genotypes over time [11, 25, 26]. Based on some previous reports from Iran, subtype 1a predominated in groups receiving blood transfusion [13–15]. We predict a gradual increase in the prevalence of subtype 3a, and at the same time, a decrease in subtype 1a in the future. This change could be attributed to a combination of at least three factors: (1) a marked decline in HCV transmission through blood transfusion as a result of routine screening, (2) a continuous increase of IDU cases, and (3) HCV-3a being the most prevalent subtype among the Iranian IDUs.

The lower average evolutionary distance among the subtype 1a sequences indicates a high degree of sequence similarity in this subtype relative to subtype 3a strains in Iranian IDUs. The trend toward greater diversity for genotype 3a in comparison with genotype 1a could be

explained by a higher efficiency of subtype 3a transmission among IDUs. Phylogenetic trees indicate that neither of these two subtypes from IDUs appears to be genetically distinct from those isolated from members of blood-transfusion-related risk groups like thalassemia, hemodialysis, and hemophilia patients. The dispersal of the sequences from transfusion-related risk groups among sequences from IDUs implies a likelihood of HCV transmission between non-drug users and IDUs. Because more than 80 % of the Iranian blood recipients are infected with the subtypes 1a and 3a, it is possible that multiple introductions have generated the related epidemics. This suggests a common origin for each HCV subtype 1a and 3a epidemic strains in Iran. Phylogenetic mixing between sequences from the cities of Kermanshah and Oromieh with those from Tehran also implies that either HCV transmission between IDU communities originated from different geographical areas or that introduction of similar lineages of the virus into different cities stemmed from an outside source. Observation of clusters formed from identical or similar strains from IDUs indicates that a local transmission among injectors from the same geographical area has occurred. In conclusion, our findings of a high prevalence of HCV among Iranian IDUs indicates the need for devising effective intervention strategies to prevent the spread of HCV infection in Iran.

Acknowledgments We would like to thank Dr. Ali Malakzadeh and his colleagues at West Health Center for their cooperation in sample collection. We thank Dr. Mehrdad Pedram (Dept. of Biotechnology and Medical Genetics, Zanjan University of Medical Sciences) for his careful editing of the manuscript and helpful comments.

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