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Research Article

Association of CRP gene polymorphism with CRP levels and Coronary Artery Disease in Type 2 Diabetes in Ahvaz, southwest of Iran

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Abstract

Introduction: We evaluated the association between four polymorphisms in the CRP gene with serum C-reactive protein (CRP) levels, prevalence and severity of coronary artery disease (CAD) in type 2 diabetes mellitus (T2DM) patients.

Methods: We performed coronary angiography for 308 T2DM patients and classified them into two groups: T2DM with CAD and T2DM without CAD. All patients were from Ahvaz, Iran. serum levels of CRP, glucose and lipid profile were measured. Genotyping was performed by PCR/RFLP, and the severity of coronary artery disease was determined by Gensini score.

Results: The GG genotype of SNP rs279421 was associated with the increased risk of CAD (OR= 2.38; 95% CI: 1.12- 5.8; p= 0.02) and CA, TT, TA genotypes and A allele of SNP rs3091244 and GA genotypes and A allele of SNP rs3093062 were significantly associated with increased CRP levels. None of genotypes or alleles was associated with Gensini score. We found that the haplotype 7 (AGCG) was associated with decreased risk of CAD (OR= 0.11; 95% CI: 0.02, 0.66; p= 0.017) and the Gensini score was correlated with increased levels of CRP, only in CAD group.

Conclusion: Although genetic polymorphisms were influenced on serum RP levels, none of the alleles and genotypes raising or falling C-reactive protein levels was consistently associated with an increased prevalence of CAD or protected from that.

Introduction

Type 2 diabetes mellitus (T2DM) represents a significant global health problem.[1] Coronary artery disease (CAD) is one of the major complications associated with T2DM. More than 50% of individuals with T2DM have coronary heart disease, stroke, or cardiac disease.[2] Hyperglycemia has been shown to be a necessary but not sufficient condition for the development of these complications. Genetic differences between diabetic patients might play an important role in determining why some diabetic patients develop these complications while others do not.[3] Considerable evidence has shown the importance of inflammation in the pathogenesis of diabetic complications, especially enhancement of atherosclerosis.[4] Both T2DM and atherosclerosis are multifactorial conditions which appear to share a common inflammatory basis.[5] Inflammation plays a key role in the pathogenesis of CAD at every stage from initiation to progression and rupture of the atherosclerotic plaque.[6]

C-reactive protein (CRP), an inflammatory biomarker, is one of the most well-documented emerging CAD risk factors.[7] CRP is produced and released by the liver under the stimulation of cytokines such as tumor necrosis factor-α and interleukin 6. Human CRP is a highly conserved protein belonging to the pentraxin family of acute phase reactants. Normal baseline serum CRP levels are ≤1 mg/L in healthy individuals,[8] but its levels are elevated in people with T2DM.[4] However, serum CRP levels can rise to 1000-fold in response to tissue injury or inflammation.[9]

In several studies, an association between high CRP levels and atherosclerosis, increased coronary heart disease (CHD),[10,11] and increased carotid intima media thickness (CIMT),[12] an indirect marker of atherosclerosis, has been reported. There is a suggestion that the association of CRP with cardiovascular disease (CVD) is modified by diabetes status;[13] however, few such studies exist. Environmental factors such as age, gender, smoking, lipid

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levels, hypertension and body mass index (BMI) have been shown to influence baseline serum CRP levels.[14]

There are also several studies which have reported an association between single nucleotide polymorphisms (SNPs) in the CRP gene, especially in the promoter region, with variation in blood levels of CRP, or with CHD events.[15-17] In addition, evidence from *in vitro* and clinical studies suggest that C-reactive protein might also be implicated in the pathophysiology of atherogenesis, which, however, still represents a controversial issue.

Scarce data exist concerning CRP polymorphisms in diabetic patients. There is conflicting evidence as to the relationship of CIMT with serum CRP levels in T2DM.[18,19] To date, however, relatively few studies have examined the associations of common variants in the promoter region of CRP gene with its serum hsCRP concentrations in type 2 diabetic patients. Based on these prior studies we hypothesized that if the CRP genotype is associated with CAD in general population, then a stronger association would likely be observed with T2DM-affected populations. In the present study, we aimed to compare the frequency of four common SNPs of CRP gene which is located in the promoter region in type 2 diabetic patients with and without CAD. These SNPs included: -757T>C(rs3093059), -717A>G(rs2794521), -409G>A(rs3093062) and -286C>T>A(rs3091244). We also examined the influence of these genetic variations at CRP SNPs on serum CRP levels and lastly investigated the correlation between serum CRP levels with presence and severity of CAD in these patient groups using Gensini score, as an atherosclerosis severity marker.

Materials and methods

Study population

We evaluated 853 T2DM patients, between 40 and 60 years of age, genetically unrelated, and with ancestry in the state of Khozestan, Iran. All patients performed carotid angiography at the Department of Cardiology, Imam Khomeini Hospital, JondiShapour University of Medical Sciences, Ahvaz, Iran.

On the basis of angiography results, patients were classified into two groups: CAD and non-CAD. Current smoking, diagnosed MI in recent 3 months, body mass index>30, and treatment for inflammatory or chronic infectious disease or malignancy were exclusion criteria. Individuals with CRP values above 10 mg/ml were excluded from the analyses, due to the possibility of an acute infection. For every participant, baseline information was taught by trained research assistants and included questionnaires related to social and medical history and physical examination. Lastly, 308 patients with complete data were included in the final analysis.

Hypertension was distinguished by systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg or taking antihypertensive drugs. Hyperlipidemia was defined as elevated one or all of plasma lipid levels in accordance with NCEP ATPIII guidelines or taking a lowering lipid drug. T2DM was defined as fasting blood sugar ≥126 mg/dl or history of using oral hypoglycemic medication.[20] And CAD was defined as the presence of at least one significant coronary artery stenosis of more than 50% luminal diameter on coronary angiography.

Coronary angiography

CAD was evaluated by coronary angiography. The standard Judkins technique was used for this purpose. Cardiologists who performed angiography, were unaware to the study protocol. Patients with stenosis ≥50% at least in one vessel were included in CAD group. Patients with lower obstruction were included in non-CAD group. Severity of CAD was assessed by Gensini score.[21]

Blood collection and biochemical analyses

In each case, after an overnight fasting, 2 mL of blood was drawn into tubes without additives for CRP determination and 8 mL in K-EDTA tubes for other biochemical marker measurement and DNA extraction. Blood cell fraction, serum and plasma were frozen at -20 °C. CRP levels were measured by commercially available sandwich ELISA (Labor Diagnostica Nord Gmbh & Co, KG). High-density lipoprotein cholesterol (HDL-c), Low-density lipoprotein cholesterol (LDL-c), total cholesterol, triglyceride (TG) and plasma glucose concentrations were determined enzymatically with a clinical chemistry analyzer (Vital Scientific, Spankeren, Netherlands).

Genotyping

DNA was extracted from leukocytes, taken from peripheral blood ,by using a salting-out procedure (Miller's method).[22]

Genotyping of the SNPs -757T>C(rs3093059), -717A>G(rs2794521), 409G>A (rs3093062) and -286C>T>A (rs3091244), in the promoter region of the CRP gene were done via PCR-restriction fragment length polymorphism (RFLP) using the appropriate restriction enzymes (Thermo scientific).

Nucleotides between -1105 and -105 to the start codon were used for primer designing and Primer 3 software was used for this purpose.

Target DNA was amplified using two different primer pairs, one for SNPs -757T>C and -717A>G and another for SNPs -409G>A and -286C>T>A, which in the latter case, reverse primer sequences was modified to create an allele-specific restriction enzyme site into the PCR product. PCR amplifications were performed in a total volume of 50 µL containing 200 ng of genomic DNA, 10x PCR buffer, 1.5 mM MgCl2, 0.3 µM of each primer, 200 µmol/l dNTP and 2.5 U Taq DNA polymerase. Thermal cycling conditions were similar in two conditions as follows: an initial denaturation step at 94 °C for 5 min, 35 cycles at 94 °C for 30 s, 53 or 55 °C for 30 s (dependant on primers) , 72 °C for 45 s and a final extension step at 72 °C for 7 min.

The amplification products were digested with an appropriate enzyme according to the manufacturer's protocols.

The digested fragments were then separated by electrophoresis in 2.5% agarose gels, followed by ethidium bromide staining and visualized under ultraviolet light. To improve genotyping accuracy, samples with known genotypes were used in each batch as positive controls to evaluate the completeness of PCR product. Details of genotyping method are shown in Table 1.

Statistical analysis

Data distributed non-parametrically (CRP, TG, Cholestrol), as determined with Kolmogorov-Smirnov analysis ($p<0.05$), were logarithmically transformed to obtain a normal distribution. Data were expressed as means ± Standard Deviation (SD), unless indicated otherwise. Significance between two groups was determined by unpaired Student's *t*-test for continuous variables and by Chi-square test for discrete variables. The independence of alleles (Hardy-Weinberg equilibrium) was ensured using the χ^2 test. The genotype distribution and the allele frequencies between patients and control subjects were compared using the χ^2 and Fisher's exact tests. The association between CAD and genotype was calculated as the odds ratio (OR) [95% confidence intervals (CIs)] using a logistic regression analysis. Pearson's correlation coefficients were used to evaluate the relationships between serum CRP level and Gensini score. The *p* values <0.05 were considered significant, and all statistical tests were 2-sided. Statistical analysis was performed using the SPSS 16.00 (SPSS Inc., Chicago, IL, USA).

Results

Clinical characteristics of study subjects

The clinical and demographic characteristics of the two diabetic patient groups are shown in Table 2. Among the 308 analyzed subjects, there were 151 individuals with and 157 without severe CAD. Diabetic patients with CAD,

Table 1. PCR primer, products and restriction enzymes for the genotyping of CRP polymorphisms

SNP	Forward and Reverse primers 5' to 3'	PCR product (bp)	Restriction enzyme	Fragments (bp)
rs3093059	F-TCAATTGGCTGAGAAAATGTGTC R-AATGGGAAATGGTAACATATTAATC	568	Mun1	568-470-98
rs2794521	F-TCAATTGGCTGAGAAAATGTGTC R-AATGGGAAATGGTAACATATTAATC	568	DraIII	568-510-58
rs3091244	FAGCGCCCAACTGAAATGTT R-TTCCCCTTCCTGTGTCCAAG	502	Taq1,Bfa1	502-475-27
rs3093062	F-AGCGCCCAACTGAAATGTT R-TTCCCCTTCCTGTGTCCAAG	502	PamII	502-456-46

Table 2. Patient characteristics

	T2DM/CAD (n=151)	T2DM/non-CAD (n=157)	p-value*
Age, years	53.6±5.3	49.6±6.3	<0.001
Men	77 (51)	57 (36.3)	0.011
Duration of Diabetes, years	7.8±7.2	6.5±5.7	0.08
Duration of CAD, years	2±3.2	-	
Hypertension	79 (52.3)	56 (35.7)	<0.001
Dyslipidemia	84 (55.6)	82 (52.2)	0.018
Body mass index, Kg/m^2	26.0±3.4	25.9±2.6	0.74
Systolic blood pressure, mmHg	133.2±25.1	124.7±17.8	0.002
Diastolic blood pressure, mmHg	82.6±13.9	77±10.7	<0.001
Current use			
Lipid lowering drug	71 (47)	56 (35.7)	<0.001
Acetyl Salisilic Acid	86 (57)	42 (26.8)	<0.001
ACE inhibitors	39 (25.8)	21 (13.4)	<0.001
FPG, mg/dl	151±53	147±51	0.18
Ln Cholestrol,mg/dl**	5.23±0.29	5.24±0.23	0.63
Ln TG, mg/dl**	5±0.55	5.22±0.54	<0.001
HDL-c , mg/dl	40.3±10.2	46.8±11	<0.001
LDL-c, mg/dl	122.9±52.	106.1±36	<0.001
Ln CRP, mg/l**	1. 46±0.55	1.06±0.63	<0.001

Values presented are means±SD or numbers (%). Numbers may not add up to the expected total due to missing data for some variables.

CRP=C-reactive protein; T2DM=type2 diabete melitus; CAD=coronary artery disease; ACE=angiotensin-converting enzyme; FPG=fasting plasma glucose; TG=triglyceride; HDL-c=high density lipoprotein cholestrol; LDL-c=low density lipoprotein cholestrol. FPG=fasting plasma glucose

*Chi –Square or unpaired-sample t-test

**Ln-Transformed variables

with the exception of plasma triglyceride and HDL-c, showed the higher values of different cardiovascular risk factors compared with patients without CAD. Mean of the natural logarithm (ln) concentration of Serum CRP was higher in CAD group (1.46 mg/L) compared with non-CAD group (1.06 mg/l) (Table 2).

Genotype distribution of the polymorphisms in CAD and non-CAD subjects

Four SNPs were successfully genotyped in the CRP gene. The characteristics of these SNPs are summarized in Table 3. The frequencies for all SNPs in both groups followed the Hardy-Weinberg equilibrium (p>0.05), except for rs3091244 among CAD patients (p= 0.03) and rs2794521 among non-CAD patients (p= 0.04). The SNP rs3093062 AA genotype was not observed in the population analyzed.

None of the polymorphisms showed significant association between the genotypes or alleles frequencies and prevalence of CAD, except for GG genotype of polymorphism rs2794521 (OR= 2.38, 95% CI; 1.12-5.08, p= 0.02). The genotypes TC of rs 3093059, TT of rs3091244 and GA of rs3093062 had higher odds ratios and genotypes CC of rs 3093059, AA of rs3091244, AG of rs2794521 and GG of rs3093062 had lower odds ratios for CAD risk.

Effect of variation in the CRP gene on CRP levels and Gensini score

The results of SNP association analysis with C-reactive protein serum concentrations are summarized in Table 4. The highest C-reactive protein concentrations were found in GA rs3093062 with a mean in concentration of 1.56 mg/L, whereas the lowest were observed in CC rs3091244 (1.06 mg/L).

In all four polymorphisms we observed that minor alleles were associated with higher C-reactive protein levels. However in none of these SNPs, the C-reactive protein genotypes and alleles were associated significantly with severity of CAD; except for carriers of the T (rs 3091244) alleles (which have increased C-reactive protein) which had a slightly lower mean severity of CAD (p= 0.08).

Table 3. Genotypic and allelic frequencies of SNPs in the CRP gene by study groups

SNP	T2DM/CAD n (%)	T2DM/non-CAD n (%)	OR (95 %CI)	p-value
rs3093059				
TT	89(58.9)	101 (64.3)	0.79 (0.5-1.26)	0.33
TC	56 (37.1)	48 (30.5)	1.34 (0.83-2.15)	0.23
CC	6 (4)	8 (5)	0.77 (0.26-2.27)	0.63
T	234 (77.5)	250 (79.6)	0.88 (0.6-1.3)	0.52
C	68 (22.5)	64 (20.4)	1.14 (0.77-1.66)	0.51
HWE: χ^2 (p)[a]	0.6 (0.44)	0.53 (0.47)		
rs3091244				
CC	46 (30.5)	53 (33.8)	0.88 (0.55-1.42)	0.61
CT	42 (27.8)	38 (24.2)	1.2 (0.72-2.01)	0.47
CA	32 (21.2)	40 (25.5)	0.78 (0.46-1.33)	0.37
TT	20 (13.2)	13 (8.2)	1.69 (0.81-3.53)	0.16
TA	9 (6)	10 (6.3)	0.93 (0.36-2.36)	0.88
AA	2 (1.3)	3 (2)	0.69 (0.11-4.18)	0.68
C	166 (55)	184 (58.7)	0.86 (0.62-1.18)	0.36
T	91 (30.1)	74 (23.5)	1.4 (0.97-2.01)	0.06
A	45 (14.9)	56 (17.8)	0.8 (0.52-1.23)	0.32
HWE: χ2 (p)[a]	9.2 (0.03)	6 (0.11)		
rs2794521				
AA	66 (43.7)	66 (42)	1.07 (0.68-1.68)	0.77
AG	62 (41.1)	80 (51)	0.67 (0.43-1.05)	0.08
GG	23 (15.2)	11 (7)	2.38 (1.12-5.08)	0.02
A	194 (64.2)	212 (67.5)	0.86 (0.62-1.2)	0.39
G	108 (35.8)	102 (32.5)	1.16 (0.83-1.61)	0.39
HWE: χ^2 (p)[a]	1.7 (0.19)	4.1 (0.04)		
rs3093062				
GG	139 (92)	151 (96.1)	0.46 (0.17-1.26)	0.13
GA	12 (8)	6 (3.9)	2.17 (0.79-5.94)	0.13
G	290 (96)	308 (98)	0.47 (0.17-1.27)	0.14
A	12 (4)	6 (2)	2.12 (0.79-5.73)	0.14
HWE: F(p)[b]	0.25 (0.6)	0.05 (0.8)		

HWE= Hardy-Weinberg equilibrium; OR= odds ratio

a: χ2 test

b: Fisher's exact test

In the present study, Gensini scores in CAD patients were positively correlated with serum CRP, (r=0.17, *p*= 0.04). Different composition of four SNPs made 24 kinds of haplotypes which only seven kinds had frequency higher than 4% (Table 5).

Discussion

CRP gene variation influences serum CRP levels and may play a potential role in the pathophysiology of carotid atherosclerosis. Increased serum CRP levels have been reported in subjects with T2DM, indicating that these individuals present a state of subclinical, low-grade inflammation that promotes the development of atherosclerosis.[23] Therefore, we designed a cross-sectional study to evaluate the association of four *CRP* SNPs on CRP levels, CAD prevalence and Gensini scores in T2DM patients. We minimized the influence of factors that modify CRP, such as age, smoking, obesity, and disease involving inflammation. In the present study, patients with CAD had higher risk factor, except for plasma triglyceride, which may be due to higher use of lipid lowering drugs by this patient group.

In our study, genotypes and alleles frequencies had not shown significant differences between two groups, except for individuals carrying the GG genotype of SNP rs2794521. The odds ratio (OR) for prevalence of CAD of this genotype was 2.38 (95% CI 1.12-5.08) compared with the AA referent genotype. Among the SNPs, only

Table 4. Effect of CRP SNPs on the concentrations of CRP or Gensini Scores

SNP	All Patients				Patients with CAD			
	n	mean Ln-CRP (mg/L) (SD)	Δ(mg/l)[a]	P-value	n	mean Gesini Score (SD)	Δ(mg/l)[a]	p-value
rs3093059								
TT	189	1.25 (0.63)	ref	ref	89	43.1 (33.7)	ref	ref
TC	105	1.27 (0.6)	0.02	0.8	56	40 (39.5)	3.1	0.63
CC	14	1.27 (0.57)	0.02	0.84	6	38.8 (32.9)	4.3	0.76
T	294	1.26 (0.62)	ref	ref	145	40.9 (35.4)	ref	ref
C	119	1.27 (0.6)	0.01	0.51	62	38 (38)	2.1	0.63
rs3091244								
CC	99	1.06 (0.61)	ref	ref	46	44.4 (40)	ref	ref
CT	80	1.21 (0.6)	0.14	0.12	42	39.1 (35.1)	5.2	0.51
CA	72	1.46 (0.61)	0.4	<0.001	32	50.2 (35.2)	5.8	0.51
TT	33	1.39 (0.61)	0.32	0.01	20	29.7 (23.6)	14.8	0.12
TA	19	1.44 (0.51)	0.38	0.01	9	27.3 (30)	17	0.23
AA	5	1.47 (0.54)	0.41	0.15	2	14 (5.6)	30.4	0.29
C	251	1.22 (0.63)	ref	ref	120	44.1 (36.8)	ref	ref
T	132	1.29 (0.6)	0.07	0.34	71	34.9 (31.7)	9.2	0.08
A	96	1.47 (0.59)	0.25	<0.05	43	43.7 (34.8)	0.4	0.95
rs2794521								
AA	132	1.26 (0.64)	ref	ref	66	43.6 (37.4)	ref	ref
AG	142	1.21 (0.61)	0.04	0.57	62	41.4 (34.2)	2.2	0.73
GG	34	1.42 (0.54)	0.16	0.19	23	37.9 (35.6)	5.7	0.53
A	274	1.24 (0.63)	ref	ref	128	41.3	ref	ref
G	176	1.26 (0.55)	0.02	0.76	85	39.7	1.6	0.74
rs3093062								
GG	290	1.24 (0.63)	ref	ref	139	42.7 (36.1)	ref	ref
GA	18	1.56 (0.3)	0.32	0.03	12	30.8 (29)	11.9	0.29
G	308	1.26 (0.62)	ref	ref	151	40.7 (35.2)	ref	ref
A	18	1.56 (0.3)	0.3	0.04	12	29.6 (28)	11.2	0.28

a: Difference from reference value

Table 5. Haplotype association with CAD (n=251)*

	SNP1	SNP2	SNP3	SNP4	Freq	OR (95% CI)	p-value
1	C	G	T	G	0.2988	1.00	-
2	C	A	T	G	0.166	0.50 (0.19-1.32)	0.16
3	T	G	T	G	0.141	1.05 (0.47-2.35)	0.91
4	C	G	C	G	0.0844	1.30 (0.37-4.58)	0.68
5	T	A	T	G	0.0615	0.72 (0.22-2.39)	0.6
6	A	G	T	G	0.0509	0.09 (0.01-1.21)	0.07
7	A	G	C	G	0.0469	0.11 (0.02-0.66)	0.017

*Adjusted by age, sex, SBP and DBP, SBP=systolic blood pressure, DBP=diastolic blood pressure

SNP rs2794521was not consistent with the most previous reports, in which GG genotype was associated with decreased prevalence of CAD.[24,25]

We also found that CA, TT and TA genotypes and A allele of SNP rs3091244 were significantly associated with higher CRP levels. These findings are in line with most of the results from previous studies.[26-28] Furthermore, GA genotype and A allele of SNP rs3093062 was significantly associated with higher CRP levels. SNP rs3093062 in comparison with other variants has been rarely studied. Two recently conducted studies revealed that this SNP had non-significant association with CRP level in healthy[29] and diabetic[30] Mexican participants. In contrast to most previous studies, none of genotypes and alleles of SNP rs3093059 were significantly influenced by CRP levels.[31] Of course, in all polymorphisms, minor alleles were associated with higher CRP levels compared to major alleles.

Most previous studies have examined the association of genetic variants in the CRP gene with severity of coronary artery disease by CIMT, but we used coronary angiography which is a gold standard for this purpose. Beyond this, coronary angiogram results were calculated as Gensini score. In fact, this is strength of our study that we avoided mis-estimation. However in most previous studies, no association was observed between CIMT and any of these four SNPs in different populations.[32] Our findings are consistent with those reports. There are conflicting data as to the relationship between CRP levels and CIMT in type 2 diabetic patients.[33,34] According to our analysis, there was significant association between CRP levels and Gensini scores only in CAD group (data not shown).

We concluded that elevation in CRP levels not only pointed to the presence of heart artery damage but also it may be associated with processes which are developing ischemic state. Based on the clinical evidences of pre and post ischemic states at cardiac tissue, our colleague cardiologist excluded patients who had these symptoms from study.

Haplotype analysis is suggested to be more powerful than individual SNP analyses. In the present study, none of the haplotypes significantly influenced the risk of CAD except for haplotype 7. Despite multivariable adjustment this haplotype was associated with decreased prevalence of CAD (Table 5).

Other studies that have examined the association of C-reactive protein gene haplotypes with CAD are not comparable to our study, because of different polymorphisms used to reconstruct the haplotypes and different populations used in terms of health status, ethnicity, or age.[35,36]

The results of previous studies are different. One reason for this discrepancy may be the distinct LD patterns among different populations. Furthermore, the potential interactive influence between CRP gene and diabetes may also partially explain the discrepancy.

Our study has few limitations: firstly, the number of subjects was relatively few. Secondly, most participants in CAD group were men and older than non-CAD group. Therefore, additional studies in larger populations in which sex and age are adequately represented are needed.

Conclusion

In conclusion, our results support most previous studies demonstrating limited association of CRP polymorphisms in promoter region with CAD. However, we identified an association between GG genotype of SNP rs2794521 with CAD in T2DM patients that has not been reported previously. We also found that individuals carrying the GA genotype and A allele of SNP rs3093062 displayed an increase in the average serum CRP levels compared with those in G carriers. Further studies are needed to confirm these relationships.

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Ethical issues

The Regional Ethics Committee of Tabriz University of Medical Sciences as well as Regional Ethics Committee for Medical Research (Ahvaz University of Medical Sciences) approved the study protocol. The study was in compliance with the declaration of Helsinki. All of the patients gave written informed consent.

Competing interests

The authors have no conflicts of interest.

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