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Association of rs7903146 polymorphism in the TCF7L2 gene with diabetic nephropathy and decreased estimated GFR in an Arab population in southwest Iran

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ABSTRACT

Background: Transcription factor 7-like 2 (TCF7L2) acts as a downstream effector in the Wnt signaling pathway. It plays important roles in the proliferation and differentiation of islet beta-cell, insulin secretion and kidney development.

Objectives: This study aimed to demonstrate whether rs7903146 variant is associated with diabetic nephropathy (DN) and measures of kidney function in a diabetic and healthy Arab population in southwest of Iran.

Patients and Methods: This study is comprised of 132 diabetic subjects (T2DM) and 66 healthy participants. The diabetic subgroups were composed of patients with DN (n=56) and early onset of diabetes (n=71). The rs7903146 polymorphism was genotyped using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method in all the participants. Blood glucose, HbA1c, blood urea nitrogen (BUN), creatinine and urinary albumin were evaluated by a biochemistry analyzer and enzyme-linked immunosorbent assay (ELISA) was employed for cystatin C measurement.

Results: The frequency of genotypes was significantly different between all the diabetic cases and control subjects ($P < 0.05$). The TT variant odds ratio (OR) versus CT/CC genotypes for diabetes was 2.47 (95% CI 1.11-5.48). An association was observed between TT homozygous and DN (OR for TT 2.78, 1.13-6.84). Early onset diabetic patients showed stronger association (OR: 4.64, 1.64-13.14, $P = 0.003$). The TT genotype was also found to be a risk variant for decreased estimated glomerular filtration rate (eGFR)_(Cys-Cr) below 60 mL/min/1.73 m² (OR: 3.36, 1.4-8.1, $P = 0.005$).

Conclusions: The results confirmed that the TCF7L2 gene rs7903146 variants are significantly associated with T2DM in Arab population of Iran. The TT genotype of this SNP is also predisposed to the risk of developing DN especially in subjects with early onset diabetes. Patients with TT genotype were also at risk of decreased GFR.

Implication for health policy/practice/research/medical education:

The results of this study confirmed the association of rs7903146 polymorphism with diabetes and diabetic nephropathy (DN) in the residents of Khuzestan province. Knowledge on the predisposing genetic factors can aid in identifying people at risk. The results can be used to better manage people predisposed to the DN.

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1. Background

Diabetes is considered as a serious health problem worldwide. Type 2 diabetes mellitus (T2DM) is related to lack of response to physiologic concentrations of insulin and β -cell impairment shown by depletion in insulin secretion and hyperglycemia (1). The incidence of diabetes mellitus has increased up to 8.5%. In the United States, 95% of diabetic patients are T2DM cases, which lead to diabetic nephropathy (DN), a significant complication of T2DM (2). It accounts for 25% cases of chronic kidney disease (CKD) and 35% of end-stage renal disease (ESRD) cases (3). Multiple environmental and genetic factors are associated with T2DM and its damages. The description of the predisposing genes to T2DM and diabetic kidney disease provides underlying mechanisms for the disease and targets for more effective treatments. Diabetes and the resultant nephropathy are both heritable (4,5). The heritability of kidney disease has been shown in individuals suffering from polycystic kidney disease (5,6). Diabetic subjects with long-term imbalanced glycemic control are at increased risk of renal complications. But many patients with weak control of diabetes do not show CKD, while others with proper glycemic control show renal complications (7). Considering these findings, it is important to find out whether genetic susceptibility to diabetes can also predispose the patient to nephropathy. Many predisposing genes with variable effects on T2DM have been revealed by genome-wide association studies (GWAS) (8,9). Transcription factor 7-like 2 (TCF7L2) is a protein encoded by chromosome 10q25.3. It belongs to the TCF/lymphoid enhancer factor (LEF) family and acts as a downstream effector of the Wnt signaling pathway which plays important roles in proliferation and differentiation of islet beta-cells, insulin secretion (10) and kidney development (11). It is known as a diabetes susceptibility gene since 2006 (12). Two intronic polymorphisms, rs7903146 (intron 3) and rs12255372 (intron 4) have significant genetic association with diabetes (12-14). The rs7903146 polymorphism was also found to be linked to DN, CKD and ESRD (15-21). It has been shown that polymorphisms of TCF7L2 gene are related to the development of CKD and indices of kidney function in other populations (18).

2. Objectives

This study aimed to demonstrate whether the rs7903146 variant is associated with DN and measures of kidney function in an Iranian-Arab population with and without diabetes in southwest of Iran.

3. Patients and Methods

3.1. Patients

The study population consisted of 132 individuals with

T2DM, referred to the Golestan hospital in Ahvaz, Iran. The patients were from Iranian-Arab population of this region. They were classified into different subgroups of clinical phenotypes: diabetic kidney disease (DN, n=56) and early onset of diabetes (<45 years) (n=71). Criteria of the American Diabetes Association were applied for diagnosis of diabetes, which are the known symptoms of high blood sugar (polydipsia, weight loss and polyuria), fasting serum glucose >126 mg/dL or random serum glucose >200 mg/dL. The duration of diabetes ranged from 4 to 25 years (mean of 12.4), which was determined from the time of the initial diabetes symptoms such as glycosuria. Persistent albuminuria (>300 mg/24 h) in two successive sampling in the absence of hematuria or infection, was considered for diagnosis of DN. The subjects with microalbuminuria or normoalbuminuric patients without DN were not included. The average duration from diabetes diagnosis to nephropathy development was 9.7 years (range of 4-19). Glycemic control was evaluated using HbA1c measurements. Other biochemical variables were assayed using standard laboratory methods. For the control group (n=66), healthy individuals without any record of diabetes or kidney disease were recruited based on laboratory data.

3.2. Genotyping

Blood samples were collected in the presence of EDTA and genomic DNA was obtained by a commercial extraction kit following the instructions of the manufacturer (Yekta Tajhiz Azma Extraction kit, Tehran, Iran). Extracted DNA was analyzed by UV absorption at 260 and 280 nm using a NanoDrop™ spectrophotometer. Genotyping was carried out using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. Touchdown PCR was performed by premix 2x master mix red containing 1.5 mM MgCl₂ (Amplicon, Denmark), 100 ng template DNA, 12 μ L master mix, 0.25 μ L of each primer (100 pmol/ μ L) and DNase/RNase free water diluted to a final volume of 25 μ L. The following modified primers were used:

Forward,

5'ACAATTAGAGAGCTAAGCACTTTTTAGGTA3',

Reverse,

5'GTGAAGTGCCCAAGCTTCTC3' (17,22).

Non-specific amplification was prevented using touchdown thermal cycling. The program included first denaturation at 95°C for 3 minutes and PCR cycles composed of denaturation at 95°C for 30 seconds, annealing at 65°C with 1°C decrease per cycle for 30 seconds, and extension at 72°C for 45 seconds, and accomplished with 24 additional cycles when annealing temperature reached 60°C. Following amplification, the 188-bp PCR products were treated with 10U RsaI restriction enzyme (Takara, Japan) by incubation at 37°C overnight. DNA fragments

were analyzed using 3% agarose gel electrophoresis. The *RsaI* enzyme cuts the restriction site 5'GT▼AC3' at the C allele of the single nucleotide polymorphism (SNP) and yields two fragments, 159 and 29 bp, but the T allele (mutant) is not digested, remaining the intact PCR product (Figure 1). Blind DNA duplicates were used to control the quality of genotyping.

3.3. Measurement of cystatin C

Levels of cystatin C, as an accurate and sensitive indicator of kidney function, were measured in serum to estimate glomerular filtration rate (GFR) (23). It was measured in samples, standards and controls diluted 1:400, using a commercially available Sandwich ELISA kit (Bio Vendor Human Cystatin C ELISA, North Carolina, USA). All the tests for the samples were performed with ELISA kit of the same lot by technicians who were unaware of whether the sample belonged to cases or controls.

3.4. Calculation of estimated GFR (eGFR)

Among the renal function indices, GFR can better reflect the kidneys performance. Variation in normal GFR is related to age, sex and body size, and decreases with aging. Based on the National Kidney Foundation recommendation, the CKD-EPI Creatinine-Cystatin C Equation (2012) for GFR estimation which is described elsewhere (24) was used:

3.5. Ethical issues

The study was approved by the Ethics Committee of the Ahvaz Jundishapur University of Medical Sciences (IR.AJUMS.REC.1395.200) and was implemented in accordance with the ethical tenets of the Declaration of Helsinki. Informed consent was obtained from all the

participants of the study.

3.6. Statistical analysis

Continuous variables with normal distribution are shown as means \pm standard deviation (SD). The chi-square test was used to analyze frequency of alleles and genotypes between groups completed by the Hardy-Weinberg equilibrium test. Student's *t* test and Mann-Whitney U-test were used for comparisons between variables/groups. Odds ratios (ORs) with corresponding 95% CIs were measured for the association between genotypes and T2DM and DN. Logistic regression was applied to adjust for the background variables in analysis of TT genotype as an independent risk factor for T2DM, DN and early onset of diabetes. The SPSS 18.0 for Windows was employed for statistical analysis (SPSS, Inc., Chicago, IL, USA).

4. Results

Genotyping of the rs7903146 polymorphism was assessed in 66 healthy subjects and 132 patients with T2DM. The demographic and clinical data of the studied individuals are shown in Table 1.

4.1. Association of rs7903146 polymorphism with T2DM

The allele frequency among the participants is in accordance to the Hardy-Weinberg equilibrium. The frequency of rs7903146 genotypes in T2DM and control subjects are shown in Table 2. Significant differences in distribution of genotypes and alleles were found between the whole diabetic group and healthy subjects ($P < 0.05$). The OR for TT genotype versus CT/CC genotypes before

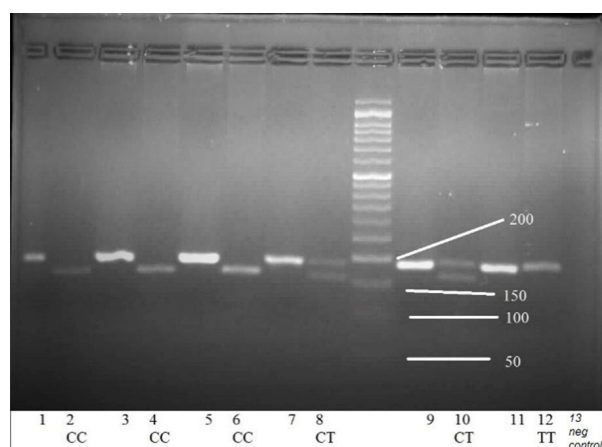


Figure 1. Electrophoresis image of PCR products. Lanes 1, 3, 5, 7, 9 and 11 are PCR products without *RsaI* digestion. Lanes 2, 4, 6, 8, 10 and 12 are PCR products with *RsaI* incubation. Lane 13 is negative control.

Table 1. Demographic and clinical characteristics of the studied subjects

Variable	Controls	T2DM	<i>P</i>
N	66	132	
Male/Female	38/28	53/79	
Age (y)	52 \pm 8	55 \pm 8	0.014
Diabetes duration	NA	12.3 \pm 3.8	
Diabetic nephropathy (n)	0	56	
FBS (mg/dL)	86.8 \pm 12.3	227.8 \pm 69.3	<0.001
HbA1c (%)	4.8 \pm 0.5	7.9 \pm 1.2	<0.001
BMI (kg/m ²)	26.9 \pm 4.6	28.7 \pm 4.9	0.015
eGFR (mL/min/1.73m ²)	79.5 \pm 16.8	55.4 \pm 31.9	<0.001
Cystatin C (mg/L)	1.16 \pm 0.35	1.96 \pm 1.48	0.019
Albuminuria (mg/24h)	6 \pm 2	225 \pm 244	<0.001
Cr (mg/dL)	0.83 \pm 0.15	1.79 \pm 1.38	<0.001
BUN (mg/dL)	13.2 \pm 2.3	27 \pm 17.5	<0.001

BMI, body mass index; FBS, fasting blood sugar; HbA1c, hemoglobin A1c; eGFR, estimated glomerular filtration rate; BUN, blood urea nitrogen; Cr, creatinine.

Data are mean \pm SD.

Table 2. Genotype and allele distribution of rs7903146 SNP of *TCF7L2* gene in the diabetic and control subjects

Subjects	CC	CT	TT	MAF	OR (95% CI, P) for TT genotype	OR ^a (95% CI, P) for TT genotype
T2DM (n=132)	38 (28.8)	57 (43.2)	37 (28)	0.496	2.47 (1.1-5.5, 0.024)	2.61 (1.1-6, 0.023) ^a
Controls (n=66)	28 (42.4)	29 (43.9)	9 (13.6)	0.356	1.0 (ref.)	1.0 (ref.)
Early onset of T2DM (n=71)	19 (26.8)	28 (39.4)	24 (33.8)	0.535	1.88 (0.9-4.1, 0.1)	2.86 (0.9-9.6, 0.087) ^a
Late onset of T2DM (n=61)	19 (31.1)	29 (47.5)	13 (21.3)	0.451	1.0 (ref.)	1.0 (ref.)

MAF minor allele frequency.

Data are n (%). HWE test: T2DM $\chi^2 = 2.45, P=0.117$, Controls $\chi^2 = 0.11, P = 0.734$.

^a Adjusted for age, sex, BMI.

and after adjustment for age, gender and body mass index (BMI) were 2.47 (95% CI 1.11-5.48) and 2.61 (95% CI 1.14-5.97), respectively. The ORs with regards to CC genotype were 3.03 (95% CI 1.26-7.28, $P=0.011$) and 1.8 (95% CI 1.12-2.79, $P=0.014$). The OR for T allele was high (1.82, 95% CI 0.98-3.38) although not considered significant ($P=0.055$) and after adjusting for age, sex and BMI, the OR for T allele was 1.75 (95% CI 0.92-3.35) (data not shown). In Table 2, genotype distribution and allele frequency were compared in subgroups of diabetic patients with early diagnosis of diabetes (<45 years). The frequency of TT genotype was higher in patients with early onset diabetes than those with late onset diabetes, although the difference was not statistically significant ($P=0.087$).

4.2. Association of rs7903146 polymorphism with DN

The OR for TT genotype in the DN patients vs. diabetic patients without any microvascular complication after more than 10 years history of the disease was 2.78 (1.13-6.84) ($P=0.02$), and after adjusting for age, gender, BMI and HbA1c, OR for TT still remained significant (2.77, 95% CI 1.08-7.11, $P=0.03$) (Table 3). In subjects with early onset of diabetes, the TT genotype of the polymorphism was even more strongly associated with DN. The frequency of TT genotype and T allele were 48.1 and 68%, respectively, as compared to 16.7 and 41% in no microvascular subgroup, and OR for TT genotype and T allele were 4.64 (95% CI 1.64-13.14) and 4.34 (95% CI 1.16-16.32) ($P=0.003$ and 0.022, respectively). The OR for TT genotype increased when adjusting for age, gender,

BMI and HbA1c (OR: 5.4, 95% CI 1.7-17.3) ($P=0.005$) (Table 3). Following adjustments in logistic regression, the OR for TT genotype still increased (OR=6.5, 95% CI 1.35-31.73) (data not shown). These results showed a strong association between polymorphism and DN in patients with diabetes onset <45 years.

4.3. Association of rs7903146 polymorphism with decreased eGFR

In order to evaluate the influence of rs7903146 genotypes on glycemic and kidney function parameters, fasting blood sugar (FBS), serum HbA1c, BUN, creatinine, cystatin C and albuminuria in patients with TT and CC homozygous in all the groups were compared. Increased levels of the parameters and decreased eGFR were found in diabetic patients with TT genotype (Table 4). The association of rs7903146 SNP with DN was further confirmed by measuring the OR for TT homozygous diabetic subjects to have an estimated GFR <60 mL/min/1.73 m². In T2DM patients, the TT genotype was associated with CKD and decreased eGFR <60 ml/min/1.73 m². The OR value of association was 3.36 (95% CI 1.4-8.1) ($P=0.005$; Table 5). Multivariate logistic regression test was performed to study the association of the polymorphism in the presence of other coexisting factors such as age, sex, BMI, HbA1c and diabetes duration. These adjustments did not significantly change the OR estimates. Association of T2DM and diabetic kidney disease with the TT genotype of the polymorphism remained significant in logistic regression, adjusting for other covariates which are risk factors for T2DM and DN, including age, sex and BMI and HbA1c

Table 3. Genotype and allele distribution of rs7903146 SNP in the diabetes patients with and without diabetic nephropathy

Subjects	CC	CT	TT	MAF	OR (95% CI, P) for TT genotype	OR ^a (95% CI, P) for TT genotype
DN (n=56)	12 (21.4)	24 (42.9)	20 (35.7)	0.57	2.78 (1.1-6.8, 0.023)	2.77 (1.1-7.1, 0.034) ^a
Early onset T2DM with DN (n=27)	3 (11.1)	11 (40.7)	13 (48.1)	0.68	4.64 (1.6-13.1, 0.003)	5.4 (1.7-17.3, 0.005) ^a
No complication (n=54) ^b	19 (35.2)	26 (48.1)	9 (16.7)	0.41	1.0 (ref.)	1.0 (ref.)

MAF minor allele frequency.

Data are n (%). ^a Adjusted for age, sex, BMI, HbA1c; ^b No microvascular complications after ≥10 years of T2DM duration.

Table 4. Comparison of the glyceamic and kidney function parameters in subjects with CC and TT genotypes

	Healthy			Diabetes without nephropathy			Diabetes with nephropathy		
	CC	TT	P	CC	TT	P	CC	TT	P
FBS (mg/dL)	84 ± 16	87 ± 9	0.46	199 ± 49	245 ± 76	0.04	213 ± 75	267 ± 77	0.037
HbA1c (%)	4.8 ± 0.4	4.9 ± 0.6	0.68	7.5 ± 0.8	8.5 ± 1.8	0.04	7.35 ± 0.8	8.2 ± 1.1	0.01
eGFR (mL/min/1.73m ²)	80 ± 18	69 ± 17	0.14	78 ± 15	77 ± 21	0.81	41 ± 24	19 ± 12	0.002
BUN (mg/dL)	13 ± 2	13 ± 3	0.67	13 ± 3	13 ± 3	0.95	33 ± 10	46 ± 15	0.007
Cr (mg/dL)	0.8 ± 0.1	0.8 ± 0.2	0.35	0.9 ± 0.1	0.9 ± 0.2	0.66	2.1 ± 1	3.3 ± 2	0.008
Cystatin C (mg/L)	1.1 ± 0.4	1.4 ± 0.4	0.12	1 ± 0.3	1.2 ± 0.5	0.29	2.1 ± 1.4	3.5 ± 1.6	0.01
Albuminuria (mg/24h)	6 ± 2	6 ± 2	0.93	10 ± 4	11 ± 5	0.6	357 ± 120	468 ± 115	0.006
BMI (kg/m ²)	27 ± 5.4	26 ± 4	0.38	31 ± 6	30 ± 5	0.535	28 ± 4.4	28 ± 4	0.981

Data are in mean ± SD.

Table 5. Association of TT genotype with eGFR <60 mL/min/1.73 m²

	OR (95% CI, P value) for TT genotype	OR (95% CI, P value) for TT genotype ^a
Diabetic Subjects		
eGFR <60 mL/min/1.73m ²	3.36 (1.4-8.1, 0.005)	3.34 (1.3-8.5, 0.011) ^a
eGFR >60 mL/min/1.73m ²	1.0 (ref.)	1.0 (ref.)

^a Adjusted for age, BMI, sex, HbA1c and diabetes duration.

(OR 2.61, 95% CI 1.14-5.97, $P=0.023$ for T2DM and 2.77, 95% CI 1.08-7.11, $P=0.03$ for DN).

5. Discussion

TCF7L2 is the strongest predisposing gene for T2DM and previous studies have shown the effect of rs7903146 polymorphism of this gene on T2DM development in different populations (12,25-27). In this study, the potential association of T2DM with the rs7903146 SNP in a group of Iranian population with T2DM was investigated. It was confirmed that rs7903146 is a significant susceptibility polymorphism for T2DM in the southwest of Iran, where people with Arab ethnicity comprise the majority of the population, similar to previous data on Iranian population (28-30). A significant association between the rs7903146 polymorphism and T2DM was found in this study subjects. The T allele frequency of the SNP in the current study population was high, similar to other studies on Iranian and Arab populations (28,31-33). The T allele frequency in non-diabetic people in this study was 35.6%, while it was 29.3, 37.2 and 40.5% in Palestinian, Emirati and Saudi populations, respectively. In the present study, the frequency of the T allele in T2D patients was 49.6%, while it was 49.7, 40.0 and 41.5% in Palestinian, Emirati and Saudi populations, respectively (31-33). Therefore, these findings support the association between this SNP and T2DM. The OR of TT genotype and T allele for diabetes in the present study were similar to that of another study in Poland (16). Other studies have also demonstrated allele-specific islet enhancer activity as a functional role for this polymorphism (34). Villareal et al (35) proposed that the

TCF7L2 rs7903146 polymorphism affect insulin secretion and the risk of T2DM, partly by incretins changes. Up-regulation of *TCF7L2* expression up to 5-fold in islet cells of diabetic people with TT genotype has been reported, which was related to impaired glucose-stimulated insulin secretion, incretin modifications and increased hepatic glucose production (36).

In addition, it was found that the diabetic subgroup with an early onset of diabetes with TT genotype are at higher risk of diabetic kidney disease, suggesting increased risk of microvascular complications of type 2 diabetic patients for the TT genotype. Previous studies have also reported the correlation of the rs7903146 polymorphism with DN in other populations (16,37). An Italian study suggested that the T allele carriers have an association with renal dysfunction (37). In an African-American population, another study showed the association of candidate gene variant with T2DM and ESRD (20). Similarly, in a Polish population, a study showed a strong association of rs7903146 with diabetic kidney disease (16). The results of the present study are in accordance with earlier reports on the association of rs7903146 polymorphism with kidney function and development of CKD (16,19). *TCF7L2* might increase the risk of nephropathy through its effects on diabetes, glycation and also some tissue-specific changes.

Logistic regression models were used to analyze interactions with risk factors of DN such as age, BMI, sex, HbA1c and diabetes duration. The results suggest that effect of the polymorphism on nephropathy might be independent of its effect on diabetes. Wnt signaling

pathway is important in the development of kidney through TCF4, a product of the TCF7L2 (10). Mutations in the Wnt signaling pathway were reported in hereditary kidney diseases (38). Studies on animal models of DN have shown that Wnt signaling via β -catenin is upregulated in podocytes and mesangial in kidney, which caused injury and mesangial lesion (11). Based on the functional studies that showed increased TCF7L2 expression in the human islet of TT genotype carriers (36), it is suggested that the same TCF7L2 increase and up-regulated Wnt signaling in podocytes of kidney might account for the DN in TT genotype carriers. Besides, it has been shown that Wnt signaling pathway is in relation to inflammatory responses. The inflammatory status is common and well established in T2DM and CKD. Accordingly, the rs7903146 variant was associated with decreased eGFR in healthy subjects, but the decrease was not statistically significant. In addition, as shown in Table 4, BUN, creatine, cystatin C and albuminuria as markers of kidney damage, increased variably in people with TT homozygous genotype in all the groups. Similar evaluations in healthy people with higher sample size and in non-diabetic CKD patients can better reveal the involvement of this variant in kidney damages. Apart from the increased risk of T2DM by 2.47 and augmented risk of DN by 2.78 in TT homozygous of rs7903146 SNP, similar to previous reports, the effect of genotypes on cystatin C and eGFR was evaluated and a significant association was demonstrated between the TT genotype and decreased eGFR (eGFR <60 mL/min/1.73 m²).

6. Conclusions

The results further showed that the TT genotype of TCF7L2 gene rs7903146 polymorphism is strongly associated with T2DM, even after adjusting for the background covariates. The risk allele of the polymorphism was also predisposed to developing DN, especially in patients with early onset diabetes. The TT genotype was also associated with decreased eGFR.

Limitations of the study

The study was conducted on a limited number of diabetic patients. In addition, only Arab people of the region were included. A study on a larger sample size including all ethnicities of the Khuzestan province is suggested.

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Authors' contribution

AKA and RT carried out the experiment and prepared

the draft. MZ contributed to data collection and patient selection. HY and NMT supervised the project and edited the final manuscript. All the authors read, revised and approved the final manuscript.

Conflict of interests

There is no conflict of interests to declare.

Ethical considerations

Ethical issues (including plagiarism, data fabrication and double publication) were completely observed by the authors.

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