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Association of AGTR1 and ACE2 gene polymorphisms with structural atrial fibrillation in a Chinese Han population

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The renin-angiotensin system (RAS) is thought to play an important role in atrial fibrillation (AF). The RAS contains the ACE/AngII/AGTR1 axis and the ACE2/Ang(1-7)/MAS axis, which restrict each other *via* mutual antagonism and regulate myocardial hypertrophy, fibrosis and remodelling. The aim of our study was to investigate the association between single nucleotide polymorphisms (SNPs) in angiotensin-II type-1 receptor (AGTR1) and angiotensin-converting enzyme 2 (ACE2) and structural AF in a Chinese Han population. The SNPs (rs1492100, rs1492099, rs1492097, rs3772616) in AGTR1 and the SNP rs6632677 in ACE2 were compared in 300 structural AF patients (67.61±12.56 years) and 300 controls (66.08±12.47 years). The genotype frequencies of SNP rs1492099 in AGTR1 in the structural AF cohort vs controls were as follows: GG, 72.7 *vs* 83.0%; AG 26.0 *vs* 16.3%; AA 1.3 *vs* 0.7% (P=0.009). The frequency of the minor allele of SNP rs1492099 in AGTR1 was 14.2% in the structural AF group compared with 8.8% in the controls (t=0.004; odds ratio [OR], 1.727; 95% confidence interval [CI]: 1.154–2.487). In addition, the genotype frequencies of SNP rs6632677 in ACE2 in the structural AF male patients *vs* male controls were as follows: GG, 70.5 *vs* 83.1%; CG 26.3 *vs* 15.6%; and CC 3.2 *vs* 1.3% (P=0.029). The frequency of the minor allele of SNP rs6632677 in ACE2 was 16.3% in structural AF male patients compared with 9.1% in male controls (P=0.008; OR, 1.954; 95%CI: 1.196-3.192). Furthermore, we found an interaction between the SNP rs6632677 in ACE2 and the SNPs (rs1492100/rs1492099 / rs3772616) in AGTR1 in structural AF patients by the multifactor dimensionality reduction (MDR) method. The results indicate that polymorphism rs1492099 in the AGTR1 gene is associated with structural AF in a Chinese Han population. It was hypothesized that the ACE2 gene, which maps to the X chromosome, may be correlated with the risk of structural AF in a Chinese Han male population. Furthermore, we found an interaction between ACE2 and AGTR1 in structural AF patients in a Chinese Han population.

1. Introduction

Atrial fibrillation (AF) is the most common sustained cardiac arrhythmia in the general population and leads to significant cardiovascular morbidity and mortality (Chang et al. 2012). AF increases thrombosis especially cerebral embolism, risk, and causes chronic heart failure, tachycardia-induced cardiomyopathy and angina pectoris. Pathogenesis and underlying mechanisms of AF have been the subject of intense research (Nattel 2002). The RAS plays a significant role in the structural and electrical remodelling of the atrium and plays an important role in the pathophysiology of AF (Liu et al. 2011). The RAS consists of the ACE/AngII/AGTR1 axis and the ACE2/Ang(1-7)/MAS axis, which restrict each other via mutual antagonism and regulate myocardial hypertrophy, fibrosis and remodelling (Ferrario 1990; Lindan et al. 2013; Nicholls et al. 1998).

The ACE/AngII/AGTR1 axis is involved in the pathogenesis of AF. Classically, angiotensin converting enzyme (ACE) converts angiotensin I (AngI) into the biologically active octapeptide angiotensin II (AngII). AngII, the most vasoactive component of the RAS, can contribute to AF by causing an increase in myocardial fibrosis and hypertrophy (Madrid et al., 2002; Pedersen et al. 1999; Van Den Berg et al. 1995). AngII is the major signaling molecule of the RAS and its major cardiovascular effects are mediated by AGTR1. AGTR1, a G-protein coupled receptor, has been studied in a number of medical conditions, including heart failure, prehypertension and stroke (Fung et al. 2011; Wu et al. 2009). It has been indicated that the levels of AGTR1 are increased in the left atrium of AF patients (Boldt et al. 2003; Cong et al. 2010; Goette et al. 2000a).

The ACE2/Ang(1-7)/MAS axis is involved in the pathogenesis of AF. Originally, RAS was considered to be an endocrine system with circulating AngII as its functional effector hormone. However, the advent of new molecular techniques in the past decade has significantly changed our view of this system, and a new axis, the ACE2/Ang(1–7)/Mas receptor, was established. Accumulating evidence has indicated that ACE2 may play a pivotal role in counterbalancing the vasoconstrictive actions of the ACE/AngII/AGTR1 axis by cleaving AngII into Ang(1–7), which may be beneficial for the cardiovascular system (Danilczyk et al. 2003; Donoghue et al. 2000). In patients with chronic atrial fibrillation, ACE2 expression was significantly decreased, and atrial tissue AngII levels were significantly increased (Hu et al. 2007). Accordingly, we studied the associations between the AGTR1 and ACE2 genes, their gene-gene interactions and the risk of structural AF in a Han population.

2. Investigations and results

We performed genetic analysis of the 300 structural AF patients *vs* 300 control subjects in the cardiovascular ward at the First Affiliated Hospital of Harbin Medical University. The general and clinical characteristics of the structural AF patients and controls are listed in Table 1. There were no significant differences between structural atrial fibrillation and controls regarding age, sex, smoking, significant valvular heart disease, or common cardiovascular risk factors, including diabetes, hypertension, coronary heart disease and heart failure. With respect to echocardiographic parameters, the left ventricular ejection fraction, left ventricular end diastolic

Table 1: Baseline characteristics of structural AF patients

	AF(n=300)	Control(n=300)	P value
Age (years)	67.61±12.56	66.08±12.47	0.133
Male (%)	155 (51.7%)	153 (51.0%)	0.935
Smoking (%)	81 (27%)	78 (26%)	0.853
Diabetes (%)	47 (15.7%)	52 (17.3%)	0.660
Hypertension (%)	48 (16.0%)	43 (14.3%)	0.649
Coronary heart disease (%)	116 (29.0%)	100 (31.3%)	0.202
Heart failure (%)	87 (31%)	94 (24%)	0.374
LVEDD	52.6±4.7	51.8±5.9	0.079
LVESD	35.3±4.1	35.7±4.4	0.215
LEF(%)	55.7±11.3	55.6±13.7	0.894
LV mass(g)	243±97	252±112	0.320

Values are mean ± SD or numbers (%). Probabilities determined by Student unpaired t test or X2 tests. AF, atrial fibrillation; LVEDD, left ventricular end diastolic dimension; LVESD, left ventricular end systolic dimension; LVEF,left ventricular ejection; LV left ventricular; NS, not significant.

Table 2: AGTR1 SNP genotype frequencies and Hardy-Weinberg equilibrium in structural AF patients and normal controls

SNP	allele	geno-type	AF (%)	Control (%)	P	P-HWE (AF/Control
rs1492097	C/T	CC	260 (86.7)	267 (89.0)	P=0.294	0.993 /0.587
		CT	38 (12.7)	33 (11.0)		
		TT	2 (0.6)	0 (0)		
rs1492099	G/A	GG	218 (72.7)	249 (83.0)	P=0.009	0.746 / 0.994
		AG	78 (26.0)	49 (16.3)		
		AA	4 (1.3)	2 (0.7)		
rs1492100	A/T	AA	251 (83.7)	258 (86.0)	P= 0.305	1.000 / 0.348
		AT	47 (15.7)	42 (14.0)		
		TT	2 (0.7)	0 (0)		
rs3772616	G/A	GG	216 (72.0)	212 (70.7)	P= 0.512	0.996 / 0.512
		AG	78 (26.0)	85 (29.3)		
		AA	6 (2.0)	3 (0)		

SNP,single nucleotide polymorphism;AGTR1,Angiotensin II Receptor 1 ; AF,atrial fibrillation; HWE, Hardy-Weinberg equilibrium.

Table 3: AGTR1 SNP allele frequencies in structural AF patients and normal controls

SNP		allele	AF (%)	Control (%)	P	OR	95% confidence intervals
rs1492097	C/T	C	558 (93)	567 (94.5)	0.286	1.290	0.808-2.071
		T	42 (7)	33 (5.5)			
rs1492099	G/A	G	514 (85.7)	547 (91.2)	0.004	1.727	1.154-2.487
		A	86 (14.2)	53 (8.8)			
rs1492100	A/T	A	549 (91.5)	558 (93.0)	0.388	1.234	0.807-1.888
		T	51 (8.5)	42 (7.0)			
rs3772616	G/A	G	510 (85.0)	509 (84.8)	1.000	1.013	0.738-1.390
		A	90 (15.0)	91 (15.2)			

SNP, single nucleotide polymorphism; AGTR1, Angiotensin II Receptor 1; AF, atrial fibrillation.

dimension, left ventricular end systolic dimension and ventricular septal thickness in diastole also did not differ significantly between structural AF patients and controls.

We observed that the genotype frequencies of the polymorphisms (rs1492100, rs1492099, rs1492097, rs3772616) in the AGTR1 gene were not significantly different from Hardy-Weinberg equilibrium in either the structural AF patients or the controls (P>0.05).

Table 4: Genotype frequencies of SNP rs6632677 in ACE2 and Hardy-Weinberg equilibrium in structural AF patients and normal controls

SNP	allele	genotype	AF (%)	Control (%)	P	P-HWE (AF/Control)
rs6632677	G/C	GG	230 (76.7)	250 (83.3)	P=0.121	0.312 / 0.184
		CG	60 (20.0)	42 (14.0)		
		CC	10 (3.3)	8 (2.7)		
rs6632677 (female)	G/C	GG	120 (83.3)	122 (83.6)	P= 0.941	0.295/ 0.166
		CG	19 (13.2)	18 (12.3)		
		CC	5 (3.5)	6 (4.1)		
rs6632677 (male)	G/C	GG	110 (70.5)	128 (83.1)	P= 0.029	0.922 / 0.812
		CG	41 (26.3)	24 (15.6)		
		CC	5 (3.2)	2 (1.3)		

SNP, single nucleotide polymorphism; ACE2, angiotensin-converting enzyme 2; AF, atrial fibrillation; HWE, Hardy-Weinberg equilibrium.

Table 5: Allele frequencies of ACE2 SNP rs6632677 in structural AF patients and normal controls

SNP		allele	AF(%)	Control(%)	P	OR	95% confidence intervals
rs6632677	G/C	G	520 (86.7)	542 (90.3)	0.057	1.438	1.004-2.058
		C	80 (13.3)	58 (9.7)			
rs6632677 (femal)	G/C	G	259 (89.9)	262 (89.7)	1.000	0.978	0.571-1.676
		C	29 (10.1)	30 (10.3)			
rs6632677 (male)	G/C	G	261 (83.6)	280 (90.9)	0.008	1.954	1.196-3.192
		C	51 (16.3)	28 (9.1)			

SNP, single nucleotide polymorphism; ACE2, angiotensin-converting enzyme 2; AF, atrial fibrillation.

The genotype frequencies of SNP rs1492099 in AGTR1 in the structural AF cohort *vs* controls were as shown in Tables 2 und 3. The frequency of the minor allele of SNP rs1492100 in AGTR1 was 8.5% in the structural AF group compared with 7.0% in the controls (t=0.388; OR, 1.234; 95%CI: 0.807-1.888).

The observed genotype frequencies of the polymorphism rs6632677 in the ACE2 gene were not significantly different from Hardy-Weinberg equilibrium in either the structural AF patients or the controls (P>0.05).The genotype frequencies of SNP rs6632677 in ACE2 in the structural AF male patients vs male controls were as shown in Tables 4 und 5.

In patients with structural AF, there were 4 models with AGTR1 and ACE2 genes with MDR analysis. In patients with structural AF, MDR analysis revealed the 4-locus model (rs1492100/rs1492099/rs3772616/rs6632677; P=0.001, prediction error 41.83%; cross-validation consistency 9.8) and the 3-locus model (rs1492099/rs3772616/rs6632677; P=0.27, prediction error 43.83%; cross-validation consistency 10). The 4-locus model had the highest cross-validation consistency and lowest prediction error. Therefore, we chose the 4-locus model as the best model (Table 6).

3. Discussion

Our current study is the first clinical investigation of the significance of the polymorphisms of the AGTR1 and ACE2 genes and their interaction with structural AF.

The RAS is involved in many cardiovascular diseases, including myocardial fibrosis and hypertrophy in hypertensive heart disease (Brilla et al. 1990), congestive heart failure (Weber et al. 1993), myocardial infarction (Hanatani et al. 1995), and cardiomyopathy

Table 6: Multilocus interaction model by the MDR method

Locus No. And Combination	Cross Validation Consistency	Prediction Error,%
2-locus : rs3772616/rs6632677	3.7	49.67
3-locus : rs1492099/rs3772616/ rs6632677	10*	43.83*
4-locus : rs1492100/rs1492099/ rs3772616/rs6632677	9.8#	41.83#
5-locus : rs1492100/rs1492099/ rs1492097/rs3772616/rs6632677	10	45.00

P=0.027 based on 1000 permutations.
#P=0.001 based on 1000 permutations.
MDR ,Multifactor Dimensionality Reduction.

AGTR1 in the structural AF cohort *vs* controls were as follows: GG 72.7 vs 83.0%; AG 26.0 vs 16.3%; AA 1.3 vs 0.7% (P=0.009). In addition, the frequency of the minor allele of SNP rs1492099 in AGTR1 was 14.2% in the structural AF group compared with 8.8% in the controls (t=0.004; OR, 1.727; 95%CI: 1.154–2.487). Our sensitivity analysis showed a significant correlation between polymorphisms of AGTR1 with structural atrial fibrillation in a Chinese Han population. AngII plays a significant role in the structural and electrical remodelling of the atrium, and it therefore contributes to the development of arrhythmia (Li et al. 2009). The major cardiovascular effects of AngII are mediated by AGTR1 (Tsai and Lai 2004). Furthermore, it has been shown that AGTR1 levels are increased in the left atrium of AF patients (Boldt et al. 2003).We hypothesized that the effects of the AGTR1 gene might be one of the molecular mechanisms involved in structural AF.

Table 7: Primer sequences used for multiplex PCR amplification panels

Gene	SNP (rs)	Forward (5' →3')	Reverse (3' →5')	Amplicon size(bp)
AGTR1	rs1492100	TTCAATAACAGATTCCCAGAG	CCACCTCAACTTGCCTGTG	460bp
AGTR1	rs1492099	TTCAATAACAGATTCCCAGAG	CCACCTCAACTTGCCTGTG	460bp
AGTR1	rs1492097	TTCAATAACAGATTCCCAGAG	CCACCTCAACTTGCCTGTG	460bp
AGTR1	rs3772616	TGATAATTTATGTACTCCCTC	CAAAGCATAAGTGTCAACAGA	251bp
ACE2	rs6632677	CTGACTTGTTGCAGCAAGATGC	TAGGAGTCCAGGCACAGTTCAG	218bp

PCR, polymerase chain reaction; SNP, single nucleotide polymorphism; AGTR1, Angiotensin II Receptor 1; ACE2, angiotensin-converting enzyme 2 .

Table 8: Extension probes used for multiplex primer extension

Gene	Positionchang	Variation	Extension probe (5' →3')
AGTR1	rs1492100	[A/T]	TTTTTTTGAGCTCAGCGATTGGATGGCT
AGTR1	rs1492099	[A/G]	GCATTTCAAGCAGATTCCCCAG
AGTR1	rs1492097	[C/T]	GTTATTAAAGTAAAAGAGCAAA
AGTR1	rs3772616	[A/G]	TATCATAGCATGTTGTCGACCT
ACE2	rs6632677	[C/G]	TTTTTTGAAGAGACCATAGCTCTAGCCA

AGTR1, Angiotensin II Receptor 1; ACE2, angiotensin-converting enzyme 2.

(Urata et al. 1993). Reports suggest that RAS activation induces AF (Goette et al. 2000b) in humans and in a dog model of AF. The RAS contains the ACE/AngII/AGTR1 axis and the ACE2/Ang(1-7)/MAS axis, which restrict each other via mutual antagonism to regulate myocardial hypertrophy, fibrosis and remodelling (Ferrario 1990; Lindan et al. 2013; Nicholls et al. 1998).

Originally, the RAS was considered to be an endocrine system, with the ACE/AngII/AGTR1 axis as its functional effector hormone. AngII is the major signalling molecule of the RAS, and its major cardiovascular effects are mediated by AGTR1, including atrial structural and electrical remodelling, to provide substrates for the development of AF (Liu et al. 2011; Madrid et al. 2002; Pedersen et al. 1999; Tsai and Lai 2004). Expression of angiotensin II receptor type 1, but not type 2, is highly upregulated only in the left atrial tissue of patients with rheumatic valvular disease with AF (Cong et al. 2010). Moreover, angiotensin-converting enzyme inhibitors (ACE-Is) and angiotensin II receptor blockers (AIIRBs), which are thought to reduce atrial dilatation, dysfunction and fibrosis, may reduce the propensity for developing AF (Heckbert et al. 2009; Nakashima et al. 2000; Schneider et al. 2010; Wachtell et al. 2005). Recently, Liu et al. (2011) conducted a comprehensive meta-analysis of all available data regarding the association between ACE I/D gene polymorphisms and AF risk. However, no study has been performed to investigate the AGTR1 gene in AF. Our study investigated the association between SNPs (rs1492100, rs1492099, rs1492097, rs3772616) in AGTR1 and structural atrial fibrillation in a Chinese Han population. The genotype frequencies of SNP rs1492099 in

Originally, the functional effector hormone of RAS was AngII. However, the advent of new molecular techniques in recent decades has significantly changed our view of this system, and a new axis, the ACE2/Ang(1–7)/Mas receptor, was established. ACE2 can directly metabolize AngII to generate Ang(1–7). By acting through the receptor Mas, Ang(1–7) promotes vasodilation, antiproliferation and antihypertrophy and has opposing properties to that of AngII (Jessup, 2005). Accumulating evidence indicates that by cleaving AngII into Ang(1–7), ACE2 may play a pivotal role in counterbalancing the vasoconstrictive actions of the ACE/AngII/AGTR1 axis and may be beneficial for the cardiovascular system (Xu et al., 2011). The overexpression of ACE2 inhibits atrial collagen accumulation and improves left atrial remodeling and function in a canine model of atrial fibrillation (Zhou et al. 2015). However, the results of ACE2 genetic studies have been inconsistent. In the current control subjects, the frequency of the allele T of SNP (rs2106809) in ACE2 was approximately 10% in Chinese people, which is much higher than the allele frequency of 3% in USA control subjects in the NCBI dbSNP database. These results indicate that polymorphism (rs2106809) at the ACE2 gene is associated with male lone AF in a Chinese Han population (Wang et al. 2013). No study has investigated the ACE2 gene in nonfamilial structural AF. The heterogeneity test in our analysis showed that genotype frequencies of SNP rs6632677 in ACE2 in the structural AF male patients *vs* male controls were as follows: GG 70.5 *vs* 83.1%; CG 26.3 *vs* 15.6%; CC 3.2 *vs* 1.3% (P=0.029). The frequency of the minor allele of SNP rs6632677 in ACE2 was 16.3% in structural AF male patients compared with 9.1% in male controls

(P=0.008; OR, 1.954; 95%CI: 1.196-3.192). It was hypothesized that the ACE2 gene may be correlated with the risk of structural AF in a Chinese Han male population. In this study, we only found an association of the rs6632677 polymorphism in the ACE2 gene with structural atrial fibrillation in men. The mechanisms by which ACE2 SNPs increase the structural AF risk in males and the exact roles that the ACE2 variants play in AF pathogenesis remain unknown. The potential reasons are as follows. Men are more likely to have a coronary event than women (Talebizadeh et al. 2006). Moreover, the gender-specific effects may be explained by the ACE2 gene mapping to the X chromosome, which may have different effects on women and men.
Furthermore, we found an interaction between the SNP rs6632677 in ACE2 and the SNPs (rs1492100/rs1492099/rs3772616) in AGTR1 by the MDR method in structural AF patients in a Chinese Han population. These two loci were located within two different chromosomes, indicating that the interaction crossed chromosomal boundaries between the ACE2 and AGTR1 genes. The AGTR1 and ACE2 genes restrict each other via mutual antagonism to regulate myocardial hypertrophy, fibrosis and remodelling, and they participate in the occurrence of structural AF. This study suggested that the disequilibrium of AGTR1 and ACE2 might play an important role in the process of AF. In the future, it will be necessary to elucidate how AGTRI and ACE2 contribute to the pathophysiology of structural AF to advance the current understanding of the mechanism of structural AF and provide an opportunity for the future development of anti-arrhythmic drugs.
One limitation of our study should be noted. The sample size is still relatively small and may not provide sufficient power to estimate the association between the ACE2 and AGTR1 genes and structural AF.
In conclusion, these results indicate that polymorphism rs1492099 in the AGTR1 gene is associated with structural AF in a Chinese Han population. It was hypothesized that the ACE2 gene, which maps to the X chromosome, may be correlated with the risk of structural AF in a Chinese Han male population. Furthermore, we found an interaction between ACE2 and AGTR1 in structural AF patients in a Chinese Han population.

4. Experimental

4.1. Study subjects

We enrolled 300 subjects with structural AF and 300 controls for genetic analysis. Patients were recruited from cardiology departments in the First Affiliated Hospital, Harbin Medical University, China between January 2011 and April 2015. The presence of AF was determined by taking the patient's history and a standard 12-lead ECG and/or Holter, in accordance with established guidelines (Boldt et al. 2003). We defined structural AF as described in the study. Patients who had a history of myocardial infarction and significant coronary artery disease, echocardiographic evidence of left ventricular hypertrophy, hypertension, reduced left ventricular systolic function (left ventricular ejection fraction <45%) or severe valvular heart disease were categorized as having structural AF. Exclusion criteria were age <18 or >85 years and hyperthyroidism. Patients with lone AF, familial AF or first episode AF were also excluded. For every AF patient, a matched control without a history of AF was selected from the same geographical area. The controls were individually matched with cases according to the following criteria: age (difference ≤5 years), presence of left ventricular dysfunction (ejection fraction <55%), presence of significant valvular heart disease (at least moderate to severe) and the presence of hypertension or diabetes. All individuals agreed to participate in the study.

4.2. TagSNP selection

The first step of this study was to find SNPs associated with AF. The RAS was associated with AF according to the Genetic Association Database (GAD) and the Online Mendelian Inheritance in Man (OMIM). We used the HapMap (http://hapmap.ncbi.nlm.nih.gov/biomart/martview/) obtained tagging SNPs (rs1492100, rs1492099, rs1492097, rs3772616) in AGTR1 and the SNP rs6632677 in ACE2 for the Chinese Han subjects using a minor allele frequency (MAF) cutoff of 0.05.

4.3. Blood sample measurement

Fasting blood samples (5 mL) were collected from participants at baseline. Plasma and serum were separated from blood cells after 1 h of collection and stored at -80 °C until assay. Blood samples for blood lipids, blood glucose and homocysteine concentrations were collected in empty tubes and analysed by a biochemical laboratory (Shanghai Generay Biotech Co, Ltd.).

4.4. DNA extraction

Genomic DNA was extracted from EDTA-anticoagulated peripheral blood leucocytes by the salting-out method. Briefly, 3 mL of blood was mixed with Triton lysis buffer (0.32 M sucrose, 1% Triton X100, 5 mM $MgCl_2$, H_2O, 10 mM Tris–HCl, pH 7.5). Leucocytes were spun down and washed with water. The pellet was incubated with proteinase K at 56 °C and subsequently salted out at 4 °C using a saturated NaCl solution. Precipitated proteins were removed by centrifugation. The DNA in the supernatant fluid was dissolved in 300 µl water.

4.5. Multiplex PCR amplification

The first step of this study was to design a multiplex PCR assay that included all 5 SNPs among the two genes (Tables 7 and 8). Multiplex PCRs were performed in 15 µl total volume with a Type-it® Mutation detect PCR kit protocol. The thermal cycler conditions were: initial denaturation step at 95 °C for 3 min, 11 cycles of 94 °C or 60 °C for 15 s and 72 °C for 30 s, then 24 cycles at 94 °C (panel 1) or 54 °C (panels 2 and 3) for 15 s and 72 °C for 3 min, followed by a final extension at 72 °C for 3 min. Then, 3 µl PCR products were treated with a mix of 7 µl Exonuclease I (ExoI, Fermentas) and 1 U of Fast AP™ Thermosensitive Alkaline Phosphatase (SAP, Fermentas) at 37 °C for 15 min, and the enzyme was then inactivated at 80 °C for 15 min. PCR quality was evaluated after electrophoresis on a 3% agarose gel.

4.6. SNaPshot analysis

SNaPshot analysis was performed using a SNaPshot Multiplex Kit protocol (Applied Biosystems). Two µl of PCR products were treated with a mix of 6 µl followed by 60 °C for 30 s, then 30 cycles at 96 °C for 10 s or 52 °C for 5 s and 60 °C for 30 s. Detection was carried out using 1 µl of SNaPshot products mixed with 10 µl of HiDi™, 95 °C for 3 s and then an ice water bath. Data were generated after capillary electrophoresis on an automated sequencer (ABI 3730 Genetic Analyser, Applied Biosystems).

4.7. Statistical analysis

SPSS 19.0 was used for statistical analysis. Data with a normal distribution were presented as means ± SD. Characteristics of the data between the case and control groups were compared with Student's unpaired t-test for continuous data and with the chi-squared test for categorical data. Hardy-Weinberg equilibrium of the genotype distribution of the polymorphisms was tested using the chi-squared test. The genotype and allele frequencies between the cases and controls were tested using the chi-squared test. We used multifactor-dimensionality reduction (MDR) for evaluation of gene-gene interactions. $P<0.05$ was used to determine statistical significance, and the OR relative to the allele frequencies and its 95% confidence interval (CI) were calculated.

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Conflicts of interest: None declared.

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