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Original Article

Arg753gln and Arg677 Trp Polymorphisms of Toll-Like Receptor 2 In Acute Apical Abscess

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KEY WORDS
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ABSTRACT

Statement of the Problem: Genetic polymorphisms can alter immunity response against pathogens, which in turn influence individuals' susceptibility to certain infections.

Purpose: Our aim was to determine the association of Arg753Gln (rs5743708) and Arg677Trp (rs12191786) polymorphisms of toll like receptor-2 gene with the two clinical forms of apical periodontitis: acute apical abscess (AAA) and asymptomatic apical periodontitis (AAP).

Materials and Method: There were 50 patients with AAA as case group and 50 with AAP as control group. Genotyping was done using Tetra-ARMS (amplification refractory mutation system) PCR.

Results: Heterozygous genotype of Arg677Trp polymorphism was associated with risk of AAA (OR=1.9, 95% CI: 0.7-5.5, p= 0.05). Although statistically insignificant, Arg677Trp polymorphism promoted the risk of AAA in dominant model (OR=2.1, 95% CI: 0.7-5.9, p> 0.05). The frequency of mutant allele (T) of Arg677Trp polymorphism was higher in AAA (14%) than AAP (7%) subjects (OR=1.7, 95% CI: 0.6-4.7). For Arg753Gln polymorphism, wild homozygous (GG) represented the dominant genotype in both cases (96%) and controls (100%). Variant allele (A) of Arg753Gln polymorphism was identified in 2% of AAA, while no individual represented with this allele in AAP subjects. Individuals with Arg753Gln; Arg677Trp (GG; TC) combination showed an elevated risk of AAA (OR=1.6, 95% CI: 0.5- 4.2, p> 0.05).

Conclusion: Arg677Trp polymorphism of TLR-2 rendered a higher risk for the development of abscesses in apical periodontitis. It is recommended to explore role of this polymorphism in other populations.

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Introduction

Acute apical abscess (AAA) is a severe symptomatic inflammatory condition, which is triggered by bacterial infection of the root canal system. The condition mainly results from the microbial invasion of periapical tissues originated from infected root canal. Dissemination of bacterial toxins and their metabolites into periapical tissues activates a dynamic inflammatory response by

host defense mechanisms. [1] Such extra-radicular infections further spread into sinuses and other spaces within the face, head, and neck exacerbating the clinical presentation. The strength and quality of host's defense mechanisms against pathogens is dependent on both acquired and genetic which alter the susceptibility of individuals to specific types of infections. [2-4]

Some recent studies have evaluated the potential role of genetic variations of genes in immune system in the risk of acute periodontitis (AP), [2, 5-6] external apical root resorption, [7] caries [8] and adult periodontitis. [9] Amaya *et al.* [6] reported that functional polymorphisms in IL-8 gene might contribute to AP pathogenesis. In addition, clinical presentation of AAA has been closely related to IL-6 gene polymorphisms. In addition, polymorphisms of IL-1β were reported to be involved in preservation of periapical lesions in humans and rats models. [9] An association has also been described between persistent post-treatment AAA and the polymorphisms of immunoglobulin receptor genes, Fc**γ**RIIa and Fc**γ**RIIIb. [3] There have been suggestions indicating a role for genetic variations in genes encoding pro inflammatory cytokines, [6, 9-11] and pattern recognition receptors (PRRs) including Toll-like receptors (TLRs) [12-13] in pathogenesis of AP.

TLRs are a family of receptors with at least 13 members (TLR 1-13) which are coded by separate genes. TLRs are expressed by most tissues and cells that have antigen recognition activities. TLR-2 is a subtype of TLR family participating in acute phase response of inflammation. The receptor is highly expressed on the surface of a variety of immune cells [14-17] and it interacts effectively with other TLRs to recognize various microbial antigens. [18] In heterodimer combination with either TLR-1 or TLR-6, TLR2 can recruit nuclear Factor (NF)- κB transcription factor, and induce expression of a wide-range of inflammatory genes. [18-19] From a total of 175 single nucleotide polymorphisms (SNP) identified within TLR-2 gene, only 9 are functional polymorphisms altering the activity of the receptor. [18] Two of these SNPs are Arg753Gln (rs5743708) and Arg677Trp (rs12191786). The both of these polymorphisms reside in cytoplasmic signaling domain, and reduce the receptor activity. [19] These polymorphisms have been reported in association with impaired receptor-linked signal transduction and increased susceptibility to various infections. [14, 20] Furthermore, TLR-2 signaling has been suggested to influence the both duration of inflammation and persistence of bacterial load in periodontal infections. [21] In line, the size of periapical lesions has been greater in experimental TLR-2 gene knocked out rats. [22] These studies suggest a potential role for TLR-2 in influencing biologic features of periodontitis infections.

By taking into consideration the role of genetic polymorphisms in susceptibility to infections, variations in host genotypic signature may be an important predictor of individuals' risk to inflammatory conditions. [5, 23] However, the role of Arg677Trp and Arg753Gln polymorphisms of TLR-2 gene has not been assessed in AAA. In present study, we aimed to evaluate correlation between the Arg753Gln and Arg677Trp polymorphisms of TLR-2 gene and the risk of AAA in an Iranian population.

Materials and Method

This study was a case-control study conducted in 2014-2015. All participants signed an informed consent before entering into the study. The study was approved by the Ethical Committee of Research of Zahedan University of Medical Sciences.

A total of 100 individuals were recruited. The participants were categorized according to radiographic findings and clinical manifestations. Fifty Patients with AAA and 50 age, sex, and ethnic matched patients with asymptomatic apical periodontitis (AAP) served as case and control groups respectively. Sample size was determined based on a previous study on the selected polymorphisms. [24]

Inclusion criteria

The case group complaining of symptomatic dental abscesses, as a result of endodontic infection, and soft tissue swelling, with or without systemic manifestation such as fever; redness; malaise and lymphadenopathy. The involved tooth exhibited varying degree of mobility without response to thermal and electrical stimulus because of necrotic pulp. In radiological study, they showed a slight thickening of the periodontal ligament space (PDL) to a radiolucent lesion. Patients in the control group consisted of individuals with no signs and symptoms and had no history of a previous exacerbation. The teeth presented a thickening of PDL compatib-

Table 1: Primer sequences for amplification of Arg753Gln and Arg677Trp polymorphisms

SNPs	Sequences primers	Products Size
Arg753Gln (G2258A)	F Outer: 5'-GTT GTG TCT TCA TAA GCG GGA CT -3' R Outer: 5'- AGT CCT CAA ATG ACG GTA CAT CC –3' F Inner: 5'- AGC GCT TCT GCA AGC TTC G –3' R Inner: 5'-GTA GGT CTT GGT GTT CAT TAT CTG CT-3'	Outers: 459 A Allele: 271 G Allele: 234
Arg677Trp (C2029T)	F Outer: 5-CTG TGC TCT GTT CCT GCT GAT C-3 R Outer: 5-TGA GAA TGG CAG CAT CAT TGT T- 3 F Inner: 5-CCC TTC AAG TTG TGT CTT CAT ACG T-3 R Inner: 5-TTG CCA GGA ATG AAG TCA CG-3	Outers: 419 T Allele: 200 C Allele: 264

le with periapical lesion; without sensitivity to percussion and did not respond to thermal or electrical stimulation. [6, 10]

Exclusion criteria

Ever smoked patients or those who received prior endodontic treatment or antibiotic treatment within the preceding 3 months were excluded. Furthermore, teeth with an intra or extra oral sinus tract, systemic disease, history of corticosteroid or non-steroidal anti-inflammatory drug (NSAID) usage, hepatitis or HIV infection, receiving previous chemotherapy, radiation or transplant, bleeding and immunodeficiency disorders were also considered as excluding criteria.

Sample collection

Sample collection and genomic DNA extraction were carried out as previously described. [25] Participants were asked to scrape their oral mucosa by moving their tongue throughout the oral cavity. Then, exfoliated mucosal epithelial cells were expectorated into sterile micro tubes including 3 ml TNE solution (17 mM Tris/HCl (pH 8.0), 50 mM NaCl, and 7 mM EDTA) diluted in 66% ethanol. The samples were subsequently transferred to the laboratory for DNA extraction.

DNA extraction

Genomic DNA was obtained from oral cavity epithelial cells. Briefly, tubes including mucosal epithelial cells were initially centrifuged (10 min, 3000 rpm), and supernatant was removed. Then, 1 ml of TNE buffer was used to suspend the pellet containing epithelial cells. Following a second centrifugation (5 min, 2000 rpm), precipitates were shaken intensely, and admixed with 1.3 ml of lysis buffer (10mM Tris (pH 8.0), 0.5% SDS, 5 mM EDTA) and 10 µl of proteinase K., and then incubated for 12 h at 55°C. After that, the solution (1.4 ml) was combined with 500 µL of 8 M ammonium acetate and 1 mM EDTA. Micro tubes were vigorously shaken and centrifuged (10 min, 4000 rpm). Two volumes (900 µl) of supernatant were separately collected into two new 1.5 ml micro tubes containing 540 µl isopropanol. Then, the tubes were inverted at least 20 times, and centrifuged again at 4000 rpm for 5 min. The pellet was recovered and allowed to air-dry before adding 2 ml of 70% ethanol. After a further centrifugation (5 min, 4000 rpm), precipitated DNA was dissolved in 100 µl of TE buffer (10 mM Tris (pH 7.8) and 1 mM EDTA). Presence of the DNA was confirmed by 2% agarose gel electrophoresis. The quantity and purity of the DNA were checked by spectrophotometry and determination of 260/280 nm ratio (Biochrome WPA Biowave II, Cambridge, UK). DNA was labeled as good quality if the ratio was within 1.6-2. After overnight storage in refrigerator, DNA was kept at -20°C until use for PCR experiment.

Polymorphism genotyping

Tetra-ARMS (Amplification Refractory Mutation System) PCR was method of choice to detect the polymorphisms. Four pairs of primers (two outers and two inners) were designed by blasting against NCBI nucleotide databases. Sequences of primers have been presented in Table 1. Arg677Trp polymorphism was further confirmed by direct sequencing (ABI 3730xl sequencer (Applied Biosystems).

PCR reaction

The amount of 50 ng genomic DNA, 1 µl of each pair of primers (1 µMol) and 12.5 µl Taq DNA polymerase master mix (Pishgam, Iran) were mixed in a reaction tube, and net volume was reached to 25 µl by addition of distilled water. PCR reaction was performed under the temperature profile mentioned ahead. Initially, a phase of denaturation at 95°C was done for 5 min. After that, 30 thermal cycles of 95°C for 60 seconds (denaturation), 60°C for 45 seconds (annealing) and 72°C for 60 seconds (extension) were carried out. At the end, a final 5 minutes of extension phase at 72 °C was considered to complete the amplification process. PCR products were electrophoresed on 2% agarose gel to identify the electr-

Table 2: General demographic features of two clinical form of AP; Acute Apical Abscess (AAA) as case group and Asymptomatic Apical Periodontitis (AAP) as control group

Characteristics		Total N=100	Case N=50 n (%)	Control N=50 n (%)	*p* Value
Age(years)	Mean (SD)	31.6 (12.5)	31.4 (12.8)	31.7 (12.3)	0.8*
Sex (%)	Male	50	26 (52)	24(48)	0.8
	Female	50	24(48)	26(52)	
Vacancy (%)	Rural	23	15(34)	8(16)	0.07**
	Urban	77	35(70)	42(84)	
Jaw position (%)	UP	35	17(34)	18(36)	0.5
	Down	65	33(66)	32(64)	
Teeth type (%)	Anterior	14	7(14)	7(14)	0.7
	Premolar	25	14(28)	11(22)	
	Molar	61	29(58)	32(64)	

* Independents sample t-test
** Fischer exact test

ophoretic patterns.

Statistical analysis

Chi-square test, independent sample t test, and logistic regression were used as statistical procedures using SPSS 19 software. We used additive and dominant models in regression analysis. Age, sex and smoking status were treated as covariates when performing logistic regression for obtaining odd ratios. In appropriate cases, Fischer exact test was considered to evaluate the association between nominal variables. Statistical significant threshold was considered at 0.05 level.

Results

Table 2 represents demographic information of the studied population. Frequency of the wild-type homozygous (GG) genotype of Arg753gln polymorphism was 96% in cases and 100% in controls. Wild (G) and mutant (A) alleles of this polymorphism were detected in 98% and 2% of AAA patients respectively. On the other hand, all controls were identified with G allele of Arg753gln polymorphism. For Arg677Trp polymorphism, variant allele (T) showed higher occurrence in AAA (14%) than AAP patients (7%, $p > 0.05$). Genotype frequencies were in Hardy-Weinberg equilibrium for the both polymorphisms. Moreover, Arg753gln: Arg677Trp (GG; CC) represented as the most common genotypic combinations in our study (72% and 84% in AAA and AAP respectively). Other relatively common combination was Arg753gln: Arg677Trp (GG; CT) in both cases (22%) and controls (16%). Table 3 shows distribution of genotypes, alleles and genotypic combinations for Arg 753gln and Arg677Trp polymorphisms. The genotypes and allele frequencies were in Hardy-Weinberg equilibrium. Based on agarose gel electrophoresis, the most of the participants showed heterozygote (CT) pattern for Arg677Trp polymorphism in both case and control groups (94% and 98% respectively). However, because of the possibility of the false electrophoretic patterns re-

Table 3: Frequencies of genotypes, alleles, and genotypic combinations of Arg753Gln and Arg677Trp polymorphisms of TLR-2 gene in Acute Apical Abscess (AAA) patients as case group and Asymptomatic Apical Periodontitis (AAP) as control group

TLR-2 polymorphisms	Genotypes Alleles Haplotypes	Total N=100	Case N=50	Control N=50	*p* Value
Arg753Gln (%)	GG	98	48(96)	50(100)	0.2*
	AG	2	2(4)	0(0)	
Allele	G	98%	98(98)	100(100)	0.2*
	A	2%	2(2)	0(0)	
Arg677Trp (%)	CC	80	37(74)	43(86)	0.2
	TC	19	12(24)	7(14)	
	TT	1	1(2)	0(0)	
Allele	C	89.5%	86(86)	93(3)	0.2*
	T	10.5%	14(14)	7(7)	
Genotypic combinations Arg753Gln: Arg677Trp (753;677)	(GG;CC)	78	36(72)	42(84)	0.4
	(GG;TC)	19	11(22)	8(16)	
	(GG;TT)	1	1(2)	0(0)	
	(AG;CC)	1	1(2)	0(0)	
	(AG;TC)	2	2(4)	0(0)	

* Fischer exact test

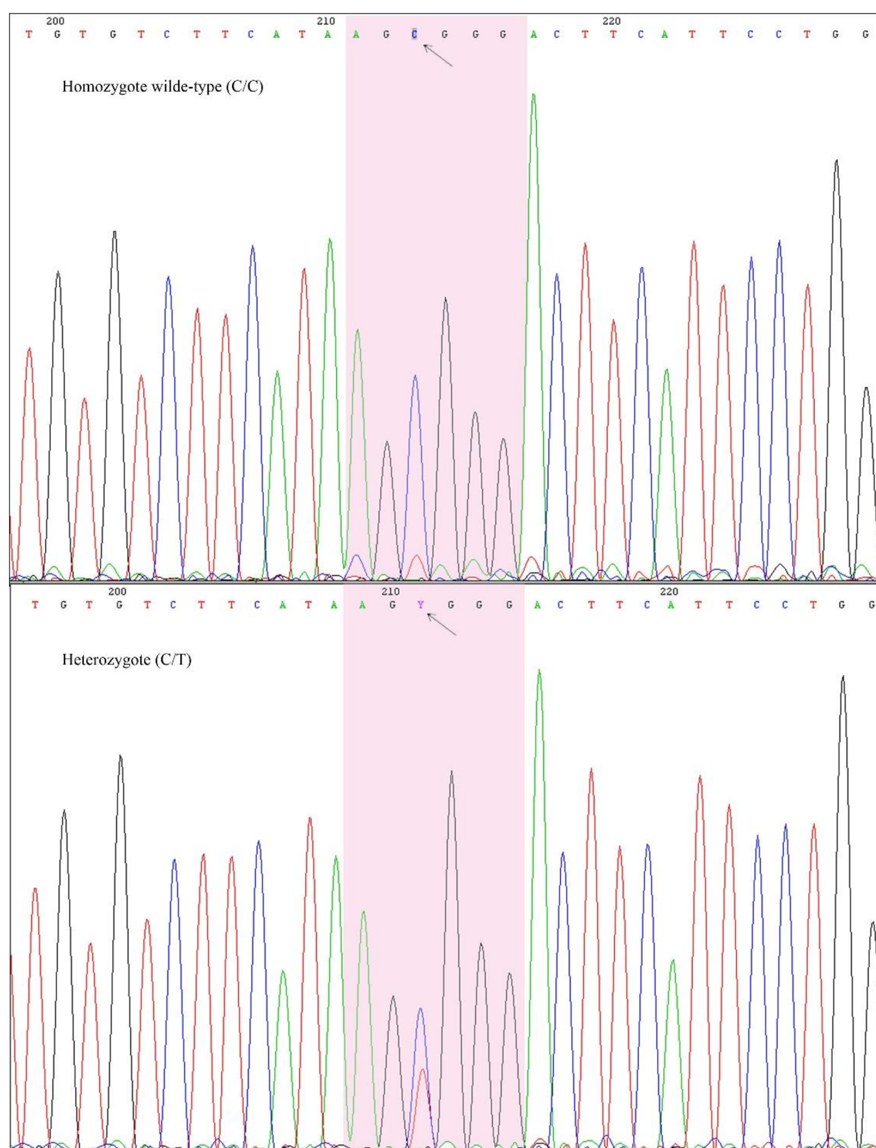

Figure 1: Sequence analysis of Arg677Trp polymorphism of TLR-2 gene. Despite mutated representation of the allele in electrophoretic patterns, sequencing was revealed that the most seemingly heterozygous genotypes were actually wild-type allele homozygotes. Figure shows sequences of wild-type ("C" nucleotide, arrow) and mutant ("Y" ambiguity code, IUPAC nucleotide ambiguity codes, [37] arrow) alleles

ported for this polymorphism, [26-27] we genotyped all of these samples by direct sequencing (Figure 1). Interestingly, wild-type allele homozygous (CC) was revealed as the most recognized genotype (74% and 86% in cases and controls respectively) based on the sequencing results. After sequencing; however, 24% of cases and 14% of controls were confirmed to have true heterozygote (CT) genotype. Overall, mutant homozygote (TT) genotype of Arg677Trp polymorphism was only identified in one individual within the case group.

In logistic regression analysis, heterozygous genotype of Arg677Trp polymorphism was identified as a potential risk factor for AAA in both additive (OR=1.9, 95% CI: 0.7-5.5, p= 0.05) and dominant (OR=2.1, 95% CI: 0.7-5.9, p> 0.05) models. Although the association was not of statistical significance, the mutant (T) allele of Arg677Trp polymorphism may increase the risk of AAA (OR= 1.7, 95% CI: 0.6-4.7, p> 0.05). Table 4 represents the association of the studied variables with the risk of AAA.

Discussion

In current study, Arg753Gln and Arg677Trp functional polymorphisms of TLR-2 were evaluated in two clinical forms of AP, AAA (symptomatic, case group), and AAP (asymptomatic, control group). We observed a higher risk of AAA development in individuals with variant (T) allele of Arg677Trp polymorphism (OR=1.9

Table 4: Logistic regression analysis for estimation risk of studied variables for acute apical abscess formation (AAA clinical form of AP)

	Characteristics	Adjusted OR	(95%CI)	*p* Value
Age(years)	> 25	1.2	0.5-2.9	0.6
Sex	Female	0.8	0.3-1.8	0.6
Vacancy	Rural	2.2	0.8-5.9	0.1
Jaw	Down	1	0.4-2.8	0.8
Teeth	Premolar	1.2	0.3-4.7	0.7
	Molar	0.9	0.2-2.8	0.8
Arg677Trp	TC genotype	1.9	0.7-5.5	0.05
	TC+TT	2.1	0.7-5.9	0.1
	T allele	1.7	0.6-4.7	0.2
Genotypic combination	753;677(GG;TC)	1.6	0.5-4.2	0.3

for heterozygous CT genotype). Nevertheless, variant allele homozygous (TT) genotype was detected neither in AAA patients nor in controls; therefore, predicting of a double risk of AAA for TT genotype was inapplicable. Although our low sample size is a disadvantage and may be partly responsible for observing insignificant results, our sample population is comparable with the sample size of previous studies of these polymorphisms.

As mentioned before, both Arg753Gln and Arg677Trp polymorphisms reside in toll/IL-1 receptor (TIR) signaling domain of TLR-2, and lead to reduced signaling activity of the receptor. [19] TLR-2 signaling contributes to the efficient production of inflammatory cytokines in immune responses. [28] Activation of TLR-2 on gingival epithelial cells, the first cellular line of immunity against periodontitis infections, induces releasing of leukocyte chemo attractants by these cells. [29-30] In accordance, less frequent inflammatory cells and diminished phagocytic activity have been described at periodontitis infected tissues in TLR-2 deficient mice. [31] Arg677Trp polymorphism has also been associated with lower production of IL-12, a critical cytokine for secretion of IFN-γ and activation of T-helper 1 mediated cellular immunity. [14, 32] Regarding such critical roles for TLR-2, reduced activity of this receptor may attenuate the inflammatory immune responses against causative microorganisms in periodontitis, and subsequently facilitate acute presentation of AP.

Above-mentioned statements are in line with our results regarding the higher risk of AAA in presence of Arg677Trp polymorphism. However, these notions are opposed to some observations in precedent studies. High producer polymorphic allele of Interleukin-8 (IL-8), a chemotactic factor for phagocytic cells of immune system, was reported to increase the risk of AAA, while low-producer allele of this polymorphism was associated with AAP. [6] In another study, the higher risk of symptomatic AP was observed in cases with high-output allelic polymorphisms of IL-6 and IL-1 pro inflammatory cytokines. [10] On the other hand, risk of acute clinical presentation in AP has been described in association with low-producer polymorphisms of immune-regulatory mediators. [10] These evidences suggest a higher risk of symptomatic AP in conditions with potentially more intense immune responses. However, this is not in accordance with our results, which indicated a higher risk of AAA in the presence of low-activity polymorphism of TLR-2.

Nonetheless, the effects of a single component of immune system may not be a well representative of variations observed in susceptibility to periodontal diseases, including AP. It has been noted that both destructive and protective reactions take place simultaneously in infected periapical tissues. [33] In addition, recruitment of immune receptors and mediators against microbial agents has been proposed to be dependent on the type of microorganisms and inter-individual genetic variations. [33] In addition, some concurrent virus infections may influence the immune activities against bacterial infections in AP. [33] Regarding complex interactions between immune system components; it seems to be a plausible approach to conduct further risk association studies in AP.

Recently, a review article has been published on distribution of Arg753Gln and Arg677Trp polymorphisms in different populations across the globe. [14] It has been mentioned that these polymorphisms have very low-zero penetrance in European, Asians, Africans and Americans. [14, 34-35] Unlike to this, minor allele of Arg677Trp and Arg753Gln polymorphisms displayed respective overall frequencies of 10.5% and 2% in our studied population. In line with our results, frequency of

the heterozygous genotype of Arg677Trp polymorphism was found in 16% of healthy individuals in a recent study in our region. [36] Altogether, it seems that the penetrance of variant alleles of Arg677Trp and Arg753Gln polymorphisms are especially higher in our population than other areas of the world. This needs to be clarified in future studies.

As mentioned in the result section, the dominant genotype of Arg677Trp polymorphism was revealed heterozygous (CT) based on the electrophoretic patterns. Interestingly, direct sequencing showed that the most cases with heterozygous pattern in gel electrophoresis had actually wild-type homozygous (CC) genotype. This unexpected high prevalence of the heterozygous genotype has also been reported in some precedent studies. [26-27] In fact, presence of a duplicated upstream region homologous to exon 3 of TLR-2 gene has been responsible for the observed false results in electrophoretic patterns. [26-27, 37] As we noted, sequencing analysis for Arg677Trp polymorphism revealed that homozygous wild-type allele rather than heterozygous was the true genotypic signature. Therefore, it is highly recommended to evaluate the Arg677Trp polymorphism of TLR-2 gene by means of high sensitive sequencing procedures.

Conclusion

Our findings suggested that Arg677Trp TLR-2 polymorphism might contribute to the higher risk of AAA. Arg753Gln polymorphism was not as penetrant as Arg677Trp, so possible impact of this polymorphism remained unclear in this study. Larger studies are suggested to reveal a possible significant association between these polymorphism and AAA.

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Conflicts of Interest

None declared.

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