Angiotensin-converting enzyme gene insertion/deletion polymorphism in Saudi patients with rheumatic heart disease

Khalid M. Al-Harbi, MD, *Ibrahim S. Almuzaini,* MD, *Mohamed M. Morsy,* MD, *Nada A. Abdelaziz,* MD, *Alia M. Al-Balawi,* BSc, *Atiyeh M. Abdallah,* MSc, PhD.

ABSTRACT

الأهداف: دراسة مدى العلاقة بين تعدد الشكل الجيني لإنزيم محول الأنجيوتنسين وظهور مرض القلب الروماتزمي بين المرضى السعوديين.

الطريقة: شملت هذه الدراسة المقطعية المرضى السعوديين المصابين بمرض القلب الروماتزمي والذين قسموا إلى مجموعة الشاهد ومجموعة الدراسة. وبلغ عدد عينة الدراسة 99 مريضاً ممن كانوا يرتادون عيادة قلب الأطفال التابعة لمستشفى النساء والولادة، المدينة المنورة، المملكة العربية السعودية وذلك خلال الفترة من مارس 2013م إلى يونيو 2014م. ولقد قمنا بمراجعة السجلات الطبية للمرضى من أجل المطابقة مع معيار جونز لتشخيص المرض، وبعدها تم التأكد من تشخيص المرض باستخدام تخطيط صدى القلب. وقمنا بتحديد نمط تعدد الشكل الجيني لإنزيم محول الأنجيوتنسين بواسطة تفاعل البلمرة المتسلسل.

النتائج: أشارت نتائج الدراسة إلى ظهور فرق في توزيع حامل أليل د لإنزيم محول الأنجيوتنسين وذلك بين مجموعة الدراسة المصابة بمرض القلب الروماتزمي ومجموعة الشاهد (p=0.02, odds ratio = 3.6, 95% confidence interval: 1.2-10.8). ولقد كان هناك ارتباط واضح من الناحية الإحصائية بين ظهور حامل أليل د وظهور أمراض الصمام المترالي (p=0.03).

الخاتمة: أظهرت الدراسة بأن هناك ارتباط بين ظهور تعدد الشكل الجيني لإنزيم محول الأنجيوتنسين وزيادة خطر الإصابة بمرض القلب الروماتزمي بين المرضى السعوديين. لذلك فنحن بحاجة لمزيد من الدراسات لتأكيد هذه النتائج، وتفسير ألية هذا الشكل الجيني.

Objectives: To investigate the association between angiotensin-converting enzyme (ACE) insertion/deletion (I/D) polymorphism and rheumatic heart disease (RHD) in Saudi patients.

Methods: A case-control study was conducted in Saudi RHD patients. Genomic DNA was isolated from 99 RHD patients attending the Pediatric Cardiology Clinic at the Maternity and Children Hospital, Al-Madinah, Saudi Arabia from March 2013 to June 2014, and from 145 age- and gender-matched controls. Patient clinical records were reviewed to report major and minor modified Jones' criteria for diagnosis. The diagnosis was confirmed by echocardiography. The ACE I/D polymorphism was identified by polymerase chain reaction.

Results: A significant difference in ACE D allele carriage (DD+ID) distribution between RHD cases and controls was identified (p=0.02, odds ratio = 3.6, 95% confidence interval: 1.2-10.8). The D allele carriage was significantly associated with development of mitral valve lesions alone (p=0.03).

Conclusion: The ACE I/D polymorphism is associated with an increased risk of RHD in the Saudi population. Further studies are needed to confirm our findings and to understand the molecular mechanisms underlying this association.

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From the Cardiogenetics Unit (Al-Harbi, Abdelaziz, Al-Balawi, Abdallah), Center of Genetics and Inherited Diseases, Taibah University, the Department of Pediatric Cardiology (Almuzaini), Children and Maternity Hospital, the Department of Cardiology (Morsy), Madinah Cardiac Center, Al-Madinah, Kingdom of Saudi Arabia, and the Department of Microbiology and Immunology (Morsy, Abdelaziz), Sohag University, Sohag, Egypt.

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Address correspondence and reprint request to: Dr. Atiyeh M. Abdallah, Cardiogenetics Unit, Center for Genetics and Inherited Diseases, Taibah University, Al-Madinah 30001, Kingdom of Saudi Arabia. E-mail: amabdallah@gmail.com

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Rheumatic fever (RF) is a systemic autoimmune disease that can develop after infection with Group A Streptococcus (GAS). Abnormal immune responses to GAS, most often during childhood, can cause valvular heart damage and lead to rheumatic heart disease (RHD).[1] Rheumatic fever remains one of the most common global causes of acquired heart disease in pediatric populations, especially in developing countries.[2] In Saudi Arabia, Al-Sekait et al[3] assessed the prevalence of RHD among 9,418 school children in the western district of the country. After rigorous clinical and cardiac examination, they found that the prevalence of RHD in this cohort was 2.4 per 1000 children aged 6-15 year old.[3] In another retrospective study in Riyadh covering a period between 1994 and 2003,[4] RF/RHD was still reported as a serious complication despite socioeconomic progress in Saudi Arabia, especially in rural areas with limited medical resources. Carditis is the most serious complication of RF that significantly contributes to morbidity and mortality. Worldwide, 30-50% of patients with RF develop carditis.[5] However, in Saudi Arabian patients, carditis has been reported to occur in 53-65% of patients with RF and, of those, mitral regurgitation occurred in 93.3%.[4,6] Carditis results from chronic inflammation after several attacks of acute RF, causing fibrosis and valve damage.[5] It has been shown that in human hearts, angiotensin-converting enzyme (ACE) mRNA is significantly increased in patients with aortic valve stenosis, which leads to increases in collagen I and III and fibronectin activity.[7] Plasma ACE levels are genetically controlled by an insertion/deletion (I/D) polymorphisms (287bp) in intron 16 of the ACE gene that functions as a quantitative trait locus (QTL).[8] This locus (rs4340) controls up to 44% of the variability in plasma ACE levels.[8] Other genetic variations in ACE have been reported to influence ACE protein levels in different populations, but the functional role of these variations has not be confirmed.[8] The I/D polymorphism in intron 16 of ACE therefore remains an important genetic variation associated with ACE protein levels. The DD genotype carriers have the highest plasma levels of ACE protein, while those with the II genotype have the lowest levels. Heterozygous individuals (ID genotype) have intermediate protein plasma levels, indicating co-dominancy.[8] The ACE I/D polymorphism has been associated with an increased risk of RHD in different populations.[9-11] With respect to Arab populations, only one study from Egypt has reported that the ACE DD genotype is associated with an increased risk of RHD.[12] Since there is a lack of data regarding the role of this polymorphism in the Saudi population, we evaluated the ACE I/D polymorphism in Saudi patients with RHD.

Methods.

Study population. This case-control study was conducted at Taibah University, Al-Madinah, Kingdom of Saudi Arabia. Ninety-nine unrelated RHD patients attended the Pediatric Cardiology Clinic at the Maternity and Children Hospital, Al-Madinah region, Saudi Arabia between March 2013 and June 2014. Using the terms (rheumatic heart disease AND ACE polymorphism), PubMed/MEDLINE, SCOPUS, and Google Scholar were searched for all related literature. Complete and thorough clinical and laboratory assessments were performed in all patients. Diagnosis was made according to modified Jones' criteria at initial diagnosis,[13] and the diagnosis was confirmed by echocardiography. Patients were sub-grouped according to echocardiographic findings into "mitral valve lesion alone" (MVL) or multiple valves lesions including the mitral valve, termed the "combined valve lesion" (CVL). Patients with suspected or confirmed rheumatic fever without valve involvement, other heart complications, and/or other inflammatory conditions were excluded from the study. One hundred and forty-five age-, gender-, and ethnically-matched unrelated healthy volunteers without evidence or family history of cardiac illness or history of rheumatic fever or autoimmune disease were studied as the control group. All participants were of Saudi Arabian ethnicity. The Center for Genetics and Inherited Diseases (CGID) research ethical committee and the Maternity and Children Hospital ethical committee approved the study, and the authors followed the norms of the World Medical Association Declaration of Helsinki.[14] All adult patients and donors and the parents/guardians of children participants (<18 years old) signed fully informed and written consent approved by the committees.

Genotyping of the ACE I/D polymorphism. Genomic DNA was prepared from 2 ml of whole peripheral blood using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Extracted DNA was quantified by spectrophotometry (MaestroNano, MaestroGen, Las Vegas, NV, USA). Samples were stored at -20°C until used. The ACE I/D polymorphism was assessed by detection of the presence or absence of the 287 bp sequence in intron 16 of the gene. The I/D region-flanking primers were used as previously described.[8] This method has been shown to give accurate results compared with other reported methods.[15] Reactions were performed with 10 pmol of each primer: forward primer (5`-CTG GAG ACC ACT CCC ATC CTT TCT-3`) and reverse primer (5`-GAT GTG GCC ATC ACA TTC GTC AGA T-3`).[8] The amplification was carried out in a total of 12.5 µL reaction volume containing 30 ng of genomic DNA added to 2x

colorless Go-Taq master mix that included $MgCl_2$, 10x PCR buffer, dNTPs, and 10 units of Taq DNA polymerase (Cat # M7132, Promega, Madison, WI, USA). Samples were amplified using the Veriti thermal cycler (Life Technologies, Forester City, CA, USA). The cycling and amplification conditions were as follows: an initial denaturation at 95°C for 2 minutes (mins) followed by 35 cycles with denaturation at 95°C for 30 seconds (s), annealing at 58°C for 15 s, and extension at 72°C for 30 s. The polymerase chain reaction (PCR) products were separated on 2% agarose gels after staining with ethidium bromide and visualized with UV light (G:BOX, Syngene, Cambridge, UK). Samples with an insertion allele (I) produced a 490 bp PCR fragment, while the deletion allele (D) was identified by a 190 bp product without the 287 bp Alu insertion. In heterozygous samples, 2 PCR fragments (490 and 190 bp) were detected. Randomly selected samples were repeated to confirm the accuracy of the method. Genotypes were determined by direct counting and labeled as follows: DD homozygous when only the 190 bp fragment was present, II homozygous when only the 490 bp fragment was present, and ID heterozygous when both fragments were present.

Statistical analysis. Statistical analysis of the data was performed using the Statistical Package for Social Sciences (IBM SPSS Statistics for Windows, IBM Corp., Armonk, NY, USA) version 21. All results were confirmed using an online freely available statistical tool (www.vassarstats.net). The unpaired Student's t test was used to compare the mean age between groups. Genotype and allele frequencies were determined by direct counting in patients and controls. The differences in genotype distributions of the polymorphism between cases and controls were analyzed using chi-squared contingency table analysis or Fisher's exact test as appropriate. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated. A *p*-value of <0.05 was considered statistically significant. All genotyping data were checked for any deviation from Hardy-Weinberg equilibrium (HWE) using the chi-squared test.

Results. ***Patient characteristics.*** Two hundred and forty-four Saudi Arabian individuals (145 controls and 99 patients) participated in the study. The baseline characteristics of the RHD patients at disease presentation are shown in **Table 1**. There was no statistically significant difference between the groups in terms of age and gender (**Table 1**). The MVL group included 48 patients, and the CVL group included 51 patients. Carditis was found in 79.8% of patients, and arthritis was found in 63.6%.

Disease susceptibility. The distributions of ACE I/D polymorphism genotype and allelic frequencies

Table 1 - Demographic characteristics and clinical manifestations of the Saudi RHD patients and controls (controls N=145 and patients N=99).

Parameter	Values	*P*-value
Average age (mean ± SD years)		
Controls	20.6±4.5	0.06
Patients	19.4±5.2	
Gender: male/female (%)		
Controls	49.6/50.4	0.45
Patients	54.5/45.5	
Clinical manifestations of patient group		
Valvular lesion: n (%)		
Mitral valve lesion	48 (48.5)	
Combined valve lesion	51 (51.5)	
Carditis	79 (79.8)	
Arthritis	63 (63.6)	
Chorea	8 (8.1)	
Skin rash	3 (3.0)	
Subcutaneous nodules	2 (2.0)	
Recurrence	*NA*	

RHD - rheumatic heart disease, SD - standard deviation, NA - not available

Table 2 - Distribution of angiotensin-converting enzyme I/D polymorphism genotypes and allele frequencies between Saudi rheumatic heart disease patients and controls.

Genotype	Control (N=145)		Patients (N=99)		X^2	*P*-value	OR (95% CIs)
	Count	Frequency (%)	Count	Frequency (%)			
DD	64	(44)	50	(51)	5.7	0.06	
ID	62	(43)	45	(45)			
II	19	(13)	4	(4)			
D	190	(66)	145	(73)	3.2	0.07	1.4 (0.97 - 2.1)
I	100	(34)	53	(27)			
DD+ID	126	(87)	95	(96)	5.7	0.02	3.6 (1.2 - 10.8)
II	19	(13)	4	(4)			
II+ID	64	(56)	49	(49)	0.0	0.96	1 (0.6 - 1.7)
DD	81	(44)	50	(51)			

D - deletion allele , I - insertion allele, OR - odds ratio, CI- confidence interval

Table 3 - Comparison of angiotensin-converting enzyme gene insertion/deletion genotype in mitral valve lesion (MVL) and combined valve lesion (CVL) Saudi rheumatic heart disease patients and controls.

	Control (N=145)	Patients (N=99)		*P*-value†	*P*-value‡	*P*-value§
		MVL (n=48)	CVL (n=51)			
Genotype	Count (%)	Count (%)	Count (%)			
DD	64 (44)	25 (52)	25 (48)	0.07	0.6	0.6
ID	62 (43)	22 (46)	23 (45)			
II	19 (13)	1 (2)	3 (7)			
D	190 (66)	72 (75)	73 (72)	0.09	0.4	0.5
I	100 (34)	24 (25)	29 (28)			
DD+ID	126 (87)	47 (98)	48 (93)	0.03*	0.2	0.3
II	19 (13)	1 (2)	3 (7)			

*Fisher's exact test, †analysis between controls and MVL subgroup, ‡analysis between controls and CVL subgroup, §analysis between MVL and CVL subgroups. D - deletion allele , I - insertion allele

in RHD patients and controls are shown in **Table 2**. Both populations were in Hardy-Weinberg equilibrium for all genotype frequencies. The genotype frequencies between RHD cases and controls were not significantly different, despite a trend towards higher DD genotype and D allele frequencies in patients (**Table 2**). Interestingly, considering a dominant model, patients with DD+ID genotypes had a 3.6-fold increase in risk compared with patients with the II genotype (p=0.02, OR = 3.6, 95% CI: 1.2 - 10.8; **Table 2**).

The ACE I/D polymorphism was also analyzed between the controls and male/female subgroups of RHD patients. No significant differences were observed in the distribution of ACE polymorphism genotype frequency (p=0.1), or allele frequency (p=0.1) in the male subgroup. Likewise, in the female subgroup, no significant difference was observed in the distribution of the genotype frequency (p=0.4) or the allele frequency (p=0.8).

Disease subgroups and severity. To clarify whether the ACE D allele carriage acted as an indicator of disease severity, the possession of a D allele was investigated in relation to disease subgroups. The distribution of ACE I/D genotypes in patient subgroups (MVL and CVL) and the control group is shown in **Table 3**. Considering a dominant model, the frequency of DD+ID in patients with MVL was significantly higher than in healthy controls (p=0.03) (**Table 3**). However, no significant difference was found in the CVL subgroup (**Table 3**).

Discussion. In this case-control study from Al-Madinah, Saudi Arabia, we investigated the association between the ACE I/D polymorphism and RHD. Patients with RHD tended to have higher DD genotypes frequencies than controls; however, the difference did not reach statistical significance. Interestingly, D allele carriage (DD+ID genotype dominant model) was significantly greater in patients than in controls. In addition, D allele carriage was significantly greater in the MVL subgroup compared with controls.

The association of the ACE I/D polymorphism with RHD has previously been reported in several populations. However, the results are inconsistent. With respect to Arab populations, only a single study from Egypt has reported an association between the DD genotype and RHD.[12] Two studies from Turkey[10] and Taiwan[9] have reported an association with the II genotype. This inconsistency may be due to ethnic factors, since the distribution of the ACE I/D polymorphism is known to be different between various ethnic populations.[16] Curiously, a recent meta-analysis of 636 RHD cases and 533 controls that included studies from India, Taiwan, Turkey, and Egypt showed a significant association between ACE D allele carriage and an increased risk of RHD.[11] Moreover, in our cohort, we found that the D allele was associated with the MVL subgroup, whereas the D allele was found to be associated with CVL in an Indian study.[11] However, no significant differences in the distribution of the I/D polymorphism between MVD and CVD subgroups have been observed in the Egyptian and Taiwanese populations.[9,12] It is important to note that in patients with RHD, the cardiac lesions may progress over time to involve more valves and the condition develops from MVL to CVL.

Therefore, we believe that the association between the ACE I/D polymorphism and RHD in different populations and the inconsistent results regarding the association of ACE with MVL and CVL indicate that ACE is linked to the presence instead of the number of valves involved; it may therefore play an important role in the inflammatory stage of the disease. Other genetic factors may modulate tissue damage of the heart valves and disease progression to different subgroups and severity.

Since the identification of the ACE I/D polymorphism as a QTL in 1990,[8] considerable effort has been made in trying to determine whether the I/D polymorphism

is itself responsible for plasma ACE activity or whether it merely represents a surrogate marker.[8] Different methods have been used in different populations to determine the QTL of ACE gene activity.[17] In a recent study, Chung et al[18] identified 4 major haplotype blocks in the ACE gene in the Taiwanese population that were significantly associated with ACE plasma protein levels. Interestingly, they found that the region between exon 13 and intron 18 and a region between intron 20 and the 3` UTR had the most QTL of ACE activity.[18] This indicates that the I/D polymorphism in intron 16 remains an important genetic locus for ACE gene activity. High levels of ACE mRNA and protein have been found in stenotic heart valves.[19] Interestingly, in an animal model, ACE inhibition attenuates aortic valve thickening.[20] Moreover, hypertensive patients carrying the D allele are less responsive to ACE inhibitors, which indicates the strong influence of the D allele in gene expression and disease course.[21] The effect of the ACE I/D polymorphism in patients with RHD requires further study. This may open up the possibility of using ACE inhibitors in the management of RHD and provide an opportunity for pharmacological intervention.

This study has some limitations. It was a case-control observational study with a relatively small sample size that limited the statistical power of the study. Also, we selected only one ACE polymorphism to study. Further work is needed in a larger sample size from different areas of Saudi Arabia that includes screening additional known QTLs in the ACE gene for haplotype analysis. This will be useful for future meta-analyses.

In conclusion, our data suggest that the ACE I/D polymorphism is associated with an increased risk of RHD in the Saudi population. We believe that the ACE I/D polymorphism is linked to the presence of the disease, and may play an important role in the inflammatory phase of RHD. Further studies are needed to understand the molecular mechanisms underlying this association.

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