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Association between the ACE insertion/deletion polymorphism and pterygium in Sardinian patients: a population based case–control study

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ABSTRACT

Objective: The purpose of the study was to examine whether the insertion (I) and/or deletion (D) polymorphism of ACE confers susceptibility to primary pterygium in Sardinian patients in a case–control study.

Methods and results: Polymorphism genotyping was performed by nested PCR using genomic DNA extracted from the whole peripheral blood of participants with (n=251) and without (n=260) pterygium. DD, ID and II genotype frequencies were: 48%, 39% and 13%, respectively, for patients with pterygium, and 15%, 40% and 44%, respectively, for the control group. A statistically significant difference was found between the pterygium and control groups for the *ACE* I/D polymorphism (p<0.001). Moreover, a statistically significant difference was found between the DD and II groups (p<0.01; OR=10.49; 95% CI 6.18 to 17.79), DD+ID versus II group (p<0.01; OR=5.23; 95% CI 3.37 to 8.13) and DD versus ID groups (p<0.01; OR=3.21; 95% CI 2.04 to 5.04).

Conclusions: Statistical analysis showed that the DD genotype is associated with an increased risk of developing pterygium, and with a good chance that the D allele may play an important role in the development of disease.

Strengths and limitations of this study

- In our investigation, we studied the *ACE* I/D polymorphism in pterygium in a pool of Sardinian patients.
- For the first time, we provided an evidence of linkage between *ACE* I/D polymorphism and pterygium risk. This result may be potentially useful in terms of screening and/or prevention.
- In our study, we performed a nested PCR that enhances the sensitivity and specificity of the results.
- Owing to technical and clinical restrictions, we were unable to obtain ACE plasma level. ACE level is well known to be elevated in patients with homozygous DD compared with patients with heterozygous ID or homozygous II.
- In this study, we involved 251 confirmed cases of participants affected by pterygium. This number is enough to get a significant statistical study, but in order to obtain more accurate results, the authors would need to increase the number of participants involved in the study.

INTRODUCTION

The ACE I (or ACE (kininase II, EC 3.74.15.1)) is a zinc metalloproteinase whose main known functions are to convert angiotensin I (Ang I) into a potent vasopressor and aldosterone-stimulating octapeptide angiotensin II (Ang II) and to convert the vasodilator enzyme bradykinin (BK) into the inactive metabolite bradykinin 1–5 (BK1–5). The *ACE* gene is located on chromosome 17q23 and consists of 26 exons and 25 introns spread over ~24 kb. Intron 16 contains a restriction fragment length polymorphism based on the presence (insertion I) or absence (deletion D) of a 287-base pair (bp) non-sense DNA domain *alu* repeat sequence (NCBI ref. SNP ID: rs1799752), resulting in three different genotypes: DD and II homozygote, and ID heterozygote.[1 2]

Over the past decade, studies concerning the possible linkage of the *ACE* I/D polymorphism and the risk of developing several diseases, such as heart failure, breast cancer and diabetic nephropathy (DN), have received considerable attention.[3] The DD genotype is associated with higher levels of circulating ACE than the ID and II genotypes, leading to increased levels of circulating Ang II which are commonly found in patients with myocardial infarction and hypertension. On the other hand, the high

levels of Ang II associated with cardiac hypertrophy and ventricular remodelling in DD patients may be due to its role as a growth factor and immunomodulator.[3–6] The presence of the D allele has been associated with other diseases, including cerebral infarction, atherosclerosis, hypertension, DN, immunoglobulin A nephropathy, pneumonia risk and diabetic retinopathy.[7] Investigations on the presence of the *ACE* I/D polymorphism have also been performed in lung, prostate, breast, gastric and endometrial carcinomas with controversial findings.[8 9]

A recent investigation from our laboratory reported the involvement of ACE I in the pathogenesis of pterygium in the Sardinian population.[10] Pterygium is a chronic, degenerative and hyperplastic disease with inflammatory features, characterised by angiogenesis, cellular proliferation and extracellular matrix remodelling. Pterygium also displays tumour-like features, such as the propensity to invade normal tissue, which are associated with high recurrence rates following resection and may coexist with secondary premalignant lesions.[11]

This study evaluated the association of the *ACE* I/D polymorphism with primary pterygium in a case–control study in a Sardinian population. To the best of our knowledge, there are no data available in the literature regarding the association of the *ACE* I/D polymorphism and pterygium. At the start, our findings demonstrate evidence of the linkage of the *ACE* I/D polymorphism and the susceptibility of developing pterygium in the Sardinian population.

METHODS

Geographic location of the study: outlines

Situated to the west of mainland Italy, Sardinia (population density of 69/km^2) is an island in the Mediterranean sea, located between 38° 51′ and 41° 15′ north latitude and 8° 8′ and 9° 50′ east longitude, with a high ultraviolet radiation exposure.

Patients and control subjects

The study protocol for the use of human subjects in research was approved by the Human Study Ethic Committee of the Medical School, University of Cagliari, Italy. Two hundred and fifty-one patients (age 25–73 years; mean±SD 52.9±11.98) with a surgically and histologically confirmed unilateral primary pterygium were admitted and treated at the Department of Surgical Science, Eye Clinic, University of Cagliari, Italy. The size of the pterygium varied from 4 to 8 mm with a median size of 5 mm. Moreover, only patients with a body translucency (grade T) from T1 to T2[12] were enrolled in this study. Two hundred and sixty-one volunteers (age 33–76 years; mean±SD 50.2±9.02) were considered eligible as normal control subjects for this study. They were recruited from the Department of Surgical Science and University of Cagliari employees. They had to have no history of pterygium and/or other ocular surface diseases, a normal blood pressure in accordance with the WHO criteria[1] and an absence of acute or chronic history of heart or kidney disease. A specific questionnaire was used to obtain all the information listed above. Informed consent was obtained from each participant.

Table 1 Demographic data for the entire cohort

Characteristic	Pterygium n=251	Control n=260	p Value
Baseline mean age (SD)	52.9 (±11.98)	50.2 (±9.02)	0.20
Sex (%)			
Male	129 (51)	134 (51)	>0.05
Female	122 (49)	126 (49)	
Type of lesion			
Primary (♂/♀)	121 (57♂/64♀)	–	>0.05*
Recurrent (♂/♀)	130 (61♂/69♀)	–	
Eye			
Right (♂/♀)	123 (65♂/68♀)	–	>0.05*
Left (♂/♀)	128 (63♂/65♀)	–	
Location of the lesion			
Nasal (♂/♀)	124 (60♂/64♀)	–	>0.05*
Temporal (♂/♀)	127 (65♂/62♀)	–	
Translucency grade			
T1 (♂/♀)	132 (61♂/71♀)		>0.05*
T2 (♂/♀)	119 (65♂/54♀)		

*Comparison between men and women: p>0.05.

The characteristics of the two subject groups are summarised in table 1.

Genotyping

The phenol-chloroform extraction method was performed to obtain genomic DNA starting from 2 mL of peripheral blood leucocytes. Final DNA was diluted into 200 µL of diethylpyrocarbonate water and assessed for spectrophotometric quantification using a Pearl NanoPhotometer (Implen GmbH, Münich, Germany) to determine DNA concentration and purity.

For the detection of the *ACE* I/D polymorphism, we used a nested PCR method. The theoretical melting temperatures (Tms) of all the primers used in this work, the formation of possible oligonucleotide dimers, and self-complementarity were evaluated by the Oligo Program V.7 (MedProbe, Oslo, Norway),[13] and with module 1 of the HYTHER programme (http://ozone3.chem.wayne.edu/),[14] using the subsequent conditions : [DNA] 10-7 M, [Na+] at 5×10^{-2}.

The first-round PCR was carried out using 5′-GACTGCTGAGGCCCTGCAGG-3′ (sense) and 5′-GGACGTGGCCATCACATTCG-3′ (antisense) primers. Samples were subject to an initial start denaturating at 94°C for 2 min, followed by 28 cycles of 94°C for 1 min, 59°C for 1 min and a final extension at 72°C for 3 min.

The second round used the primers pair 5′-CTGTAAGCCACTGCTGGAGAGC-3′ (sense) and 5′-TAGCTCACCTCTGCTTGTAAGGG-3′ (antisense) to amplify a product of 145 bp for the D allele and 432 bp

for the I allele. PCR conditions were the same as in the first round, except that the annealing temperature was 59°C. PCR was initiated starting from 1 µg of DNA for both amplifications in the PCR mixture containing Go Taq Reaction Buffer (1.5 mM $MgCl_2$)[2], 0.25 mM of dNTP and 5 U of Go Taq DNA polymerase (Promega, Madison, Wisconsin, USA). Ultrapure DNase and RNase free water was added to a final volume of 50 µL. Amplification was performed using a GeneAmp PCR System 9700 (Applied Biosystems). Negative control reactions included omitting the DNA template. Amplificons were resolved by agarose gel (2%) electrophoresis and visualised by staining with ethidium bromide. Amplicons for the D allele was 145 bp and for I allele 432 bp band.

Statistical analysis

Genotype frequencies in the patient and control groups were analysed using the standard χ^2 statistics test. The OR was estimated by the logistic regression model adjusted for sex and age, assuming 95% as the CI, to measure the strength of the association between the frequencies of the genotype and the pterygium. Values p<0.05 (two-tailed) were considered statistically significant. Using standard χ^2 statistics, it was tested if the genotype frequencies were deviated from Hardy-Weinberg equilibrium. All data were analysed using GraphPad (V.6.01) computer software and R (V.2.15.2) for Windows.

RESULTS

Figure 1 shows the PCR products of the *ACE* I/D polymorphism. The frequency of the *ACE* I/D genotype is reported in table 2. As regards the DD, ID and II genotypes, they were as follow: 48%, 39% and 13%, respectively, for patients with pterygium, and 15%, 40% and 44%, respectively, for controls. The distribution of genotypes was in the Hardy-Weinberg equilibrium. Statistically significant differences were found between the pterygium and control groups for the *ACE* I/D polymorphism in regard to distribution (p<0.001). As widely reported about the relationship between the D allele and diseases risk,[3] we assumed the D allele as the most favourable allele for the pterygium risk. We compared the DD genotype versus the II genotype. As shown in table 3, logistic regression analysis revealed a significant difference between the two groups (p<0.01, OR=10.49). Moreover, to emphasise our hypothesis about the D allele, we merged the DD and ID groups into a unique group named 'group non-II' and compared it with 'group II'. We found a significant difference with a p<0.01 and an OR of 5.23. Furthermore, we also compared the DD genotype versus the ID genotype. In this case, we found a significant correlation with a p<0.01 and an OR of 3.21.

Moreover, we were not able to find any statistical difference between the studied group in relation to the gender and age in the case group and in relation to the type, location of the lesion and eye involved (p>0.05).

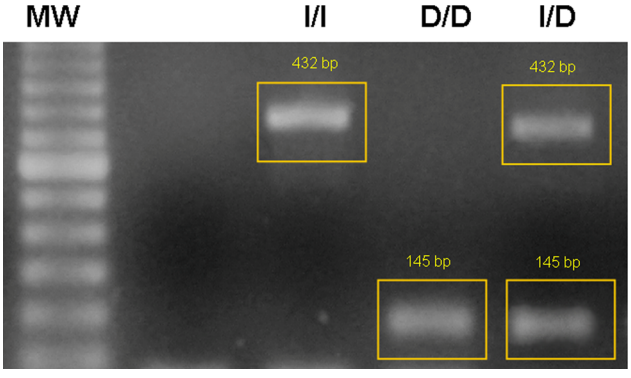

Figure 1 PCR products of *ACE* I/D polymorphism. Specific bands are shown for the I/I genotype (432 bp), D/D genotype (145 bp) and I/D genotype (432, 145 bp).

DISCUSSION

To the best of our knowledge, this is the first study to investigate whether the *ACE* I/D polymorphism is correlated with a risk of pterygium occurrence in a Sardinian population.

The ACE enzyme plays a pivotal role in the renin-angiotensin system (RAS). In the past few decades, the RAS system has expanded beyond its classical and historical roles. It plays a crucial role in the blood pressure control and water and salt homoeostasis.[10] Moreover, it is involved in the pathophysiology of hypertension and structural alterations of the vasculature, kidney and heart, including neointima formation, nephrosclerosis, postinfarction remodelling and cardiac left ventricular hypertrophy (LVH).[15 16] Functional genetic polymorphic variants have been identified for most components of RAS. One of the best known and studied among these is the *ACE* I/D polymorphism. In several studies, the D allele and the DD homozygosity have been associated with an increased risk in different diseases, such as end-stage renal disease (ESRD) in patients with DN, myocardial infarction, coronary disease and atherosclerotic plaque calcification, left ventricular dysfunction after myocardial infarction, lung cancer and colorectal cancer, and in proliferative retinopathy in type 1 diabetes.[1 7 8 17–19]

Our results are consistent with these studies. We verified that the DD genotype was associated with a high risk of developing pterygium compared with the II

Table 2 Comparison of frequency of *ACE* genotypes

Polymorphisms *ACE* I/D (%)	Total n=512	Pterygium n=252	Control n=260	p Value
DD	160 (31)	120 (48)	40 (15)	<0.001
ID	203 (40)	98 (39)	105 (40)	
II	148 (29)	33 (13)	115 (45)	

Table 3 Multivariate logistical regression analysis of case and control groups

ACE Genotype	OR (95% CI)	p Value
DD vs II	10.49 (6.18 to 17.79)	<0.01
group non-II (DD+ID) vs group II	5.23 (3.37 to 8.13)	<0.01
DD vs ID	3.21 (2.04 to 5.04)	<0.01

genotype (OR=10.49). The statistical analysis between the DD and ID groups showed an OR that was clearly lower than that for the DD versus II group (OR=3.21). This result may suggest that patients with an ID genotype may have a lower chance to develop pterygium than patients with an II genotype. Finally, we merged the groups DD and ID, named 'group non-II', and correlated with the group II. Also, in this case, we were able to find a significant statistical correlation (OR=5.23). Observing these data, patients with the DD genotype seem to be more inclined to develop pterygium than patients with the II genotype. Moreover, the correlations observed between DD versus ID and group non-II versus II led us to speculate about a possible 'protective' role played by the ID genotype. Interestingly, in both cases, the OR is substantially lower than the OR relative to DD versus II. Particularly in group non-II versus II, the ID genotype would appear to halve the chances of disease risk compared with the DD genotype versus the II genotype. We do not know the manner in which the ID genotype can decrease the disease risk, but we could hypothesise a possible synergic effect due to the D and I alleles being taken together. This effect may be reflected in a 'protective' role against the disease risk. However, what emerges strongly from the data is the probable central role played by the DD genotype in the disease risk and the possible unfavourable genetic factor represented by the D allele.

The exact mechanisms of how and why the DD genotype may represent a favourable genetic factor for the onset of the disease still remain unknown. Butler[7] provides three plausible explanations on the DD-*ACE* genotype in cardiovascular disease. The author suggests that this genotype may cause alterations in endothelial function, influencing the underlying metabolic control mechanisms, such as insulin resistance, and also the vascular responsiveness to Ang II. In view of this hypothesis, it is possible that a similar explanation may be repurposed in pterygium disease. Some papers showed the relationship between the DD genotype and increased plasma levels of Ang II.[20 21] Increased Ang II levels may lead to vasoconstriction and enhanced peripheral vascular resistance, promote trophic cell changes that influence cell proliferation, apoptosis and tissue fibrosis, and participate in inflammatory responses.[22] Interestingly, these features are characteristic of pterygium. Our data are in agreement with this study;[22] nevertheless, there are several reports that have not found a correlation between the *ACE* I/D polymorphism and disease risk.[23] It is difficult to establish the reason for these discrepancies, but we must certainly consider some aspects in our study in order to explain this heterogeneity. In our investigation, we considered a pool of Sardinian people; the Sardinian population is an ancient genetic isolate with a peculiar distribution of alleles at multiple loci and, as such, offers a unique opportunity for comparative studies on human genome diversity. The history of the Sardinian population is characterised by a prolonged isolation with a small number of founders and by a long history of settlements. The pattern of genetic polymorphisms provides a record of the demographic history of populations.

These factors, together with the pressure of selecting factors (such as malaria), made the island a genetic isolate with a unique and stable distribution of alleles.[24 25]

Some limitations of our study merit emphasis. Owing to the technical clinical restrictions, it was not possible to obtain the serum ACE concentrations; plasma ACE levels are known to be elevated in patients with homozygous DD compared with patients with heterozygous ID or homozygous II.[1 26] A cross-investigation between the ACE I/D polymorphism and ACE plasma levels may help to enhance the strength of our hypothesis about the D allele as an unfavourable genetic factor. In the future, it will be interesting to expand this point in relationship to the increased Ang II levels potentially involved in the pathogenesis of pterygium. Furthermore, in this study, we considered a cohort of 251 pterygium cases and 260 normal control which represent the minimum starting point for a statistical analysis. However, a bigger population would be useful to define more accurately the role of ACE I/D in pterygium.

Despite these limitations, to enhance the method of investigation, for *ACE* I/D genotyping we performed a nested PCR. In the literature, not all studies of the *ACE* I/D polymorphism included a second PCR; this fact may lead to imprecise results. We performed a second amplification where a second set of primers intended to amplify a secondary target within the first run product, which led to enhanced sensitivity and specificity.

In conclusion, this is the first evidence of the linkage of the *ACE* I/D polymorphism and pterygium. What has emerged is that the DD genotype is significantly associated with an increased risk of developing pterygium than the II and ID genotypes. Furthermore, the ID genotype would seem to reduce the disease risk through a feasible synergic action mediated by single alleles. The DD genotype could play an important role overall in the disease risk, although the exact mechanism of how it acts still remains unknown. This study is to be considered as a preliminary 'good start', and further investigations are required to elucidate the exact mechanisms behind the association of the *ACE* I/D polymorphism and susceptibility of developing pterygium in the peculiar Sardinian population.

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Contributors PD designed the research project, studied the research approach method, monitored the data collection, cleaned and interpreted the data, and drafted and revised the paper. GO studied the approach method and revised the draft paper. PC monitored data collection for the whole paper and designed experiments collection tools. LM wrote the statistical analysis plan and revised the draft paper. MC implemented the experiments and monitored the data collection. PS, CM, FP and DM monitored the data collection. IZ selected the patients who were eligible in the study and recruited the biological materials. ED selected the patients who were eligible in the study and recruited the biological materials. SL implemented the experiments and monitored the data collection. MTP analysed the data, and drafted and revised the paper.

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Competing interests None.

Patient consent Obtained.

Ethics approval Faculty of Medicine and Surgery, University of Cagliari, Sardinia, Italy.

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