

# Angiotensin I-converting enzyme ACE 2350\*G and ACE-240\*T-related genotypes and alleles are associated with higher susceptibility to endometriosis

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Endometriosis displays features similar to malignancy, ranging from neovascularization to local invasion and aggressive spread to distant organs. The altered vascular-related genes might be related to the development of endometriosis. This study investigates whether angiotensin I-converting enzyme (ACE) \*A2350G and A-240T gene polymorphisms could be used as markers of susceptibility in endometriosis. Women were divided into two groups: (1) endometriosis group ( $n = 150$ ) and (2) non-endometriosis group ( $n = 159$ ). Genomic DNA was obtained from peripheral leukocytes. ACE A2350G and A-240T gene polymorphisms were amplified by PCR and detected after restriction enzyme digestion with *Bst*UI and *Xba*I. Genotypes and allelic frequencies in both groups were compared. We observed that genotype distribution and allele frequency of ACE 2350 and ACE-240 gene polymorphisms in both groups were significantly different. Proportions of ACE 2350\*A homozygote/heterozygote/G homozygote in both groups were: (1) 66.7/29.3/4% and (2) 96.2/3.1/0.7%. Proportions of ACE-240\*A homozygote/heterozygote/T homozygote in both groups were: (1) 43.3/46/10.7% and (2) 62.9/35.8/1.3%. We concluded that ACE 2350\*G and ACE-240\*T-related genotypes and alleles are associated with higher susceptibility to endometriosis. ACE A2350G and A-240T gene polymorphisms might be associated with endometriosis development.

**Key words:** angiotensin I-converting enzyme/ACE/endometriosis/polymorphism/SNP

## Introduction

Endometriosis, a polygenic/multi-factorial disease, is associated with complex interactions between hormone and cytokine activation, immunoinflammatory processes and genetic factors (Vigano *et al.*, 1998). Endometriosis displays some features of malignancy, including local invasion and aggressive spread to distant organs. Similar to tumour metastases, endometriotic implants require neovascularization to become established, grow and invade tissues. Neovascular processes are prominent in the endometrial tissue. Heritable genetic factors may contribute to the initiation and progression of endometriosis (Treloar *et al.*, 1999).

Cardiovascular genes play a role in the regulation and growth of tumour and altered vascular-related genes might be related to the development of endometriosis. The renin–angiotensin system (RAS) regulates blood pressure through its effects on vascular tone, renal haemodynamics and fluid–electrolyte balance (Fornage *et al.*, 1998). Angiotensin I-converting enzyme (ACE) regulates systemic circulation through angiotensin II formation and kinin metabolism. ACE cleaves angiotensin I to angiotensin II, which is the key component in RAS (Berge *et al.*, 1994). The ACE and RAS genes are related to the regulatory pathway in cardiovascular disease (Zhu *et al.*, 2001), while the ACE gene is implicated as a risk factor in coronary artery disease and myocardial infarction (Zhu *et al.*, 2001).

Gene polymorphisms are useful tools in the study of multi-factorial disorders (Anderson *et al.*, 1994). The analyses of single nucleotide polymorphism (SNP) can be implemented to determine the mechanisms of complex genetic disorders. Numerous chronic disorders, such as endometriosis, osteoporosis, hypertension, diabetes and asthma, have been attributed to genetic susceptibility. Most studies on ACE gene polymorphisms have been focused on their associations with cardiovascular diseases, serum ACE level and blood pressure (Zhu *et al.*, 2001).

Angiogenesis and vascular remodelling play critical roles in the growth, invasion and regression of endometriosis (Donnez *et al.*, 1998). The presence of angiotensin receptors has been demonstrated in the endometrial tissue. Angiotensin II in endometrial stromal cells was mediated via angiotensin I receptors (Braileanu *et al.*, 2002). Angiotensin II could increase the intracellular calcium concentration by interaction with angiotensin receptor in endometrial stromal cells (Braileanu *et al.*, 2001). Vasopressin also stimulates phospholipase C activity in endometrial explants (Braileanu *et al.*, 2001). These findings suggest an underlying contribution of ACE for the development of endometrium and endometriosis. In this study, we aimed primarily to evaluate whether ACE A2350G and A-2240T gene polymorphisms are attractive markers for moderate/severe endometriosis susceptibility. To the best of our knowledge, this is the first survey in this field.

**Table I.** The primer sequences and PCR conditions for ACE A2350G and A-240T gene polymorphisms

Polymorphisms	Primer sequences (5' → 3')*	PCR conditions (°C/s)			Restriction enzyme digestion	Allele	DNA fragment size (bp)
		Denature	Annealing	Extension			
ACE A2350G	F-CTGACGAATGTGATGGCCGC R-TGATGAGTTCCACGTATTTCCG	94/30	62/30	72/45	<i>Bst</i> UI at 60°C for 30 min	A G	122 103+19
ACE A-240T	F-TCGGGCTGGGAAGATCGAGC R-GAGAAAGGGCCTCTCTCTCT	95/30	58/30	72/45	<i>Xba</i> I at 37°C for 30 min	A T	137 114+23

\*F and R indicate forward and reverse primers

## Materials and methods

Premenopausal Taiwan Chinese women with surgically diagnosed endometriosis and non-endometriosis were included. All patients were divided into two groups: (1) endometriosis stage III/IV ( $n = 150$ ); (2) non-endometriosis group ( $n = 159$ ). All individuals with endometriosis accepted laparoscopy or laparotomy management and endometriosis was confirmed pathologically. The non-endometriosis status in the control group was confirmed by sonography and clinical evaluation. All patients had normal blood pressure without obvious cardiovascular disease. There were non-significant differences between both groups in age ( $34.5 \pm 4.2$  versus  $36.2 \pm 5.1$  years), weight ( $51.4 \pm 3.5$  versus  $53.8 \pm 4.2$  kg) and height ( $159.4 \pm 2.5$  versus  $157.4 \pm 3.8$  cm). In clinical practice, most women with minimal/mild endometriosis accept conservative medication instead of invasive management. Therefore, we only recruited the moderate/severe endometriosis women for the survey. All women had consented to peripheral blood sampling for genotype analyses. The studies were approved by the ethical committee and institutional review board of the China Medical University Hospital. Informed consents were signed by all women who donated their blood.

The ACE A2350G genotypes (intron 17) were determined as previously described (Zhu *et al.*, 2001). The genomic DNA was prepared from peripheral blood leukocytes by the use of a genomic DNA isolation kit (Blossom, Taipei, Taiwan). A total of 50 ng genomic DNA was mixed with 20 pmol of each PCR primer in a total volume of 25  $\mu$ l containing 10 mM Tris-HCl pH 8.3, 50 mM potassium chloride, 2.0 mM magnesium chloride, 0.2 mM each deoxyribonucleotide triphosphate and 1 U DNA polymerase (Amplitag; Perkin-Elmer, Foster City, CA, USA). A total of two gene polymorphisms was surveyed, including ACE A2350G and ACE A-240T. The SNP information for the genes involved was obtained via the Internet (<http://www.ncbi.nlm.nih.gov/LocusLink/>).

The PCR primer sequences and conditions for each primer are listed in Table I. The PCR amplification was performed in a programmable thermal cycler GenAmp PCR system 2400 (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). After PCR amplification, the individual gene polymorphisms were analysed by restriction digestion with restriction enzyme (New England Biolabs, Inc., Beverly, MA). The base pairs for their wild, heterozygote and SNP types are listed in Table I.

The PCR products were mixed together and 10  $\mu$ l of this solution was loaded into 3% agarose gel containing ethidium bromide for electrophoresis. Genotypes and allelic frequencies for ACE A2350G and A-240T gene polymorphisms in both groups were compared. Correlation between the ACE A2350G genotype and moderate/severe endometriosis was evaluated. Allelic frequencies are expressed as a percentage of the total number of alleles. The SAS system with  $\chi^2$  and Fisher's exact tests were utilized for statistical analyses. A  $P$ -value of  $<0.05$  was considered statistically significant.

## Results

Genotype distribution and allele frequency of ACE 2350 and ACE-240 gene polymorphisms in both groups were significantly different (Tables II and III). Proportions of ACE 2350\*A homozygote/heterozygote/G homozygote in endometriosis and non-endometriosis populations were (1) 66.7/29.3/4% and (2) 96.2/3.1/0.7%, respectively ( $P < 0.0001$ ; Table II). The percentage of ACE 2350\*A/G alleles in both groups were (1) 81.3/18.7% and (2) 97.8/2.2%,

**Table II.** Genotype and allelic frequencies for ACE A2350G gene polymorphism in individuals with and without endometriosis

Genotype	Endometriosis ( $n = 150$ )	Non-endometriosis ( $n = 159$ )	$P$
A/A	100 (66.7)	153 (96.2)	$<0.0001^a$
A/G	44 (29.3)	5 (3.1)	
G/G	6 (4.0)	1 (0.7)	
Allelic frequency			
A	244 (81.3)	311 (97.8)	$<0.0001^b$
G	56 (18.7)	7 (2.2)	

<sup>a</sup> $P$ -value was calculated by Fisher's exact test.

<sup>b</sup> $P$ -value was calculated by  $\chi^2$ -test.

**Table III.** Genotype and allelic frequencies for ACE A-240T gene polymorphism in individuals with and without endometriosis

Genotype	Endometriosis ( $n = 150$ )	Non-endometriosis ( $n = 159$ )	$P^a$
A/A	65 (43.3)	100 (62.9)	$<0.0001$
A/T	69 (46)	57 (35.8)	
T/T	16 (10.7)	2 (1.3)	
Allelic frequency			
A	199 (66.3)	257 (81.8)	$<0.0001$
T	101 (33.7)	61 (18.2)	

<sup>a</sup> $P$ -values were calculated by  $\chi^2$ -test.

respectively ( $P < 0.0001$ ; Table II). Proportions of ACE-240\*A homozygote/heterozygote/T homozygote in both groups were (1) 43.3/46/10.7% and (2) 62.9/35.8/1.3%, respectively ( $P < 0.0001$ ; Table III). The percentage of ACE-240\*A/T alleles in both groups were (1) 66.3/33.7% and (2) 81.8/18.2%, respectively ( $P < 0.0001$ ; Table III).

The most common genotype and allele for ACE 2350 and ACE-240 gene polymorphisms in both groups were A homozygote and allele. However, the ACE 2350\*G-related genotypes (A/G heterozygote, G homozygote, G allele) and ACE-240\*T-related genotypes (A/T heterozygote, T homozygote, T allele) were strikingly higher among the endometriosis population (Tables II and III). These findings indicated that ACE 2350\*G and ACE-240\*T-related genotype and allele were associated with higher susceptibility to endometriosis.

## Discussion

The endometrium, which has prominent blood vessels and blood flow, is one of the few adult tissues that exhibits regular periods of rapid growth. Angiogenesis, therefore, is an important component for the growth and function of these tissues. Endometriosis is a disease of endometrial tissue shedding outside the uterus during

menstruation. These explants require a rich blood supply which enables them to survive and grow. The activation of angiogenesis, therefore, might be a key factor in pathogenesis of endometriosis (Inan *et al.*, 2003).

ACE activity is associated with angiogenesis. ACE inhibition by perindopril improves myocardial angiogenesis (Toblli *et al.*, 2004). Angiotensin II, a key regulator of blood pressure and body fluid homeostasis, exerts mitogenic effects on endothelial cells. Angiotensin II is also a humoral regulator of peripheral angiogenesis (Walther *et al.*, 2003). ACE catalyses angiotensin II formation; therefore, the ACE activity is positively correlated with angiotensin II production.

Recently, ACE inhibitors were found to significantly inhibit tumour growth and angiogenesis along with suppression of the vascular endothelial growth factor (VEGF) level (Yasumatsu *et al.*, 2004). Angiotensin II might have an important role in carcinogenesis and the anti-angiogenic activity is partly mediated by angiotensin II and ACE inhibition. ACE and angiotensin II inhibitors might be considered as useful anti-tumour agents. However, the mechanisms of suppression of the VEGF level are still unclear. Furthermore, in our previous survey, we also observed a correlation between VEGF and endometriosis (Hsieh *et al.*, 2004). Considering all these observations ACE appears to have angiogenic and tumorigenic effects upon endometriosis.

The ACE gene, which is located on chromosome 17q23, contains some gene polymorphisms and candidate markers for hypertension and related diseases (Doria *et al.*, 1994). The ACE gene polymorphism located on intron 17 of the ACE gene might be in linkage disequilibrium with other important gene variants. ACE gene polymorphisms are related to numerous diseases, including carotid artery wall thickness (Sayed-Tabatabaei *et al.*, 2003), post-transplant erythrocytosis (Yildiz *et al.*, 2003), diabetic nephropathy (Chang *et al.*, 2003), Alzheimer's disease (Kehoe *et al.*, 2003), ischemic cerebrovascular disease (Um *et al.*, 2003), dementia (Choi *et al.*, 2003), segmental glomerulosclerosis (Dixit *et al.*, 2002), cystic fibrosis (Arkwright *et al.*, 2003), etc. ACE insertion/deletion (I/D) polymorphism affects the uteroplacental and umbilical flow as well as the recurrence of an adverse pregnancy outcome in women with pre-eclampsia (Mello *et al.*, 2003). The ACE I/D and M235T polymorphisms are associated with an increased risk of developing coronary heart disease, hypertension and ventricular hypertrophy (Alvarez *et al.*, 2000; Sethi *et al.*, 2003). Three ACE gene polymorphisms [Alu insertion/deletion, 23949 (CT), 10698 (G)] might influence the development of systemic lupus erythematosus and nephritis (Parsa *et al.*, 2002).

In contrast, some investigators have demonstrated the non-association between the ACE gene polymorphisms with individual diseases, including hypertension (Harrap *et al.*, 1993), left ventricular hypertrophy (West *et al.*, 1997), pregnancy outcomes, pregnancy-induced hypertension (Tamura *et al.*, 1996) and nephronophthisis (Omran *et al.*, 1999). ACE-5466C and 4656 gene polymorphisms are not directly related to the occurrence of sarcoidosis (Schurmann *et al.*, 2001). However, the genotype frequencies of the insertion/deletion polymorphisms of the ACE gene and the M235T polymorphism for the angiotensin gene might not contribute to hypertension. This discrepancy might be due to different illness classifications, or racial and disease variations. In fact, different ethnic groups might influence the ACE gene distributions (McKenzie *et al.*, 2001). Ethnic variation plays a major role in the genetic regulation of serum ACE activity and ACE gene polymorphism for cardiovascular disease (Bloem *et al.*, 1996).

Zhu *et al.* (2001) demonstrated that ACE A2350G and A-240T polymorphisms are significantly associated with blood pressure

and ACE concentration. The G allele for the ACE 2350 gene polymorphism is significantly associated with higher blood pressure and ACE concentration (Zhu *et al.*, 2001). They suggested that allelic interaction of these gene polymorphisms might play an important role in the dissection of complex traits such as blood pressure. They also indicated that these associations were more obvious in female individuals than in male patients. Therefore, the gender-specific influence of these gene polymorphisms should be addressed in these studies.

In this study, we noted that the genotype distributions for ACE A2350G and A-240T gene polymorphisms were significantly different between the individuals with and without endometriosis. This finding is the first indicating that ACE gene polymorphisms might predispose to endometriosis development. We also observed that the ACE 2350\*G-related genotypes and G allele appeared in a higher percentage among the moderate/severe endometriosis populations than the controls. This finding is compatible with the result of Zhu *et al.* (2001), who suggested that the ACE 2350\*G allele might be associated with a higher risk of vascular lesion (higher systolic blood pressure) and higher ACE concentrations. Therefore, our data strongly suggest that the ACE gene polymorphisms might substantially contribute to the pathogenesis of endometriosis. The data also suggest that RAS might be involved in the pathogenesis of endometriosis.

The mechanisms of SNPs on individual disease remain uncertain. Despite SNPs not altering transcript levels, some investigators have demonstrated that the disequilibrium effects of certain genotypes might influence the related three-dimensional structure and efficiency of the transcripts (Shintani *et al.*, 1999; Kennon *et al.*, 2004; Shirasawa *et al.*, 2004). Presumably, the distinct biological condition caused by ACE is among numerous contributions that influence endometriosis development. These contributions include genetic, dietary and environmental factors regulating hormonal and non-hormonal conditions. Furthermore, the ACE polymorphisms might be in linkage disequilibrium with other unidentified functional polymorphisms, which co-operatively influence the susceptibility to endometriosis.

In conclusion, associations of moderate/severe endometriosis with ACE\*A2350G and A-240T gene polymorphisms exist. ACE 2350\*G and ACE-240\*T-related genotypes and alleles increase the susceptibility to endometriosis. The ACE gene polymorphisms likely contribute to the pathogenesis of endometriosis. Although the real role and mechanism of ACE gene polymorphisms has not yet been clarified, this polymorphism deserves more attention for realizing its importance to endometriosis development. Furthermore, this study could be extended to determine whether the RAS and its related gene polymorphism also affect the endometriosis formation. After the clarification of its role in endometriosis, ACE gene polymorphism may become a useful marker to predict the future development of endometriosis and to permit early therapeutic intervention in women at high risk of endometriosis.

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