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ACE and *TGFBR1* genes interact in influencing the susceptibility to abdominal aortic aneurysm

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Abstract

A role of *ACE* I/D polymorphism in the pathogenesis of abdominal aortic aneurysm (AAA) has been demonstrated, possibly due to the effect of angiotensin II on vascular tissue remodelling. Angiotensin II exerts profibrogenic effects through the local induction of TGF-β. Dysregulated TGF-β signalling may result from mutations in *TGFBR1* and *TGFBR2* genes, thus resulting in degenerative changes in the vessel wall. We performed a case-control study in order to investigate the role of *TGFBR1* 9A6A polymorphism as predisposing factor to AAA *per se*, and in the presence of *ACE* DD and *AT1R* 1166 CC genotypes in 201 AAA patients (mean age ± S.D., 71.5 ± 6.9) referred to the Unit of Vascular Surgery of the University of Florence, compared with 252 healthy controls (mean age ± S.D., 70.6 ± 8.6). A significant difference in genotype distribution and allele frequency between patients and controls was found for *ACE*, but not for *AT1R* and *TGFBR1* polymorphisms. At univariate analysis a significant association between *ACE* DD, but not *AT1R* CC and *TGFBR1* 6A allele, and the susceptibility to the disease was found [*ACE* DD OR = 1.86 (95% CI 1.26–2.76), $p = 0.002$]. After adjustment for age, gender, traditional cardiovascular risk factors, and CAD, PAD and CVD, *ACE* DD genotype still affected the susceptibility to AAA [OR = 2.13 (95% CI 1.06–4.28), $p = 0.03$], and the contemporary presence of *ACE* DD genotype and *TGFBR1* 6A allele, increased the predisposition to the disease [OR = 5.09 (95% CI 1.44–18.02), $p = 0.01$]. This study, which demonstrates an interaction between *ACE* and *TGFBR1* genes in predisposing to AAA, may provide further information on the mechanisms contributing to AAA susceptibility, and offer a topic for future larger studies.

Keywords: *ACE*; *AT1R*; *TGFBR1* genes; Abdominal aortic aneurysm; Genetic predisposition

1. Introduction

Abdominal aortic aneurysm (AAA), a chronic degenerative condition, is associated with atherosclerosis and characterized by dilation of the aortic wall and segmental weakening.

The pathogenesis of this complex disorder is the result of the interaction between multiple genes and multiple environmental factors, and remains not yet completely defined. Actually, there is increasing information on the relevance of investigating polymorphisms in candidate genes for AAA, in order to evaluate their influence on susceptibility to this condition.

We previously demonstrated a role for the angiotensin converting enzyme (*ACE*) I/D, but not angiotensin II type 1 receptor (*AT1R*) 1166A>C polymorphism, as predisposing factor to AAA [1], probably related to the increased

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expression of *ACE* in human aneurismal aorta. This observation has been strengthened by experimental data, which demonstrated that angiotensin II infusion produces large AAA in apo-E deficient mice [2], and, in a Marfan mouse model, angiotensin II type 1 receptor blockers treatment prevented the aortic aneurysm development [3]. Furthermore, clinical studies demonstrated that in patients with established AAA, ACE-inhibitors augment systemic collagen synthesis and reduce stiffness of the aortic wall [4], and, most importantly, are able to reduce the risk of ruptured AAA [5]. Angiotensin II may affect the vascular tissue remodelling by activation of growth factors and inflammation, through the local induction of TGF-β [6]. Gray et al. [7] showed that angiotensin II stimulates cardiac myocytes to release TGF-β1, thus contributing to cardiac myocyte hypertrophy, and in a mouse model of Marfan syndrome increased TGF-β signalling was demonstrated to contribute to the formation of aortic aneurysm [3]. Really, treatment with TGF-β antagonists, such as TGF-β neutralizing antibody, or angiotensin II type 1 receptor (AT1) blockers, was shown to prevent the dilatation of aorta [3].

Dysregulated TGF-β signalling results from mutations in *TGFBR1*, and *TGFBR2* genes codifying for the serine-threonine protein kinase receptors of TGF-β (TGFβR type I and type II), so leading to an altered phenotype of TGF-β, and in turn resulting in degenerative changes in the vessel wall, which lead to aneurysm formation [8]. A polymorphism (*TGFBR1* 9A6A), which consists of 3 alanines deletion within a polyalanine sequence repeated 6 or 9 times, at the 3′-end of exon 1 in the *TGFBR1* gene has been identified [9]. The 6A rare allele encodes a transmembrane receptor that lacked three alanine residues. The 6A and 9A alleles differ in their signal sequences and not their mature receptors. The altered TGF-β signalling due to 6A allele could influence the pathways that regulate TGF-β signalling [9].

At the best of our knowledge, no data are available concerning the role of the *TGFBR1* 9A6A polymorphism as predisposing factor to AAA. Angiotensin II high levels, related to the presence of *ACE* D allele, may affect vascular tissue remodelling, by activating growth factors, such as TGF-β. Therefore, we carried out the present study, in the same group of AAA patients previously analysed for the *ACE* and *AT1R* polymorphisms [1], in order to investigate the role of *TGFBR1* 9A6A polymorphism as predisposing factor to AAA *per se*, and in the presence of *ACE* DD and *AT1R* 1166 CC genotypes.

2. Methods

2.1. Study population

The current study group, including 201 out of 250 consecutive patients with AAA referred to the Unit of Vascular Surgery of the University of Florence, has been previously analysed for the *ACE* I/D and *AT1R* 1166A>C polymorphisms [1].

AAA was defined as a focal dilation of the abdominal aorta at least 50% larger than expected normal diameter, according to the current report standard [10]. Exclusion criteria were the presence of familial (18%) and/or inflammatory (2%) aneurysms. Familial AAA was considered in patients with one or more first-degree family members with AAA, whereas inflammatory AAA was diagnosed on the basis of operative appearance (presence of extensive perianeurysmal and retroperitoneal fibrosis, and dense adhesion to adjacent abdominal organs).

A group of 252 healthy subjects recruited from partners or friends of patients, and comparable for age and gender was used as control group. All controls had no AAA and a detailed interview addressing personal and family history was performed within a physical examination by expert physicians, in order to identify subjects with no symptoms of vascular disease and to exclude who was suspected of having any kind of vascular disease. All subjects gave informed consent; the study was approved by the local ethics committee and complies with the Declaration of Helsinki.

All subjects underwent duplex scanning examination using an Acuson Sequoia Color Duplex System (Mountain View, CA, USA) with a multi-frequency convex probe, ranging from 5 to 2 MHz. Ultrasound scanning examination was then confirmed in patients by angio-CT scan. Digital subtraction angiography was performed only in patients with concomitant peripheral arterial disease (PAD) and in patients candidate to endovascular treatment of AAA when angio-CT study was not satisfactory to evaluate the feasibility of endovascular treatment.

Aortic diameter was measured below the origin of renal arteries in both groups. The subjects were classified as having hypertension according to the guidelines of European Society of Hypertension/European Society of Cardiology (systolic blood pressure > 140 mmHg, and diastolic blood pressure > 90 mmHg for ≥2 repeated measurements) [11], or if they reported taking antihypertensive medications, as verified by the physicians. Dyslipidemia was defined following the criteria of the ATP III Expert Panel of the US National Cholesterol Education Program, as having fasting serum total cholesterol concentrations > 220 mg/dL, LDL-C > 140 mg/dL, triglyceride > 150 mg/dL, HDL-C < 40 mg/dL [12], or if they reported taking anti-dyslipidemic medications, as verified by the physicians. Smoking status was determined at the time of blood collection: smoking history included past smoking (≥10 years) and current smoking.

In order to assess the presence of coronary artery disease (CAD) patients were evaluated according to the ACC/AHA guidelines [13]. Severe carotid stenosis, confirmed by computed tomography angiography, was defined according to the NASCET criteria [14].

PAD was documented as present if ankle/brachail pressure index was less than 0.9. On the basis of these criteria,

60 (29.8%) patients had clinical evidence of CAD, 18 (8.9%) had cerebrovascular disease (CVD) and 32 (15.9%) PAD. Aneurysmal size was measured by means of computed tomography.

2.2. Detection of ACE I/D, AT1R 1166A>C and TGFBR1 6A9A polymorphisms

Genomic DNA was extracted from peripheral blood leukocytes using a QIAmp Blood Kit (QIAGEN, Hilden, Germany).

The *TGFBR1* 6A9A polymorphism was amplified by PCR amplification using TAQ DNA polymerase (Amersham) and the following primer pair (5′-gac cat gga ggc ggc ggt c-3′ and 5′-gtc gcc ccc ggg agc ag-3′; primers modified from Pasche et al. [9], at an annealing temperature of 62 °C). PCR fragments were loaded on a 4% agarose gel and submitted to electrophoresis for 1 h to separate the different alleles.

The *ACE* I/D and *AT1R* 1166A>C polymorphisms were genotyped as already described [1].

2.3. Statistical analysis

Statistical analysis was performed by using Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA) software for Windows (Version 11.5, Copyright ©SPSS Inc.). Data are expressed as mean ± S.D. or median and range. The non-parametric Mann–Whitney test was used to test for comparison between single groups. Kruskall–Wallis test used for comparisons among different groups. The χ^2-test was used to test for deviation of genotype distribution from Hardy–Weinberg equilibrium.

The number of subjects studied was sufficient to detect, with a statistical power of 80% ($\beta = 0.8$) and significance value of 0.05 (α), absolute differences in *ACE* allele frequencies between patients and controls.

In our previous study [1] we performed the statistical analysis under a recessive genetic model of inheritance. In the present study, due to the lack of subjects (both patients and controls) homozygous for the *TGFBR1* 6A allele, the association between *TGFBR1* polymorphism and AAA was assessed by using a logistic regression analysis under a dominant genetic model, which compares individuals with one or more polymorphic alleles with a baseline group with no polymorphic alleles (e.g. *TGFBR1* 9A6A vs. 9A9A).

All variables that resulted with a p-value < 0.05 in the univariate analysis (hypertension, dyslipidemia, and smoking habit) were introduced into a multivariable model. Variables as age and gender were also included into the multivariable model. Odds ratio (OR) with 95% confidence interval was determined.

3. Results

The demographic and clinical characteristics of the study population are reported in Table 1. A significant difference between patients and controls has been observed in relation to cardiovascular risk factors, and aortic diameter. The genotype distribution and allele frequency of the *ACE* I/D, *AT1R* 1166A>C and *TGFBR1* 6A9A polymorphisms were in agreement with those predicted by Hardy–Weinberg equilibrium. A significant difference in both genotype distribution and allele frequency between AAA patients and controls was observed for *ACE* I/D, but not for the other two polymorphisms analysed (Table 2).

At both univariate and multivariate analysis, no significant association between the *TGFBR1* polymorphism and the susceptibility to AAA was found (Tables 3 and 4). As previously observed [1], at both univariate and multivariate analysis after adjustment for confounding variables, for traditional cardiovascular risk factors (hypertension, smoking habit, dyslipidemia), and the presence of other atherosclerotic disease (i.e. CAD, PAD, and CVD), a significant association between *ACE* DD genotype and the predisposition to the disease was found (OR 1.86, $p = 0.002$ and OR 2.13, $p = 0.03$, respectively) (Tables 3 and 4). Nevertheless, subjects homozygous for the *ACE* D allele and contemporary carrying the *TGFBR1* 6A allele, showed a significant increased predisposition to AAA at both univariate and multivariate analysis (Tables 3 and 4). The contemporary presence of *AT1R* 1166C and *TGFBR1* 6A alleles did not influence the predisposition to the disease (Table 4).

Table 1
Demographic characteristics and traditional cardiovascular risk factors of the study population

Variable	Patients ($n = 201$)	Controls ($n = 252$)	p-Value
Age (years)[a]	71.5 ± 6.9	70.6 ± 8.6	0.1
Males, *n* (%)	175 (87)	216 (85.7)	0.9
Females, *n* (%)	26 (13)	36 (14.3)	0.7
Hypertension, *n* (%)	145 (72.1)	21 (8.3)	<0.0001
Dyslipidemia, *n* (%)	82 (40.8)	32 (12.7)	<0 .0001
Smoking habit, *n* (%)	140 (69.6)	45 (17.9)	<0 .0001
Abdominal aortic diameter (cm)[a]	6.1 ± 1.3	1.9 ± 1.2	<0 .0001

$p < 0.05$, significant value.
[a] Mean ± S.D.

Table 2
Genotype distribution and allele frequencies of *ACE*, *AT1R* and *TGFBR1* polymorphisms

Genotype	Allele	Patients ($n = 201$), n (%)	Controls ($n = 252$), n (%)	p-Value
ACE II		34 (16.9)	59 (23.4)	
ACE ID		82 (40.8)	122 (48.4)	
ACE DD		85 (42.3)	71 (28.2)	0.006
	ACE D	0.63	0.52	0.002
AT1R AA		96 (47.8)	121 (48.0)	
AT1R AC		86 (42.8)	108 (42.9)	
AT1R CC		19 (9.5)	23 (9.1)	0.9
	AT1R C	0.31	0.30	0.9
TGFBR1 9A/9A		170 (84.6)	209 (82.9)	
TGFBR1 9A/6A		31 (15.4)	43 (17.1)	
TGFBR1 6A/6A		0 (0)	0 (0)	–
	TGFBR1 6A	0.08	0.08	0.6

$p < 0.05$, significant value.

Table 3
Univariate analysis for *ACE*, *AT1R* and *TGFBR1* polymorphisms

	OR	95% CI	p-Value
Age	1.02	0.99–1.04	0.2
Gender	0.89	0.52–1.53	0.67
Smoking habit	10.55	6.79–16.40	<0.0001
Hypertension	28.48	16.55–49.01	<0.0001
Dyslipidemia	4.73	2.97–7.54	<0.0001
ACE DD *vs.* ID + II	1.86	1.26–2.76	0.002
AT1R CC *vs.* AC + AA	1.04	0.55–1.96	0.9
TGFBR1 6A[a] *vs.* 9A[b]	0.89	0.53–1.47	0.6
Combined genotypes			
ACE DD × 6A *vs.* (ID + II) + 9A	2.38	1.07–5.28	0.03
AT1R CC × 6A *vs.* (AC + AA) + 9A	1.25	0.17–8.99	0.82

ID + II = subjects with *ACE* ID and II genotypes; AC + AA = subjects with *AT1R* AC and AA genotypes. $p < 0.05$, significant value.
[a] 6A = 6A/9A (lack of 6A/6A homozygotes).
[b] 9A = 9A/9A homozygotes.

As far as aortic diameter is considered, we found no difference among genotypes for the three polymorphisms analysed (Table 5). The contemporary presence of homozygosity for the *ACE* D and *TGFBR1* 6A allele did not affect the aortic diameter (Table 5).

Table 4
Multivariate analysis for *ACE*, *AT1R* and *TGFBR1* polymorphisms

Variable	OR[a]	95% CI	p-Value
Age	1.05	0.98–2.29	0.06
Gender	1.21	0.54–2.72	0.6
Smoking habit	15.45	8.03–29.71	<0.0001
Hypertension	42.66	20.80–87.51	<0.0001
Dyslipidemia	1.18	0.58–2.40	0.6
ACE DD	2.13	1.06–4.28	0.03
TGFBR1 6A	0.76	0.32–1.82	0.5
Combined genotypes			
ACE DD × *TGFBR1* 6A	5.09	1.44–18.02	0.01

$p < 0.05$, significant value.
[a] Adjusted for age, gender, traditional cardiovascular risk factors, CAD, PAD, CVD, and for the *ACE* and *TGFBR1* polymorphisms.

4. Discussion

In this study we contributed to better define the role of the genetic makeup in the pathogenesis of a complex and polygenic disorder, such as AAA. Our study documents a role for *TGFBR1* gene in modulating the predisposition to AAA not *per se*, but only when increased angiotensin II levels are present. Really, the 6A allele of the *TGFBR1* 9A6A polymorphism influences the susceptibility to AAA in patients who were also homozygous for the *ACE* D allele, but not in the presence of the *AT1R* 1166C allele.

We previously demonstrated that *ACE* DD genotype represented a marker of increased susceptibility to AAA [1] apart from traditional cardiovascular risk factors, and hypothesized that increased angiotensin II levels, which are known to be related with a dose dependent effect [15] to the presence of the *ACE* D allele, might lead to the remodelling of vascular tissue by activating growth factors, such as TGF-β transcription and synthesis.

Table 5
Aortic diameters according to *ACE*, *AT1R* and *TGFBR1* polymorphisms

Genotype	Patients ($n = 201$) (mean ± S.D.)	p-Value
ACE		
DD	6.0 ± 1.2	
ID	5.8 ± 1.1	0.09[a]
II	6.3 ± 1.4	
AT1R		
CC	5.6 ± 0.8	
AC	6.0 ± 1.3	0.3[a]
AA	6.0 ± 1.2	
TGFBR1		
6A/6A	–	
6A/9A	6.3 ± 1.2	0.06[b]
9A/9A	5.9 ± 1.2	
ACE DD × TGFBR1 6A combined genotype	6.25 ± 1.28	0.29[b]

Values are expressed in cm. $p < 0.05$, significant value.
[a] Non-parametric test: *p for trend* Kruskall–Wallis test.
[b] Non-parametric test: Mann–Whitney test.

TGF-β represents an important molecular determinant of wall strengthening during vascular development [16], and mediates responses of vessels submitted to injury, by promoting intimal tissue accumulation [17]. AAA shows an inexorable tendency to expand under radial hemodynamic stress [18] with clinically relevant consequences because of vessel rupture. In experimental AAAs animal models, and in an explant model, by using human atherosclerotic incubated AAA fragments, it has been demonstrated that active TGF-β over-expression is able to promote stability of expanding AAA already injured by inflammation and proteolysis [19]. Conversely, data from experimental studies showed that the inhibition of TGF-β signalling favours the development of lesions with increased inflammatory component and decreased collagen content [20], so shifting towards a phenotype depleted in extracellular matrix. TGF-β circulating concentration is dependent on *TGFBR1* gene, which has been recently investigated as a candidate gene in the sporadic AAA onset [21]. TGF-β induces extracellular matrix (ECM) formation by binding to its two receptors TGFβR type I and II [22], and mutations in genes encoding for TGF-β receptors may lead to ECM degradation, activation of matrix metalloproteinases, apoptosis and inflammation [8].

Mutations in *TGFBR2* gene are present in families with Marfan's syndrome [23] or familial thoracic aortic aneurysm and dissection [24], but no data are present concerning the role of *TGFBR1* gene in both familial or sporadic AAA. The *TGFBR1* 6A/9A polymorphism is located at the amino terminus of *TGFBR1* gene, near the cleavage signal sequence of the protein. The lack of the three alanines in the *TGFBR1* 6A allele might induce either the transcriptional inactivation or the alteration of pathways that regulate TGF-β signalling [25], so influencing the role of TGF-β signalling in triggering the responses to aneurysmal wall injury in AAA.

Angiotensin II, the final effector of the renin angiotensin system, through the activation of AT1 receptor, increases TGF-β mRNA levels [26] and up-regulates TGFBR2 expression [6]. Experimental data demonstrated that an over-expression of active TGF-β pathway has a role in re-establishing the aortic wall ability to resist wall stress due to inflammation and proteolysis [19]. Inhibition of TGF-β signalling might accelerate the development of aortic wall lesions because of increasing inflammatory components and decreasing the collagen content, so lacking of its major protective role. Emerging evidence links the renin angiotensin system to the development of aortic aneurysm, and a role for increased angiotensin II levels, related to *ACE* D allele, in influencing the remodelling of vascular tissue, thus determining AAA formation has been suggested [1]. Recently results from a meta-analysis of 26 case-control studies looking at 78 different single nucleotide polymorphisms, and performed in order to analyse candidate genes for AAA, provide evidence for a role of *ACE* gene in affecting predisposition to AAA [27]. Moreover, a large study ($n = 1226$ AAA patients and 1723 controls) investigating 3 geographically distinct cohorts, confirmed a role for *ACE* gene in modulating the susceptibility to the disease, and demonstrated that the *AT1R* 1166A>C polymorphism represented a risk factor for AAA. [28]. The present study does not provide evidence of a role for this polymorphisms in influencing the susceptibility to the disease. This conflicting finding may be due to the different genetic background of the examined populations, and to the different sample size.

Interestingly, experimental studies provided evidence of a benefit of ACE inhibitors treatment in preventing aortic expansion and rupture [29], and clinically relevant data from a large population study ($n = 15{,}326$) showed that ACE-inhibitors therapy was associated with a reduced risk of aortic rupture in patients who had AAA [5]. Moreover, in patients with established AAA, ACE inhibitors therapy is able to augment collagen synthesis and reduce stiffness of the aortic wall [4], and to attenuate angiotensin II-induced atherosclerosis and vascular inflammation [30].

Therefore, based on the above-mentioned observations, we could speculate that the decrease in function of TGFβR1, related to *TGFBR1* 6A allele, may lead to the lack of TGF-β signalling protective role, and, in the presence of high angiotensin II levels, predispose to AAA.

Our findings failed to demonstrate the influence of *TGFBR1* gene on the aortic diameter, as any difference in AAA dilation among *TGFBR1* genotypes has been observed. The same datum, concerning the *ACE* and *AT1R* genes, has been provided in our previous report [1], so strengthening the observation of a genetic role in influencing the susceptibility, but not the severity of the disease.

This study has some limitations. First, *ACE* and *TGFBR1* polymorphisms did not justify more than a part of genetic susceptibility in this complex disorder, suggesting that other candidate genes are certainly implicated in AAA determination. Nevertheless, we observed an interaction between *ACE* and *TGFBR1* genes in influencing the susceptibility to AAA, thus offering a better understanding of the hereditary contribution to the disease. Second, we did not provide information of the ACE phenotype. Genetic case-control association studies look for correlations between phenotype and genotype and represent the most common studies used to evaluate the genetic basis of disease predisposition. Really, some polymorphisms, such as the *ACE* I/D polymorphism, have functionally significant effects on the gene product, and data from experimental studies reported both a functional role for the *ACE* I/D polymorphism in modulating angiotensin II levels [31], and an increased mRNA expression in white blood cells from subjects carrying the *ACE* D allele in comparison to subjects carrying the I allele [32]. Third, the control group consists of clinically healthy subjects with no evidence of atherosclerotic disease, whereas the optimal comparable group would be represented by atherosclerotic subjects with no AAA. Finally, another limitation of the study is the lack of information concerning both the BMI and lifestyle, such as alcohol consumption, which are relevant in affecting the risk of cardiovascular disease, and inflammatory markers, which are involved in the atherosclerotic process.

In conclusion, the present study, which shows a role of *ACE* and *TGFBR1* genes in predisposing to AAA apart from traditional cardiovascular risk factors, may permit to hypothesize a possible mechanism responsible for AAA susceptibility, and may contribute to characterize a genetic pattern of population susceptibility, by using the putative functional polymorphisms in candidate genes. Finally, it might offer an interesting topic for future studies performed in both larger, as well as different ethnic populations.

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