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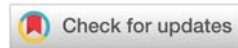
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## Research Article

# The association between copy number variant of Angiotensin Converting Enzyme gene and the risk of Ascending Aortic Aneurysm in Bicuspid and Tricuspid Aortic Valve

## Abstract

**Introduction:** The presence of ACE Insertion/Deletion polymorphism is proposed to be associated with a higher risk for abdominal aortic aneurysm. The aim of this study was to examine a relationship between the occurrence of I/D polymorphism among patients with ascending aortic aneurysm and bicuspid or tricuspid aortic valve structure.

**Material and method:** In this case-control study, 360 patients divided into two groups: 1) patients with ascending aortic aneurysm and bicuspid or tricuspid aortic valve (n= 175), and 2) those without ascending aortic aneurysm as control samples (n=185). The presence of copy number variant (CNV) patterns (Insert/Deletion) of ACE gene was analyzed using real-time polymerase chain reaction.

**Results:** The frequency of the CNV normal pattern (wild genotype) of ACE gene, among the patient with ascending aortic aneurysm with bicuspid and tricuspid aortic valve and control group was 25.4%, 30.2%, and 34.6% respectively. The frequency of CNV loss pattern among the three groups was 59.3%, 41.4%, and 49.7% respectively, while the frequency of CNV gain among those groups was 15.3%, 28.4%, and 15.7% with no statistically significant difference between aneurysm group and control (P-value = 0.117). The frequency of CNV loss genotype among case group with bicuspid aortic valve was significantly higher than tricuspid aortic valve aneurysm group. (P-value =0.031).

**Conclusion:** CNV pattern of ACE gene is not a predicting factor for occurrence of ascending aortic aneurysm with or without bicuspid aorta. However, there seems to exist a correlation between CNV loss and bicuspid aorta.

## Introduction

Ascending aortic aneurysm (AAA), first described in 1928 [1], and is an important risk factor for life threatening dissection and rupture of aorta.

Aortic dilatation is common between adults with bicuspid aortic valves (BAV) with percentage of 33-80 % [2]. The most important pathophysiological factors of dilatation and ascending aortic aneurysm among patients with bicuspid aortic valve include disruption of flow turbulence, The intima and inner medial layer alterations in all jet specimens. dilatation following stenosis and increased stroke volume caused by aortic defect. Furthermore, existence of aortic dilatation increases aortic diameter, reduces aortic wall thickness and rises wall tension culminating in aortic rupture and early death [3,4].

Aortic aneurysm in BAV may not be attributed only to dynamic disturbance in aortic wall. The role of genetic factors and heritability has also been discussed recently [5-7].

Several polymorphisms, mutations, and chromosomal loci have been identified, and additional studies to clarify the specific genes involved in the pathogenesis of AAAs are in progress.

There are recent studies have shown that mutations in NOTCH1 gene and Up-regulation of MMP-2 are involved in ascending aortic aneurysm by its effect on vascular smooth muscle cell apoptosis in BAV [8,9]. Some, reported occasional mutation in MMP9, ACE, MTHFR, and PAI-1 genes and activation of the AKT Pathway in a patient with ascending aortic aneurysm and BAV [10,11].

It has been shown that ACE I/D polymorphism is associated with a 1.33-time higher risk for abdominal aortic aneurysm [12].

Angiotensin converting enzyme ACE (Also known as DCP, ACE1, DCP1, CD143) [13], is a kind of Zinc metallopeptidase largely found on the surface of endothelial cells. ACE plays two major roles in physiological systems. The first one is associated with angiotensin production and the second is about bradykinin degeneration. The Homo sapiens (human) gene of ACE (ENSG00000159640 MIM: 106180) is located on 17q23 chromosome, and contains 26 exons and 25 introns [14]. According to NCBI 626 in/del variants and 7517 SNPs (single nucleotide polymorphism) were identified in this gene [15].

Copy number variations (CNVs) are structural genetic mutations consisting of segmental gains or losses in DNA sequence [16].

rs4646994 is one of the best-known SNPs in ACE gene exhibits an insertion/deletion in Alu repetitive portion in the 6th intron. "I" type alleles represent the insertion, and "D" type has lack of the repetitive component [17].

There are some human and experimental evidences of angiotensin-converting enzyme role in limiting abdominal aortic aneurysm and its deficiency effect on abdominal aortic aneurysm development [18-23].

Few researches have been conducted about occurrence of this polymorphism and ascending aortic aneurysm along with bicuspid aortic valve disease compared to tricuspid aortic valve [24-28].

The current study objective was to examine the correlation between copy number variant pattern (Insertion/Deletion) of ACE encoding gene and the risk of ascending aortic aneurysm among patients with bicuspid and tricuspid aortic valve.

## Materials and Method

From September 2007 to June 2015, more than 30000 open-heart surgeries were performed in Tehran Heart Center as a tertiary center in Iran. In a cross sectional study, 360 consecutive patients of ascending aortic aneurysm and coronary artery who underwent aortic reconstruction surgery by Bentall method and CABG operations included in this project.

175 cases belonged to the ascending aortic aneurysm group (including 59 patients with bicuspid aortic valve and 116 with tricuspid aortic valve) and 185 non-aneurysmal patients described as the control group.

Resorting to the surgery database of Tehran Heart Center, the patient's information including demographic data, coronary artery diseases risk factors, drug history and laboratory parameters was collected. Patient with diagnosis of Marfan syndrome or Marfanoid feature were excluded from study.

### Specimen collection

Aortic tissues were fixed in 10% neutral buffered formalin.

After dehydration in alcohols with different degrees and rehydration in xylene, embedded in paraffin, cut into 3-4- $\mu$ m sections and stained with hematoxylin and eosin (H&E) for light microscopic examination.

Aorta aneurysm diagnosis was made using clinical and imaging criteria and was confirmed by the presence of medial elastic fiber cystic degeneration in H&E and Orcein-Giemsma staining.

### DNA extraction and PCR analysis

Eight 5- $\mu$ m sections of tissue blocks used for DNA extraction based on QIAamp DNA FFPE Tissue kit with application of spin column extraction method (Qiagen Inc., Valencia, Calif.).

ACE Real-time Taq Man PCR assay was performed with the ABI (Applied Biosystem) TaqMan<sup>®</sup> Copy Number Assays Kit (ThermoFisher, USA). In summary, each PCR reaction consisted 1  $\mu$ L of extracted DNA, 10  $\mu$ L of 2x qPCR Master Mix, 2  $\mu$ L of forward and reverse primers and 1  $\mu$ L probe. The final volume was 20  $\mu$ L. Amplification performed in Corbett Rotor-Gene 6000 real-time rotary analyzer (Corbett Life Science Pty. Ltd., Mortlake, NSW, Australia) as following program: one cycle of 60 seconds at 95°C, 40 cycles of 10 seconds at 95°C and 30 seconds at 60°C summarized in Table 1.

Table 1: Conditions of PCR reaction.

Phase	number of cycles	Time (second)	Temperature (°C)
Initial denaturation	1	60	95
Denaturation	40	10	95
Annealing / extension		30	60

The Beta actin as housekeeping gene that is known to exist in two copies in a diploid genome and no template control (NTC) were used in each run of PCR for relative quantification and prevention contamination respectively. The results were interpreted by PCR Amplification plots.

Copy number variation (CNV), defined as gains or losses of a DNA segment larger than 1 kb. The number of copies of the target sequence in each test sample was evaluated by  $\Delta\Delta$ CT method. This method measured the CT difference ( $\Delta$ CT) between target (ACE) and reference (Beta actin) sequences, then compared the  $\Delta$ CT values of test samples to calibrator sample(s) known to have two copies of the target sequence. The copy number of the target was calculated to be two times the relative quantity.

$$\text{copy number}_{\text{sample}} = \text{RQ}_{\text{sample}} \times \text{copy number}_{\text{calibrator}}$$

$$\text{RQ} = 2^{-\Delta\Delta\text{Ct}} = 2^{-[(\text{CT}_{\text{ACE}} - \text{CT}_{\text{ACE Ref}}) - (\text{Ct}_{\beta\text{.Actin}} - \text{Ct}_{\beta\text{.Actin Ref}})]}$$

Finally, three patterns of copy number variants were determined and specified as: wild pattern when CN = 1, CN loss pattern when CN = 0, and CN gain pattern when CN > 1. ACE polymorphism patterns were compared in case and control groups.

## Data analysis

Mean  $\pm$  standard deviation (SD) were used for continuous variables and case and control groups comparison performed, using Student's t test for independent samples or Mann-Whitney U-test.

To assess associations between categorical variables in patient groups, and ACE genotypes, the chi-square test (or Fisher's exact, as appropriate) was used. Statistical analyses were made using the SPSS software version 20.0 and SAS version 9.1 for windows.

## Results

Of the 360 patients included in the analysis, 175 cases belonged to the ascending aortic aneurysm group (including 59 patients with bicuspid aortic valve and 116 with tricuspid aortic valve) and 185 non-aneurysmal patients described as the control group.

The mean age in the case and control group was  $57.8 \pm 13.9$  years and  $56.6 \pm 12.6$  years respectively and 68.6% and 69.2% were male with no statistically significant difference between two groups. (p-value = 0.223 and).

In univariable analyses, the ACE alleles were not statistically different in patients with and without aneurysm. Figure 1 showed three groups of patients with 1) ascending aortic aneurysm with bicuspid aortic valve, 2) ascending aortic aneurysm with tricuspid aortic valve and 3) control group. The frequency of wild pattern was 25.4%, 30.2% and 34.6% respectively, and the frequency of copy number variant loss was 59.3%, 41.4%, and 49.7%. The frequency of number variant gain was 15.3%, 28.4% and 15.7% respectively. There was a statistically significant (P-value = 0.031) difference between the three groups and the frequency of CNV loss in the group with bicuspid valve was more than what was observed in the other two groups.

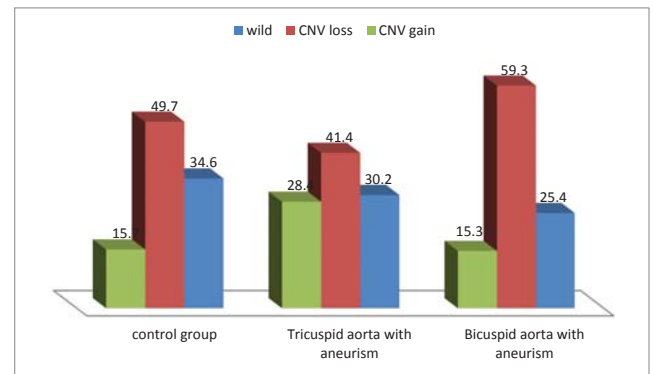
Concerning CNV patterns (I/D) of ACE gene, the frequency of wild pattern in case and control group was 28.6% and 34.6% respectively. The frequency of CNV loss was 47.4% and 49.7% respectively, while this frequency in CNV gain was 24% and 15.7% with no statistically significant (p-value = 0.117) difference between the two groups (Figure 2).

The demographic features of the persons with and without aneurysm are presented in Table 2. Patients in the aneurysm group were older with high percentage of men in Non-aneurysm group.

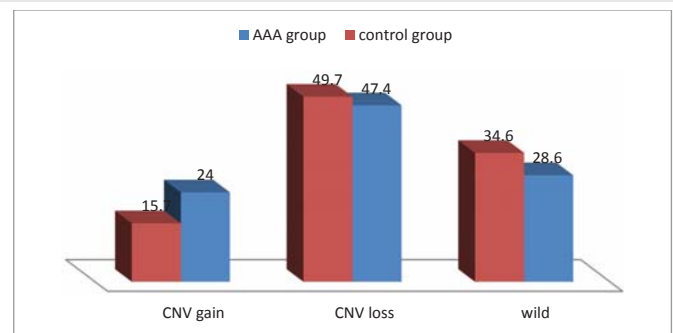
The prevalence of hypertension, hyperlipidemia and diabetes was no statistically different between two groups.

## Discussion

In the current study, we observed an association between ACE polymorphism in form of CNV loss, with the risk for the presence of ascending aortic aneurysm in patient with bicuspid aortic valve in an Iranian population. It seems that the frequency of ACE gene CNV varies widely in different



**Figure 1:** Frequency of genotype variants of ACE among three groups of patients with ascending aortic aneurysm with bicuspid aortic valve, patients with ascending aortic aneurysm with tricuspid aortic valve and non-aneurysm control group. (P-value = 0.031).



**Figure 2:** Frequency of genotype variants associated with I/D ACE polymorphism of the group with ascending aortic aneurysm (AAA) and the control group. P-value = 0.117).

**Table 2:** The demographic features of the persons with and without aneurysm.

Variables	Aneurysm group (n=175)	Non-aneurysm group (n=185)	p-value
Male sex	68.6%	69.2%	0.957
Age (years)	$57.8 \pm 13.9$	$56.6 \pm 12.6$	0.223
Hyperlipidemia	34.3%	22.7%	0.069
Diabetes mellitus	15%	14.1%	0.254
Hypertension	37.1%	25.9%	0.098

surveys with various sample sizes, between abdominal versus ascending aorta, between humans and animals, and in different geographical regions [19–23].

In Meta-analysis research by Song et al, on 3557 cases of abdominal and thoracic aorta and 5231 controls, the association between the genotype of ACE I/D and the risk of aortic aneurysm has been significant (OR = 1.30; 95% CI, 1.07–1.57; P < 0.01). This correlation was significant based upon various races among Caucasians (OR = 1.31; 95% CI, 1.07–1.61; P < 0.01). However, the results were not significant about ascending aortic aneurysm (OR = 1.33; 95% CI, 0.85–2.07; P = 0.21) [24].

Lesauskaite et al. investigated the role of angiotensin-converting enzyme and matrix metalloproteinase-3 in the development of dilatative pathology of ascending thoracic aorta in Lithuania on 107 patients and 773 controls and found

a possible protective mechanism for the combined effects of these two enzymes [25].

In a study conducted in China, ACE mRNA expression and plasma levels were intensely reduced in both thoracic ascending aortic aneurysm and dissection with ( $p < 0.05$  and  $p < 0.001$ , respectively [21]. In another research by Jing et al, on Chinese Han population, the frequency of DD genotype and D allele of ACE gene increased significantly among patients with thoracic aortic dissection (TAD) and after adjusting for conventional vascular risk factors, the association between the ACE I/D polymorphism and the susceptibility to TAD (OR 2.14, 95 % CI 1.38–3.32,  $P = 0.001$ ) confirmed [27]. Pisano et al reported a significant correlation between ACE I/D polymorphism and the risk of ascending aorta aneurysm among 24 patients with bicuspid valve [28]. In a research by Foffa et al, on 216 patients with thoracic aortic aneurysm, 312 patients with CAD and 300 healthy controls the frequency of the ACE insertion/deletion among those with thoracic aortic dissection was significantly different. And incidence of allele D was greater both in the bicuspid aortic valve patients and tricuspid aortic valve patients ( $P = .0008$ ) and ( $P < .0001$ ) respectively [29].

In contrast to some studies and in line with some other studies, we observed association between different ACE copy number variant pattern and TAA in bicuspid aorta instances.

The point of emphasize is that many previous studies worked on ACE polymorphism with respect to variation in one or more nucleotides. The present study focused on larger fragments of ACE genome using Taqman assay to increase sensitivity and specificity of test performance.

### The study limitations

Notwithstanding considerable number of case and control, lack of echocardiography and CT-angiography information for all patients is a limitation of the present study. Another is relative quantification of ACE PCR product instead of absolute quantification due to Kit and reagent selection.

### Conclusion

Existence of copy number variant (Insertion/Deletion I/D) of ACE gene is not a favorable predicting factor for occurrence of ascending aortic aneurysm with or without bicuspid aorta, but the correlation between this polymorphism and abdominal aortic aneurysm has been previously proven. As a result, ACE pattern plays different roles in various parts of aortic artery which proves the fact that the risk factors causing aneurysm in various parts of aortic artery are different. A major and noticeable finding of our research was the copy number variant loss pattern may be correlated with existence of bicuspid aorta. This claim requires further research.

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