

## Core Promoter Variants (A-20C, T-18C and G-6A) of the Angiotensinogen (AGT) Gene are not Significantly Associated with Hypertension in Patients of Tamilnadu, India

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**ABSTRACT** Hypertension is a complex multifactorial disease, which affects 10-30 % of the world population. The genetic factors of the hypertension vary from population to population. Renin angiotensin system (RAS) genes play a key role in salt-water homeostasis and blood pressure regulation. The genetic polymorphisms of the angiotensinogen (AGT) gene are associated with hypertension in different populations and some of these polymorphisms (G-6A and M235T) have a significant role in the human evolution and selection fitness. The present study was carried out to find out the role of the core promoter variants {-20(A-20C), -18(T-18C) and -6(G-6A)} in causing hypertension in rural and urban hypertensive patients of Tamilnadu. Methods: A total of 254 hypertensive and 254 normotensive subjects were screened using PCR-SSCP and RFLP methods followed by DNA sequencing. The sequences were analysed by BLAST. Results: The genotype frequencies of A-20C (AA,AC,CC), C-18T (CC,CT, TT) and -6G(AA,AG,GG) in patients/controls respectively were 63.0,33.0,4.0/67.5,28.5,4.0; 99.0,1.0,0.0/99.6,0.4,0.0 and 83.0,17.0,0.0/81.6,18.5,0.0 Observation and Conclusion: None of these promoter variants were significantly associated with hypertension. The A allele of G-6A polymorphism was found in a high frequency in patients and controls (about 91%) which correlates with frequencies observed in African and Asian ethnic populations (80-95%).

### INTRODUCTION

The renin angiotensin system (RAS) plays a central role in the pathogenesis of vascular diseases. The genes that regulate the system may contribute to the development of hypertension and end organ damage (Redon et al. 2005; Marcianti et al. 2007). The major active peptide of the RAS is angiotensin II. The precursor molecule angiotensinogen is cleaved by the enzyme Renin which produces angiotensin I. Later this angiotensin I is cleaved by Angiotensin Converting Enzyme (ACE) to produce the angiotensin II. It regulates the blood pressure and salt-water homeostasis. The majority of angiotensin II functions are mediated through

angiotensin II type 1 receptor (AT1R) (Chaves et al. 2001).

Jeunemaitre et al. (1992) first reported association between AGT gene and essential hypertension in the populations of Salt Lake city, USA and Paris, France. Later several investigators found positive correlation between AGT polymorphisms and hypertension (Bennett et al. 1993; Hata et al. 1994; Kamitani et al. 1994; Chiang et al. 1997; Inoue et al. 1997; Hegele et al. 1997; Jeunemaitre et al. 1997; Hunt 1998; Yanai et al. 1998; Zhao et al. 1999; Nakajima et al. 2004). Other investigators failed to find any association (Province et al. 2000; Shlyakhto et al. 2001; Rotimi et al. 2003) in studies carried out on Caucasians, Asians and African populations. However, due to the uneven distribution of the human AGT variants among different races and the multifactorial nature of hypertension, comprehensive knowledge on the factors contributing to hypertension remains unknown in majority of the cases.

Yanai et al. (1997) identified the critical

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promoter region between TATA box and the transcriptional initiation site, and named it as angiotensinogen core promoter element 1 (AGCE1), in which three polymorphic sites A-20C, C-18T and G-6A were identified. Several studies found that G-6A was in complete linkage disequilibrium with M235T (Methionine to Threonine missense mutation at codon 235 of the exon 2 of *AGT* gene) and that the haplotype combining both polymorphisms was associated with hypertension (Jeunemaitre et al. 1997; Nakajima et al. 2002; Nakajima et al. 2004; Li et al. 2007). Sato et al. (2000) found that C-18T polymorphism is significantly associated with hypertension in Japanese than other polymorphisms. Tiago et al. (2002) reported that A-20C polymorphism has no direct effect on either systolic or diastolic blood pressure. The *in-vitro* analyses of human *AGT* transcriptional activity suggested that the A to G change at the position -6 leads to decreased *AGT* promoter activity, indicating that this could be a phenotype causing hypertension (Cvetkovic et al. 2002). Similarly, Cui et al. (1998) reported that the A-20C polymorphism affects estrogen binding element in core promoter region. The G-6A polymorphism of the *AGT* gene has been linked with increased body weight gain in hypertensive patients (Chaves et al. 2002).

### METHODOLOGY

All the samples were selected based on the 6<sup>th</sup> (1999) and 7<sup>th</sup> (2003) JNC reports and WHO-ISH guidelines for management of hypertension (Chalmers et al. 1999). Institutional Human Ethical Committee's approval was obtained. The clinical investigations were carried out by qualified physicians and informed consent was obtained from all the patients and controls. Five ml of venous blood was collected from hypertensive patients (n = 254) and controls (n = 254) (127 males and 127 females each) between the age group of 20-80 years. Patients samples were collected from two different areas :

1. Govt. Hospital, Head Quarters Dindigul, Tamilnadu (rural patients n = 155) and
2. K.S. Hospital, Kilpauk, Chennai, Tamilnadu (urban patients n = 99).

The age and sex matched control samples (254) were collected from the same area (rural controls n = 161 and urban controls n = 93).

DNA was extracted from the buffy coat of

EDTA anti-coagulated blood by using Miller et al. (1988) salting out method. The polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis was carried out for screening the core promoter variants. The sequences of the forward and reverse primers were 5'AGAGGTCCCAGCGTGAGTGT 3' and 5'AGACCAGAAGGAGCTGAGGG 3' respectively. Reactions were performed in a final volume of 20 ml containing 100- 150 ng genomic DNA, 2.5 mM dNTP mix, 150 ng/ml of forward and reverse primers each, 10X buffer with 1.5 mM of MgCl<sub>2</sub> and 0.5 unit of the *Taq* DNA polymerase. Thermal cycling was carried out as follows using Eppendorf thermal cycler: initial denaturation at 94°C for 3 minutes followed by 30 cycles with denaturation at 93°C for 30 sec, annealing at 58°C for 30 sec and extension at 72°C for 30 sec. The final extension was carried out at 72°C for 5 min.

The PCR amplicons were subjected to the SSCP analysis by modified Peng et al. (1995) protocol. Silver staining was done by using modified Wallace et al. (1997) protocol. The SSCP was carried out in a composite gel (agarose 0.1%+acrylamide: bisacrylamide (39:1)). Each PCR amplicon 3µl was diluted with 3 µl of denaturing dye (0.1% Bromophenol blue, 0.1% Xylene cyanol, 90% Formamide, 20 mM Na<sub>2</sub>EDTA) and 12µl of autoclaved water. The mixture was denatured at 95°C for 5 minutes and then snap cooled in ice. The samples were initially electrophoresed at 150V for 1 h and at 100V for 4 hours and further run at 50V for about 10-12 hours at 28°C.

The core promoter variant allele G-6A was genotyped by Restriction Fragment Length Polymorphism(RFLP). The PCR amplified products were digested with the restriction enzyme *Bst* NI at 60°C for 1hour. *Bst* NI has two cleavage/recognition sites in the PCR amplified product. One at the -6 position (-4 to -8) and another at -56 locus (-54 to -58). Digested products were electrophoresed for 4 hours in native PAGE at 100V in 1X TBE buffer (pH 8.3). Silver staining was carried out for the native gel.

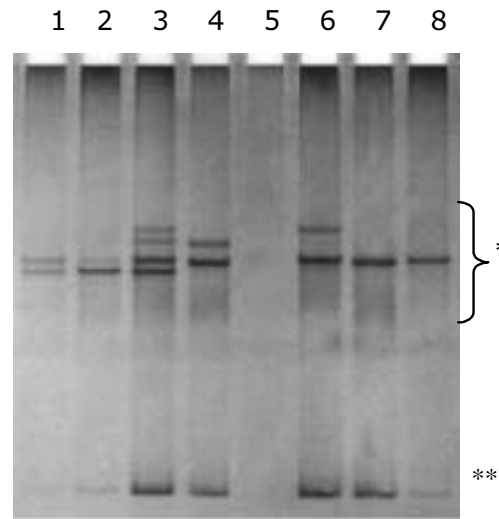
The core promoter amplicons which showed mobility shifts were sequenced commercially (Genotypic Technologies, Bangalore, India). Sequences were compared with those available in the NCBI database using BLAST. Gene frequencies were calculated by gene counting method (Li 1961). Departure from Hardy-Weinberg equilibrium was tested by Chi-square test. For

quantitative variables, mean and SE (standard error) were computed and compared between case and control groups by t-test. All statistical tests were performed using the statistical package SPSS 10.0 version (SPSS Inc., Chicago, Illinois, USA).

**RESULTS AND DISCUSSION**

Based on the different mobility patterns obtained in the SSCP gels the amplicons were classified into 6 groups. They are shown in the figure 1. One amplicon from each of these groups was sequenced. The relevant sequencing results are shown in figure 2. The genotypes of the G-6A polymorphism was confirmed by RFLP analysis wherein restriction site generation/abolition was seen figure 3. The genotypes and allele frequencies of the core promoter variants (A-20C, C-18T and G-6A) among the samples analysed are given in table 1. The frequency distributions of most of these genotypes in both patients and control groups are independent of gender and residential status ( $p \geq 0.05$ ) (data not shown).

In G-6A polymorphism the genotype GG is absent in both the groups. Allele A is more frequent in both patients and controls (0.92 and 0.91 respectively). There is no statistically significant difference between them. Based on the *in-vitro* results, Inoue et al. (1997) have shown that G to A substitution at -6 position affects the basal transcription rate of the AGT gene. In Utah and Paris studies G to A polymorphism was found to be associated with essential hypertension (Jeunemaitre et al. 1992, 1997). Inoue et al. (1997) and Nakajima et al. (2004) also found significant association of G-6A polymorphism and hypertension. Similarly Hunt et al. (1998) reported higher incidence of -6AA



**Fig. 1. SSCP analysis: Silver stained composite gel showing different mobility patterns of representative PCR samples of the core promoter region. Based on the mobility, the amplicons were classified into 6 groups. \*represents bands of single strands of PCR amplicon and \*\* represents the double stranded PCR amplicon, Lanes 1-4 were represents the group 1-4 respectively and Lane 5 is empty, 6 represent the group 5 and Lane 7 and 8 represents the group 6.**

genotype (44.6%) compared to -6GG genotype (31.5%) in hypertensive patients. The incidence of hypertension was significantly lower after sodium reduction in persons with AA genotype but not in persons with the GG genotype.

Province et al. (2000) analysed the G-6A polymorphism in different ethnic (Caucasian, African-American, Japanese and Chinese) subjects and found no significant association with hypertension. Similar results were obtained

**Table 1: Distribution of genotypes and gene frequencies of AGT gene core promoter variants (A-20C, C-18T and A-6G) in the study population**

N = sample size; EH = essential hypertension;  $\chi^2$  2df = Chi- square for independence of genotype frequencies with 2 degrees of freedom; P> 0.05 (not significant).

Variants	N	Genotypes (%)			$\chi^2$ 2df	Alleles (%)		$\chi^2$ 2df
A-6G Variant		AA	AG	GG	<b>P &gt; 0.05</b>	A	G	<b>P &gt; 0.05</b>
EH Patients	254	83.0	17.0	0.0		91.5	8.5	
Controls	254	81.6	18.5	0.0	90.8	9.2		
C-18T Variant		CC	CT	TT	<b>P &gt; 0.05</b>	C	T	<b>P &gt; 0.05</b>
EH Patients	254	99.0	1.0	0.0		99.4	0.6	
Controls	254	99.6	0.4	0.0	99.8	0.2		
A-20C Variant		AA	CC	AC	<b>P &gt; 0.05</b>	A	C	<b>P &gt; 0.05</b>
EH Patients	254	63.0	33.0	4.0		80.0	20.0	
Controls	254	67.5	28.5	4.0	81.5	19.5		

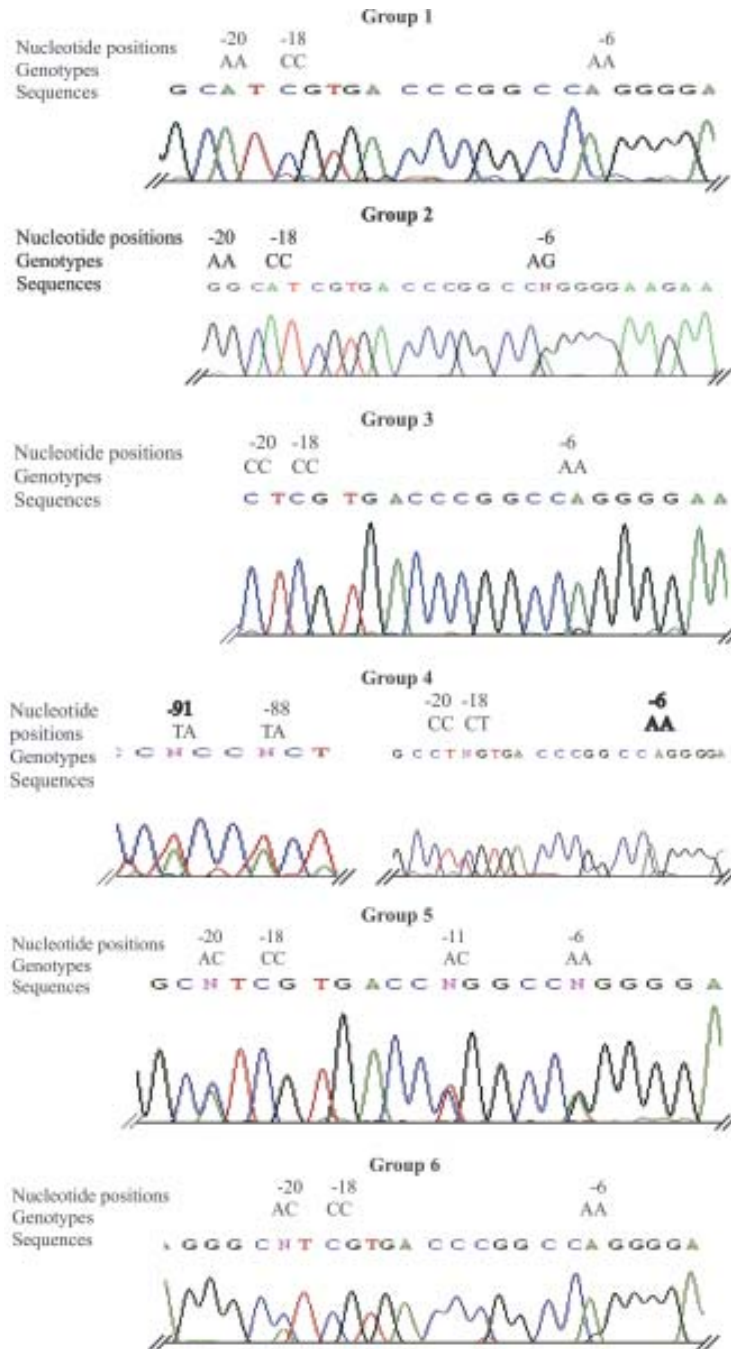


Fig. 2. Sequences of 6 different mobility groups. The core promoter regions (-20, -18, -6) are shown in the figure. The genotypes of the Group 1 is AA-20, CC-18, AA-6; Group 2 is AA-20, CC-18, AG-6; Group 3 is CC-20, CC-18, AA-6; Group 4 is CC-20, CT-18, AA-6 and TA-91, TA-88; Group 5 is AC-20, CC-18, AC-11, AA-6; and Group 6 is AC-20, CC-18, AA-6.



**Fig. 3. Genotyping of the the G-6A variant by RFLP (Restriction Fragment Length Polymorphism):** *Bst* NI digested samples were electrophoresed along with the undigested PCR amplified products. Lanes 1, 3, 5, 9 and 13: PCR amplified product 232bp (undigested positive control); Lane 4: heterozygous AG-6 (131, 101, 81, 50 bps); Lanes 2, 6, 10 and 12: homozygous AA-6 (101, 81, 50 bps); Lane 7: 100bp ladder; Lane 8 known molecular marker (PCR products; 290, 232, 226 and 107 bps).

in Spanish and Russian case-control studies (Rodriguez-Perez et al. 2000; Shlyakhto et al. 2001). The results of the present study correlate with the above studies. In Africa, Tiago et al. (2002) found that the G-6A polymorphisms had no effect on blood pressure and observed low frequency (3 to 4 %) of -6G allele. In the present study also low frequency of -6G allele was found in patients (8.5%) as well as controls (9.2%). Markovic et al. (2005) reported similar results in African-Americans. As in African studies, different Asian studies (Chinese, Taiwan and Japanese) also found negative association of G-6A polymorphism with hypertension (Sato et al. 1997; Kong et al. 2002; Wu et al. 2003).

From the analysis of the primate species (chimpanzee, gorilla, orangutan, gibbon, baboon and macaque) Nakajima et al. (2002, 2004) showed that coding region allele 235T and core promoter variant -6A were the ancestral forms of the gene and other alleles 235M and -6G are likely to have arisen during the course of human evolution. In this study we found high frequency of -6A allele, which supports the ancestral origin of this allele.

C-18T is not a common variant in several ethnic groups. Only a few populations reported the significant association of hypertension with this polymorphism. In the present study we found that there is no statistically significant association between hypertension and C-18T polymorphism.

Sato et al. (1997) showed that C-18T is an independent genetic risk factor for hypertension. No TT homozygous was found in Japanese but CT frequency was significantly higher among patients than in controls. In our study we found no TT (-18) genotype in both the patient and control groups but low frequency of the CT genotype (1.0 and 0.4 respectively in patients and controls) and high frequency of C allele (0.994 and 0.998, respectively). The Polymorphisms C-18T and A-20C affect the binding affinity of *AGT* core promoter binding factor 1 to angiotensinogen core promoter element 1 (Sato et al. 1997). This report inferred C-18T as a risk factor for hypertension in Japanese (Yanai et al. 1996; Sato et al. 1997). There is not much data available for this polymorphism in different ethnic groups. Only a few Japanese studies have shown association with hypertension.

With regard to A-20C polymorphism there is no significant difference in the distribution of genotypes between the two groups ( $p > 0.05$ ). Most of the studies have shown negative association between this polymorphism and hypertension (Jeunemaitre et al. 1992, 1997).

In the core promoter, apart from the A-20C, C-18T and G-6A polymorphisms, we found two novel variants in one patient at -88 and -91 positions (A to T transversion) (group 4, Figure 2). This novel variant in hypertensive patient was not found to be statistically significant. In one control subject at -11 position C → A transversion was observed (group 5, Fig. 2).

This study revealed that there is no significant difference between the control and patient groups with respect to any of the three core promoter markers (A-20C, T-18C, and G-6A). To elucidate the pathophysiology of the hypertension in Tamilnadu population, we are currently screening the M235T and T174M polymorphisms in the above samples.

## CONCLUSION

Gene and genotype frequencies of the core promoter variants {A-20C, T-18C and G-6A} were not significantly associated with essential hypertension in patients of Tamilnadu, India.

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