A Two-Single-Nucleotide Polymorphism Haplotype in Promoter Region of *CYP11B2* Gene Affects Plasma Aldosterone Concentration: A Matched Case-Control Study

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Abstract

Background: Aldosterone synthesized by aldosterone synthase plays an imperative role in Renin-Angiotensin-Aldosterone System (RAAS). Evidence suggested that aldosterone synthase gene (*CYP11B2*) variants are associated with the Essential Hypertension (EH).

Objective: The aim of the present study was to determine the association of -344T/C and -470C/T Single Nucleotide Polymorphisms (SNPs) of the *CYP11B2* and their resulted haplotypes with EH and Plasma Aldosterone Concentration (PAC) in southern population of Iran.

Methods: In this matched case-control study performed on southern Iranians, 45 patients with EH as well as 43 age- and sex-matched normotensive subjects were registered from September 2017 to September 2018. All the individuals were screened by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) for *CYP11B2* related genotypes and haplotypes; PAC was also measured. The gathered data were then analyzed using SPSS16 software by the independent-samples t-test and chi-square test. Deviations of genotype frequencies from Hardy-Weinberg Equilibrium (HWE) and differences between allele frequencies and genotype distributions were calculated by Chi-square test. For performing haplotype analysis and pairwise Linkage Disequilibrium (LD) among the genotypes, SNP analyzer version 2 software and SHEsis online server were utilized. The association of PAC with each one of the polymorphisms and haplotypes was also analyzed by multinomial logistic regression.

Results: There was no association among -344T/C and -470C/T variants and the susceptibility to EH (p=0.53, OR=0.72 and p=0.27, OR=1.69, respectively). Haplotype analysis has also found no association with the disease (CT: p=0.43, OR=1.26; TC: p=0.68, OR=0.88; and TT: p=0.56, OR=0.77). On the other hand, although -344T/C and -470C/T polymorphisms showed no association with PAC (p=0.97, OR=1.00 and p=0.70, OR=0.98, respectively), a significant relationship was found between the H3 haplotype (TT) and PAC (p=0.039, OR=1.29).

Conclusion: Our findings indicated that TT, as the two-single-nucleotide polymorphism haplotype in promoter region of *CYP11B2* gene, is associated with plasma aldosterone concentration in the southern population of Iran. So, these people are likely to be at higher risk of cardiovascular or kidney disease compared to the other investigated haplotypes.

Keywords: *CYP11B2*, Genetic polymorphism, Haplotype, PAC

Abbreviations / Acronyms:
AHA: American Heart Association; **BMI:** Body Mass Index; **CAD:** Coronary Artery Disease; **DBP:** Diastolic Blood Pressure; **EH:** Essential Hypertension; **HWE:** Hardy-Weinberg Equilibrium; **HRT:** Hormone Replacement Therapy; **LOD:** Logarithm of the Odds; **LD:** Linkage Disequilibrium; **OR:** Odds Ratio; **PAC:** Plasma Aldosterone Concentration; **PCR-RFLP:** Polymerase Chain Reaction-Restriction Fragment Length Polymorphism; **PRA:** Plasma Renin Activity; **RAAS:** Renin-Angiotensin-Aldosterone System; **SBP:** Systolic Blood Pressure; **SNPs:** Single Nucleotide Polymorphisms

1. Introduction
Hypertension is known as a high prevalence challenge for wellbeing, because it is associated with the increased risks of cardiovascular and kidney disease (1). In industrialized countries, the risk of getting hypertension during a lifetime exceeds 90% (2) and it is estimated that 1.56 billion individuals will be globally affected by this disease in the year 2025 (3). In 2015, the global prevalence of hypertension was about 20% in women and 24% in men aged over 18 years old, which was estimated as 18.9% and 20.4% for the Islamic Republic of Iran, respectively (4). Hypertension is considered as a genetically complex disease, on which the effect of heritability is approximately 40% and includes the allelic effects of a gene, gene-gene, and gene-environment interactions. Accordingly, genetic risk factors are most likely very heterogeneous, which challenges the identification of genes responsible for this disease (5). Between 80 and 95% cases with high blood pressure have unknown causes, which are termed as "Essential Hypertension" (EH) (6). EH candidate genes are mostly associated with sodium and electrolyte balance, sympathetic nervous system, intracellular messengers, and Renin–Angiotensin-Aldosterone System (RAAS) (7, 8). Among these, RAAS is the best-known regulator of blood pressure (9). Moreover, genetic variants in this system are associated with several cardiovascular diseases like EH (10). More than 15% of patients with EH have abnormally increased plasma aldosterone levels (11). Aldosterone is synthesized by the mitochondrial enzyme, aldosterone synthase, and *CYP11B2* (cytochrome p450 11B2), the gene encoding this enzyme is located on 8q21–22 (12, 13). Several single nucleotide polymorphisms (SNPs) have been identified in *CYP11B2* gene as well as in its promoter region (14). Two of the SNPs, which are in complete Linkage Disequilibrium (LD) are rs1799998 (T/C at -344) and rs10087214 (C/T at -470), located in the promoter region of the gene (15). Previous studies focused on the relationship between -344 variant and hypertension have reported some conflicting data (14-17), in which the -470 variant has rarely been studied (9, 18). Thus, the aim of this study, for the first time, was to evaluate the association of these two polymorphisms and their derived haplotypes with essential hypertension and Plasma Aldosterone Concentration (PAC) in southern population of Iran.

2. Material and Methods
2.1. Study design
The research protocol of the matched case-control study was approved by the Hormozgan University of Medical Sciences Ethics Committee. Due to many immigrations from different cities of Iran and mixed ethnicity of the population (19), Bandar Abbas city does not present the isolated ethical groups significantly. Therefore, in this study, we considered the origin of all the included subjects from a single population (20). After case diagnosis by a cardiologist or general practitioner, the written informed consent was completed and the checklists (including demographic characteristics, clinical information, and medical history) were filled out. In the present study, 88 subjects aged between 35 and 65 years old were registered, including 45 patients with EH and 43 age- and sex-matched healthy controls, who were referred to Shahid Mohammadi hospital and health centers in Bandar Abbas, Iran, from September 2017 to September 2018.

2.2. Inclusion and exclusion criteria
2.2.1. Cases
2.2.1.1. Inclusion criteria of the case group:
As suggested by the American Heart Association (AHA), patients with Systolic Blood Pressure (SBP) ≥140 mm Hg or Diastolic Blood Pressure (DBP) ≥90 mm Hg were selected (21).
2.2.1.2. Exclusion criteria of the case group:
We excluded those subjects diagnosed with obstructive sleep apnea, primary aldosteronism, pheochromocytoma, known adrenal mass, hyperparathyroidism, renal disease, renovascular hypertension, thyroid disease, and Cushing's syndrome. The patients who were consuming any medications (antihypertensives, spironolactone, and diuretics, and women taking contraceptive pills and using Hormone Replacement Therapy (HRT)), those with previous history of Coronary Artery Disease (CAD) and diabetes mellitus, and pregnant women were also excluded from the study.
2.2.2. Controls
2.2.2.1. Inclusion criteria of the control group:
We registered healthy controls as individuals with SBP ≤120 mm Hg and DBP ≤90 mm Hg.

2.2.2.2. *Exclusion criteria of the control group:*
We excluded the subjects with any history of essential or secondary hypertension as well as those who utilized any medications affecting the plasma aldosterone concentration or Plasma Renin Activity (PRA).

2.3. Bias and confounders
In this study, although systematic errors were minimized, some of them were not solvable that are described as limitations at the end of the discussion part. In addition, in this project, we have attempted to deal with the selection bias by randomly selecting the individuals who were matched in terms of gender and age, and to determine sample size using highly precise sample size calculation formulae. We have also used the logistic regression method and examined the effect of the variables simultaneously.

2.4. Blood pressure determination and sampling
Blood pressure was measured in the seated position using a calibrated sphygmomanometer. Each individual's blood pressure was checked at least twice and the average was finally reported (22). Six to seven ml of venous blood was taken after overnight fasting. Peripheral blood for DNA extraction and separated plasma for hormonal assay were preserved at -70 °C.

2.5. DNA extraction and genotyping
The genomic DNA of blood samples was extracted using the "DNA rapid salting out" protocol proposed by miller et al. (23).

2.6. Detection of CYP11B2 gene polymorphisms
The −344T/C and -470C/T polymorphisms were simultaneously screened by one Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) reaction. Afterward, 25μL of the reaction mixture contained 1.5 μL of DNA solution with the concentration of about 150 ng/dl, 0.5 μL of each primer (forward: 5'ATGTTGACCACCAGGAGGAGA3′; reverse: 5′ CCCAGCCAAAGGTAGATGAAGG3′, designed by OLIGO version 7 software), 12.5 μL of 2x Taq Master Mix RED (Ampliqon, Denmark), and 10 μL of sterile distilled water. With an initial denaturation for 300 seconds at 95 °C, amplification was carried out in 35 cycles containing denaturation (for 30 seconds at 95 °C), annealing (for 30 seconds at 55 °C), and elongation (for 15 seconds at 72 °C) for each cycle as well as a final extension for 350 seconds at 72 °C. PCR products were electrophoresed on a 1.5% agarose gel, stained with DNA safe stain (SMOBiO, Taiwan), and digitally photographed using a gel image system. For the detection of alleles at 2 loci on 2% agarose gel, 4.5 μL of the PCR product (421 bp) was digested with 1 μL of Lmn1 restriction enzyme, 2 μL of SE-buffer (Sibenzyme, Russia), and 7.5 μL of sterile distilled water for approximately 4 hours at 37 °C.

2.7. Hormonal assay
All the subjects rested in supine position for at least 30 min before blood sampling. PRA (μIU/ml) and PAC (ng/dl) were measured in duplicate by chemiluminescence on a LIAISON analyzer (DiaSorin, Italy) using LIAISON Aldosterone (DiaSorin, USA) and LIAISON Direct Renin (DiaSorin, Italy) assay kits. Aldosterone to Renin ratio (ARR), as an index of inappropriate aldosterone activity, was derived by dividing PAC with PRA to exclude primary aldosteronism from the study (24).

2.8. Statistical analysis
Our assembled data were carefully analyzed in SPSS16 software (SPSS Inc, Chicago). Thereafter, demographic parameters were reported as mean ± standard deviation. The independent-samples t-test and chi-square test were also used to compare the quantitative and qualitative variables, respectively. In order to calculate deviations of genotype frequencies from Hardy-Weinberg Equilibrium (HWE) and differences in allele frequencies and genotype distributions, Chi-square test was applied. Moreover, odds ratios (OR) with 95% confidence intervals (CI) were calculated. P values less than 0.05 were considered as statistically significant. SNP analyzer version 2 software (V.2) and SHEsis online server (http://analysis.bio-x.cn) were utilized for analyzing haplotypes and pairwise linkage disequilibrium (LD) between the genotypes of the studied polymorphisms. To determine the association of aldosterone concentration with each one of the polymorphisms and haplotypes, multinomial logistic regression was used.

2.9. Research ethics
All the procedures performed on human participants in this study were in accordance with the ethical standards of the Hormozgan University of Medical Sciences Ethics Committee (Ref: HUMS.REC.1396.22). Informed consent was obtained from all the participants included in the study.

3. Results
3.1. Clinical and demographic characteristics
A total of 88 subjects (59 men, 29 women) were selected, including 45 patients with EH (mean age of 42.48±7.05 years old), and 43 normal subjects (mean age of 43.14±8.52 years old). Demographic characteristics and clinical information of the patients and healthy subjects are shown in Table 1. Notably, the prevalence of three risk factors, including Body Mass Index (BMI) (p=0.001, OR=0.781), salt intake (p=0.032, OR=0.961), and family history of hypertension (p=0.012, OR=1.998) were significantly higher in the case group.

Table 1. Demographic characteristics and clinical information of the study groups

Variables		Case (n=45)	Control (n=43)	p-value*	OR^2 (95% CI^3)
Gender (%)	Male	31 (68.9%)	28 (65.1%)	0.70	1.058 (0.788-1.419)
	Female	14 (31.1%)	15 (34.9%)		0.892 (0.491-1.619)
Age (years) ± SD		42.48±7.05	43.14±8.52	0.87	1.011 (0.952-1.073)
BMI (Kg/m^2) ± SD		27.51±2.73	25.32±3.42	0.001	0.781 (0.662-0.921)
Activity	Low	16 (35.6%)	18 (41.9%)	0.78	0.635 (0.168-2.402)
	Moderate	22 (48.9%)	20 (46.5%)		
	High	7 (15.6%)	5 (11.6%)		
Smoking	No	41 (91.1%)	41 (95.3%)	0.36	1.911 (0.369-9.903)
	Yes	4 (8.9%)	2 (4.7%)		
	Passive smoker	9 (20.0%)	9 (20.0%)	0.50	
Adding salt to daily food	No	18 (55.8%)	24 (55.8%)	0.032	0.961 (0.296-3.116)
	Sometimes	21 (46.7%)	19 (44.2%)		
	Yes	6 (13.3%)	0		
Hypertension in family members	Yes	23 (51.1%)	11 (25.6%)	0.012	1.998 (1.114-3.584)
	No	22 (48.9%)	32 (74.4%)		
PAC (ng/dl) ± SD		9.99±5.35	11.24±5.41	0.43	1.014 (0.929-1.107)
PRA (µIU/ml) ± SD		1.45±0.65	1.65±0.55	0.12	1.001 (0.996-1.005)

*p-value ≤0.05 is significant; SD: Standard Deviation, BMI: Body Mass Index, PAC: Plasma Aldosterone Concentration, PRA: Plasma Renin Activity

3.2. Distribution of Genotypes and haplotypes
The −344T/C was in complete LD with -470 C/T (D=0.99, R^2 = 0.60, Logarithm of the Odds (LOD) score =75.90). Furthermore, unlike the -344 variant (p<0.05), the -470 C/T was distributed according to Hardy-Weinberg equilibrium among the control subjects (p>0.05). In addition, the frequencies of alleles and genotypes revealed no significant difference between the study groups (p>0.05). The allelic and genotype's distribution along with p-value and OR with 95% CI for alternative alleles and genotypes of the variants, are presented in Table 2 and Figures 1, 2, respectively. Furthermore, among 3 two-locus haplotypes, none of them were found to be associated with EH (p>0.05) (Table 3, Figure 3).

Table 2. The allelic and genotype frequency of the *CYP11B2* gene variants

SNP	Parameters		Case (n=45)	Control (n=43)	p-value*	OR (95% CI)**
-344T/C	Alleles	T	55 (61.1%)	50 (58.1%)	-	-
		C	35 (38.9%)	36 (41.9%)	0.68	0.883 (0.483 -1.614)
	Genotypes	T/T	12 (26.7%)	9 (20.9%)	-	-
		T/C	31 (68.9%)	32 (74.4%)	0.79	0.75 (0.088-6.388)
		C/C	2 (4.4%)	2 (4.7%)	0.52	0.727 (0.269-1.966)
		Carrier (T/C+C/C)	33 (73.3%)	34 (79.1%)	0.53	0.728 (0.271-1.955)
-470C/T	Alleles	C	45 (50%)	38 (44.2%)	-	-
		T	45 (50%)	48 (55.8%)	0.48	1.26 (0.84-1.495)
	Genotypes	C/C	10 (22.2%)	9 (20.9%)	-	-
		T/C	25 (55.6%)	20 (46.5%)	0.85	1.125 (0.384-3.298)
		T/T	10 (22.2%)	14 (32.6%)	0.47	0.683 (0.191-2.161)
		Carrier (T/C+T/T)	35 (77.8%)	34 (79.1%)	0.27	1.69 (0.654-4.365)

*p-value ≤ 0.05 is significant; **OR: Odds Ratio, CI: Confidence Intervals, SNP: Single Nucleotide Polymorphism
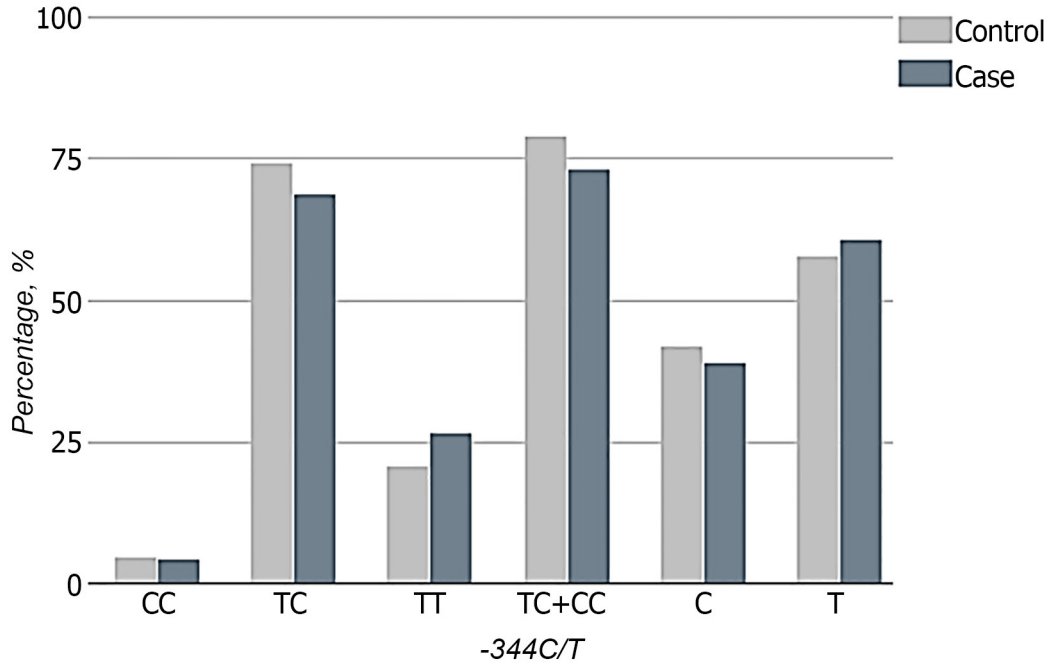
Figure 1. Comparison of the genotypic and allelic percentage of -344C/T polymorphism in both case and control groups
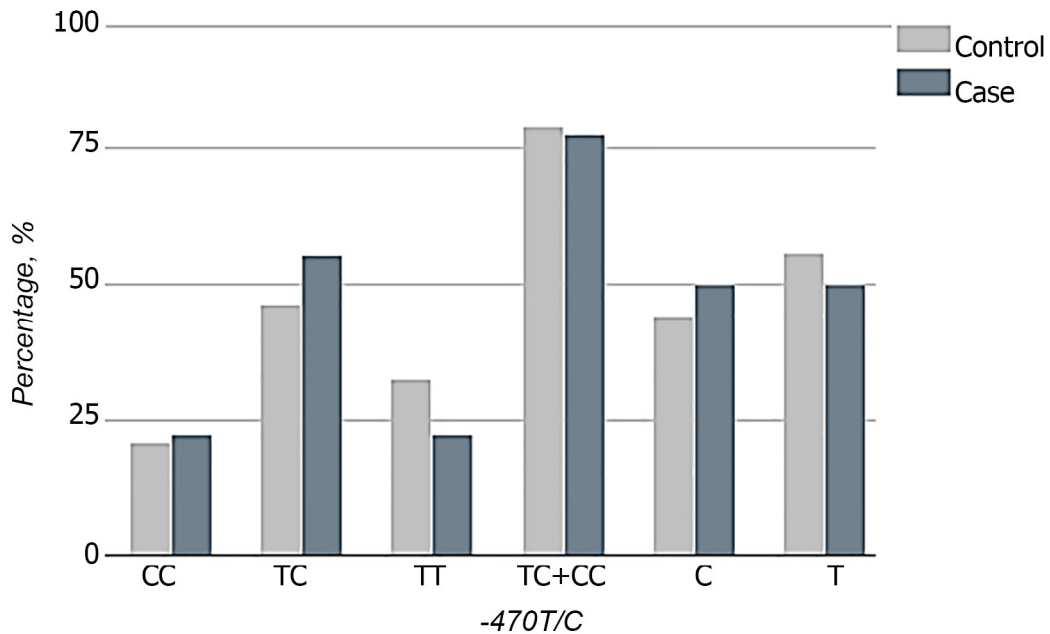

Figure 2. Comparison of the genotypic and allelic percentage of -470T/C polymorphism in both case and control groups

Table 3. *CYP11B2* gene haplotype analysis in case and control groups

Haplotype		Names	Case (%)	Control (%)	p-value*	OR (95% CI)**
-470	-344					
C	T	H1	45 (0.50)	38 (0.44)	0.43	1.263 (0.698-2.286)
T	C	H2	35 (0.389)	36 (0.41)	0.68	0.884 (0.484-1.615)
T	T	H3	10 (0.11)	12 (0.14)	0.56	0.771 (0.314-1.890)

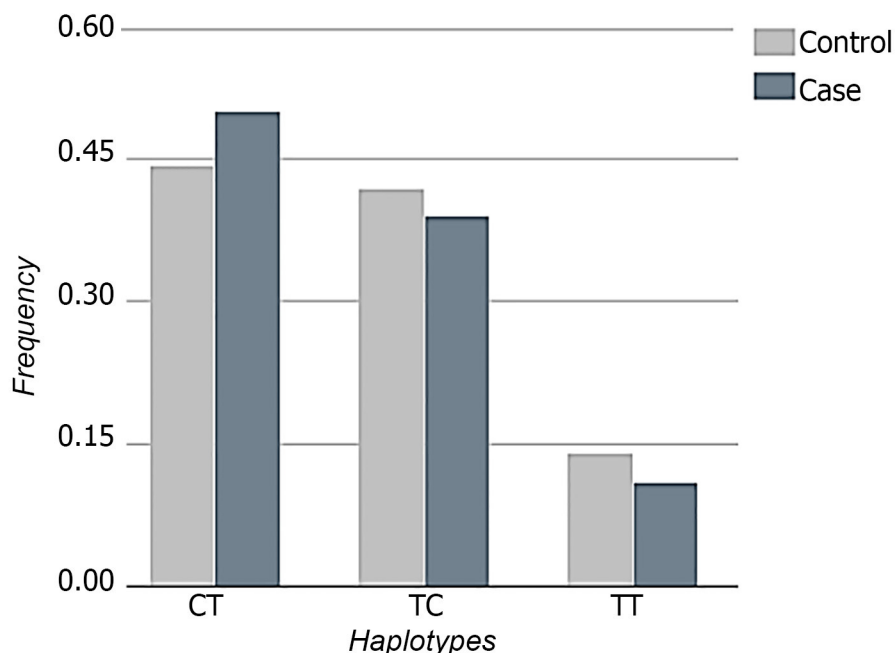*p-value ≤ 0.05 is significant; **OR: Odds Ratio, CI: Confidence Intervals

Figure 3. Comparison of the haplotype frequencies in case and control groups

3.3. Association analysis

PAC was compared on the basis of the genotypes in all the participants, which showed no significant difference (p>0.05) (Table 4). PAC was significantly higher in the subjects with TT haplotype compared to TC and CT (p=0.039, OR=1.292) (Table 5).

Table 4. Association of genotypes with plasma aldosterone concentration

SNP	Genotypes	p-value*	OR (95% CI)**
-344T/C	TT	-	-
	CT+CC	0.97	1.002 (0.914-1.098)
-470C/T	CC	-	-
	CT+TT	0.706	0.985 (0.896-1.076)

*p-value ≤ 0.05 is significant; **OR: Odds Ratio, CI: Confidence Intervals

Table 5. Association of *CYP11B2* gene haplotypes with plasma aldosterone concentration

Haplotypes	p-value*	OR (95% CI)**
CT	-	-
TC	0.53	1.0356 (0.923-1.161)
TT	0.039	1.292 (1.013-1.647)

*p-value ≤ 0.05 is significant; **OR: Odds Ratio, CI: Confidence Intervals

4. Discussion

The aim of the present study was to investigate the association of -344T/C and -470C/T SNPs of the *CYP11B2* gene and their resulted haplotypes with EH and PAC in the southern population of Iran. The importance of this study lies in the fact that it is the first one exploring the above-mentioned association in human. Our results suggest that there is no significant association between the -344T/C polymorphism of *CYP11B2* and EH or PAC. Correspondingly, this means that the two case and control groups did not differ significantly in the frequency of this polymorphism and either T or C allele in -344 site, and they also showed no significant difference in the plasma aldosterone concentration. We found several studies with conflicting results on the association of -344T/C polymorphism with high blood pressure and plasma aldosterone level. Among the studies that found no significant association in this field, in agreement with our findings, the study by Niu et al. can be mentioned in which they found no significant difference between the transcriptional activity of *CYP11B2* and the promoter alleles of -344T/C variant in a case-control study, suggesting that this polymorphism has no relationship with hypertension (14). Moreover, Cheng et al.

in their systematic review detected no association between -344T/C variant and the potential for hypertension in female and male subjects (25). In a meta-analysis, using 42 observational, case-control, and cohort studies, Sookoian et al. indicated that the subjects with CC genotype of -344T/C have a 17% lower risk of hypertension compared to those with TT. Likewise, they demonstrated that the polymorphism is not related to the aldosterone concentration (26). On the other hand, among the researches that, unlike us, found a significant association between -344T/C SNP and blood pressure or aldosterone concentration, Wang et al. in their study demonstrated that -344T/C is associated with EH, so that the CC genotype or C allele of the variant can be considered as a protective genetic factor for high blood pressure (15). In another study, Nicod et al. represented that the CC genotype at -344 is a predictive of normal aldosterone to renin ratio (ARR) in 94% of patients with hypertension (27). As indicated in a study by Tanahashi et al., the −344C promoter allele can be known to bind the steroidogenic factor-1 (SF-1) transcription factor, approximately 4 times stronger than the −344T allele. SF-1 is considered as a negative regulator for the promoter region of *CYP11B2*, so the −344C allele would be associated with lower *CYP11B2* expression due to the increased binding of SF-1 (13). Maharjan et al. in a miRNA-based study found that miR766 can reduce the expression of *CYP11B2* in subjects with T allele at -344, which may decrease blood pressure (16).

Our results also show that there is no significant association between the –470C/T polymorphism of *CYP11B2* and EH or PAC, meaning that the case and control groups do not differ significantly in the genotypic frequency of this polymorphism. Moreover, the plasma aldosterone concentration of these individuals is not apparently related to C or T allele in -470 site. Our results are in accordance with the case-control study performed by Zhang et al., in which 7 single nucleotide polymorphisms were fully checked in different sites of aldosterone synthase gene and researchers observed no significant association between rs10087214 and hypertension (18). Finally, our findings indicated that there is a significant relationship between the H3 haplotype (-470T/-344T) and aldosterone concentration in these subjects. Accordingly, individuals with TT haplotype are 1.2 times more likely to have a higher PAC than those with two other haplotypes (CT and TC). Mopidevi et al. in a study in 2015 examined the effects of -344T/C and -470C/T variants, as haplotypes, on blood pressure and plasma aldosterone concentration; however, their findings were not in line with the results of our research. Specialists examined 3 SNPs including -663T/A, -470C/T, and -344T/C. In addition, they produced transgenic mice and found that the expression of *hCYP11B2* gene (human aldosterone synthase gene) and plasma aldosterone level in mice increased with ACT haplotype, which consequently increased the blood pressure (9). Although some studies have confirmed the role of *CYP11B2* gene polymorphisms in the pathogenesis of EH, findings of various studies were conflicting. This contradiction in the outcomes can be due to the different numbers of participants in each study, as well as due to different populations with different genetic backgrounds.

5. Study limitation
Besides the few research papers available on this issue, we also had some limitations in our research. Firstly, EH is a multifactorial and polygenic disease, which challenges the impact of each variant on the pathogenesis of the disease. Secondly, small sample size of the study was due to the age range limit and several strict including and excluding criteria for sample collection. Third, hormonal assessments with managed salt intake were needed to discover the physiological impacts of this genetic variability. Moreover, we did not measure urine sodium and potassium excretion over a 24-hour period, as these variables are critical environmental factors affecting aldosterone levels. Finally, we assayed the renin hormone to exclude the primary aldosteronism from the study by aldosterone renin ratio analysis; however, confirmatory tests such as oral sodium loading, and intravenous saline infusion, were also needed for the definitive diagnosis (28). This research may have other results in other populations. Therefore, the present study should be conducted with a greater sample size in other populations and the results obtained should be compared with the findings of this examination.

6. Conclusions
To conclude, despite the finding that -344T/C and -470C/T variants of *CYP11B2* and their derived haplotypes do not have significant relationships with hypertension, the concentration of plasma aldosterone in these subjects was found to be positively associated with the TT haplotype. This finding could also suggest that these individuals may be at risk for cardiovascular and kidney disease. It is strongly recommended that confirmatory diagnostic tests such as saline infusion test and oral sodium loading should be peformed to confirm primary aldosteronism. Further studies of SNPs in coding and non-coding regions of the *CYP11B2* with larger population groups and other ethnicities are needed to provide a good basis on this topic.

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