

การศึกษาสニปของยีน angiotensin-converting enzyme (ACE) และระดับโปรตีน ACE ในซีรัมที่สัมพันธ์กับโรคความดันโลหิตสูงในคนไทย

กนกนัษฐ รัตนธาวรรณ¹ พลเพชร ทองเกตุ² เญญพร พานิชเจริญ³ วาสนา สุขุมศิริชาติ²

¹สำนักนวัตกรรมการเรียนรู้ มหาวิทยาลัยศรีนครินทรวิโรฒ

²ภาควิชาชีวเคมี คณะแพทยศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ

³ภาควิชากายวิภาคศาสตร์ คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล

บทคัดย่อ

โรคความดันโลหิตสูง เป็นปัจจัยเสี่ยงที่สำคัญของโรคหลอดเลือดในสมองและโรคหลอดเลือดหัวใจ เป็นโรคที่มีความซับซ้อน เกิดจากปัจจัยทางสิ่งแวดล้อมและพันธุกรรม โดยเฉพาะอย่างยิ่งยีนที่มีความเกี่ยวข้องกับการควบคุมความดันโลหิต ระบบเรนิน-แองจิโอเทนซิน (renin-angiotensin system; RAS) มีบทบาทสำคัญในการควบคุมความดันโลหิตและการเกิดโรคความดันโลหิตสูง เอนไซม์ angiotensin converting enzyme (ACE) เป็นเอนไซม์ชนิดหนึ่งที่อยู่ในระบบ มีหน้าที่สร้าง angiotensin II และสลาย bradykinin ซึ่งมีผลทำให้หลอดเลือดหดตัว การศึกษาครั้งนี้มีจุดประสงค์ที่จะตรวจสอบระดับของโปรตีน ACE ในซีรัมและหาความสัมพันธ์ของการเปลี่ยนแปลงเบสเพียง 1 เบส (สนิป) ในเอกซอน 17 ของยีน ACE ที่ตำแหน่ง rs4343 กับความเสี่ยงของการเกิดโรคความดันโลหิตสูงในคนไทย โดยอาสาสมัครทั้งหมดที่ถูกคัดเลือกเข้าโครงการวิจัย ถูกแบ่งเป็น 2 กลุ่มคือ กลุ่มผู้ป่วยความดันโลหิตสูง 30 คน (SBP>140 mmHg และ DBP>90 mmHg) และกลุ่มปกติ 30 คน (SBP<120 mmHg และ DBP<80 mmHg) ทำการตรวจสอบระดับไขมันในซีรัมโดยใช้วิธี enzymatic colorimetric และ homogeneous วัดระดับของโปรตีน ACE โดยใช้ชุดตรวจ ELISA วิเคราะห์จีโนไทป์โดยวิธี polymerase chain reaction (PCR) และ high-resolution melting (HRM) จากนั้นวิเคราะห์ค่าทางสถิติของระดับไขมันและโปรตีน ACE ในซีรัมโดยใช้ t-test และวิเคราะห์จีโนไทป์ของยีนโดย chi-square test จากโปรแกรม SPSS เวอร์ชัน 16.0 ผลการวิเคราะห์พบว่า ไตรกลีเซอไรด์ของกลุ่มผู้ป่วยความดันโลหิตสูงมีระดับสูงกว่ากลุ่มปกติอย่างมีนัยสำคัญทางสถิติ ($p=0.004$) ส่วนระดับไขมันอื่นๆ เช่น คอเลสเตอรอล เอชดีแอล และแอลดีแอล ไม่พบความแตกต่าง การวิเคราะห์ระดับโปรตีน ACE พบว่า กลุ่มผู้ป่วยความดันโลหิตสูงมีค่าเฉลี่ย 192.19 ± 104.33 ng/ml สูงกว่ากลุ่มปกติที่มีค่าเฉลี่ย 146.37 ± 46.65 ng/ml อย่างมีนัยสำคัญทางสถิติ ($p=0.02$) ผลการวิเคราะห์สนิป rs4343 ของยีน ACE ไม่พบความแตกต่างระหว่างกลุ่มตัวอย่างทั้งสอง ($p=0.629$) จากข้อมูลการศึกษานี้ พบความสัมพันธ์ระหว่างโปรตีน ACE กับโรคความดันโลหิตสูง แต่ไม่พบความสัมพันธ์ของสนิป rs4343 ของยีน ACE กับความเสี่ยงของการเกิดโรคความดันโลหิตสูงในคนไทย

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ผู้นิพนธ์ประสานงาน:

กนกนัษฐ รัตนธาวรรณ

สำนักนวัตกรรมการเรียนรู้ มหาวิทยาลัยศรีนครินทรวิโรฒ

114 ถนนสุขุมวิท 23 แขวงคลองเตยเหนือ เขตวัฒนา กรุงเทพฯ 10110

อีเมล: kankanit@g.swu.ac.th

Studies of single nucleotide polymorphism (SNP) of angiotensin-converting enzyme (ACE) gene and serum ACE protein level in association with hypertension in Thai patient

Kankanit Rattanathanawan¹, Polphet Thongket², Benjaporn Panichareon³, Wasana Sukhumsirichart²

¹Innovative Learning Center, Srinakharinwirot University, Bangkok, Thailand

²Department of Biochemistry, Faculty of Medicine, Srinakharinwirot University, Bangkok, Thailand

³Department of Anatomy, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

Abstract

Hypertension is a strong risk factor for stroke and cardiovascular disease. It is a complex disease resulting from the interactions of environmental factors and genetic factors, especially those genes that affect the regulation of blood pressure. The renin-angiotensin system (RAS) may play a crucial role in the regulation of blood pressure and pathogenesis of hypertension. Angiotensin converting enzyme (ACE) has a central role in this system by producing the angiotensin II and the degradation of bradykinin resulting in vasoconstriction. This study aimed to investigate serum ACE protein levels and SNP, rs4343, in *ACE* gene in association with risk of hypertension in Thai population. A total of 30 hypertension patients (SBP>140 mmHg, DBP>90 mmHg) and 30 controls (SBP<120 mmHg, DBP<80 mmHg) were recruited. Serum lipids were examined by using enzymatic colorimetric and homogeneous methods. Serum ACE levels were examined by ELISA kit. The SNP, rs4343 (exon 17), in *ACE* gene was genotyped by polymerase chain reaction (PCR) and high-resolution melting (HRM) analysis. The data were analysed using t-test and chi-square test of SPSS program version 16.0. The results showed that the triglyceride level in the hypertension group was significant higher than the control ($p=0.004$). But there was no significant differences in the level of cholesterol, HDL and LDL. The ACE levels showed significant difference between patient (192.19 ± 104.33 ng/ml) and control (146.37 ± 46.65 ng/ml) groups ($p=0.02$). However, there is no significant differences in genotype distribution of SNP, rs4343, in *ACE* gene in the subjects with and without hypertension ($p=0.629$). The data from this study suggested that there is a relationship between ACE protein levels and hypertension, but no association of SNP, rs4343, in *ACE* gene with risk of hypertension in Thai patient.

Keywords: single nucleotide polymorphism, SNP, hypertension, renin-angiotensin system, angiotensin converting enzyme

Corresponding Author:

Kankanit Rattanathanawan

Innovative Learning Center, Srinakharinwirot University

114 Sukhumvit Road, Sukhumvit 23, Wattana, Bangkok 10110, Thailand

E-mail: kankanit@g.swu.ac.th

Introduction

Hypertension is the condition of elevation leads of blood pressure above 140 mmHg systolic blood pressure and 90 mmHg diastolic blood pressure¹. The criteria of hypertension by World Health Organization, Joint National Committee and International Society of Hypertension^{2,3}. Normal hypertension exhibited less than 120 mmHg systolic blood pressure and less than 80 mmHg diastolic blood pressure. Pre-hypertension presents systolic blood pressure between 120-139 mmHg or diastolic blood pressure between 80-89 mmHg. Stage 1 hypertension harbored systolic blood pressure between 140-159 mmHg or diastolic blood pressure between 90-99 mmHg. Stage 2 hypertension have systolic blood pressure above 160 mmHg or diastolic blood pressure above 100 mmHg.

Hypertension is a chronic disease that cases health problems because it no symptoms unless can't control blood pressure level. This change manifests itself effect in each organ such as brain, heart, kidney and eyes. Hypertension is a strong risk factor for disease like stroke and cardiovascular disease. Based on a pooled analysis of available national and regional data in 2000, there were approximately 972 million hypertention patients in worldwide. It will be increasing to approximately 1.56 billion in 2025⁴. The association of hypertension with different risk factors such as obesity, stress, diabetes mellitus

and genetic factors (family history of hypertension), especially those gene that affect the regulation of blood pressure. Among these the renin-angiotensin system (RAS) may play a crucial role in the regulation of blood pressure and pathogenesis of hypertension. Several studies have identified *ACE* gene as candidate gene of hypertension in humans. *ACE* gene is located on chromosome 17q23. ACE protein has a central role in this system by producing the angiotensin II and the degradation of bradykinin resulting in vasoconstriction. Several polymorphisms were present in the *ACE* gene but the prominent of these polymorphisms are G2350A (rs4343) and I/D (rs4291)⁵⁻¹².

This study aimed to investigate the association of serum ACE levels and SNP rs4343 in *ACE* gene with risk of hypertension in Thai population.

Materials and Methods

Participants

A total of 30 patients (SBP>140 mmHg and DBP>90 mmHg) and 30 controls (SBP<120 mmHg and DBP<80 mmHg), age 25-60 years. Exclusion criteria of the subjects were (1) the one with age less than 25 years or more than 60 years, (2) SBP between 120-139 mmHg and DBP between 80-89 mmHg, (3) refuse to sign the informed consent, and (4) with secondary form of hypertension, diabetes mellitus, myocardial infraction and obstructive sleep apnea. This

study was approved by ethical committees of the Faculty of Medicine, Srinakarinwirot University, Thailand (SWUEC/E-140/2558).

Determination of serum lipid levels

Level of serum triglycerides, HDL-C, LDL-C were determined by the enzymatic colorimetric and homogenous method¹³⁻¹⁵. The analysis was performed at laboratory of HRH princess Mahachakri Sirindhorn Medical Center, Faculty of Medicine, Srinakarinwirot University. All lipid profile tests were run on Dimension RXL Chemistry analyzer (Dade Behring, USA).

ACE measurement by ELISA kit

Determination of ACE concentration was performed by the sandwich ELISA method (Boster Biological Technology, Pleasanton, CA, USA). Briefly, serum were incubated at 37°C in 96 well plates coated with antibody to ACE for 90 min. Bionylated anti-human ACE Ab was added and incubated at 37°C for 60 min. The plate was washed 3 times with 0.01M PBS. After that, Avidin-Biotin-Peroxidase Complex (ABC) (working solution) was added into each well and incubated at 37°C for 30 min and washed 5 times with 0.01M PBS. Then, TMB color developing agent was added and incubated at 37°C in dark for 20-25 min. Finally, TMB (stop solution) was added to stop the reaction. The absorbance was measured by using a microplate reader at 450 nm (BioTek Instruments, Winooski, VT, USA).

DNA extraction and genotyping

The genomics DNAs were extracted from whole blood by using Flexigene DNA kit (Qiagen, German). The concentration of the DNA was determinate by measure O.D.260 using a ND-2000C spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). The quality of genomic DNA was determined by 1% agarose gel electrophoresis. All the purified samples were stored at -80°C until used. Prior to genotype, the DNAs were diluted and adjusted to a concentration of 25 ng/μl.

The SNP rs4343 in *ACE* gene was selected from JSNP, NCBI database with a minor allele frequency (MAF) exceeding 0.2 in populations of Japan descent¹⁶. PCR primer was designed from website <http://bioinfo.ut.ee/primer3-0.40.0/primer3/>. The primer sequences were as follow: forward primer 5' AGCTCAAGGCATTCAAACC 3' and reverse primer 5' AGCCCTCCCATGCCCATATA 3'. Genotype was accomplished using real-time PCR and and high-resolution melting (HRM). The PCR reaction was performed in CFX 96 Touch real-time PCR detection system (Bio-Rad, Hercules, CA, USA). A total volume of 20 μl composed of 10 μl of supermix (2x), 2 μl of DNA (25 ng/μl), 0.6 μl of 10 μM forward primer, 0.6 μl of 10 μM reverse primer and 6.8 μl of sterile-distilled water. PCR reactions were carried out as following: pre-denaturing at 98°C for 3 min, denaturation at 98°C for 10 sec and annealing at 60°C for 15 sec for 40 cycles. HRM analysis was performed at

temperature ranging from 65°C to 95°C using a CFX 96 Touch real-time PCR detection system (Bio-Rad, Hercules, CA, USA). Nucleotide sequence of selected DNAs were confirmed by sequencing to validate the genotypes.

Statistical analysis

Clinical characteristics of hypertension patients and normal controls were compared using the t-test. The goodness of-fit test of Hardy-Weinberg equilibrium (HWE) was performed using the equation $p^2+2pq+q^2$, where p and q represented the wild-type and variant allele of a gene, respectively. Association analyses were performed using

Pearson's Chi-square test implemented in SPSS program version 16.0 for Windows. Significant level was set at $p<0.05$. Odds ratio and 95% confidence interval were calculated.

Results

The clinical characteristics of hypertension patients and normal controls are summarized in Table 1. Age, BMI and SBP, DBP were significantly higher in the hypertension patients ($p<0.0001$). Furthermore, triglyceride level was significantly higher in the hypertension patients than normal ($p=0.004$).

Table 1 Clinical characteristics of hypertension patients and normal controls

Variables	Control (n=30)	Hypertention (n=30)	p-value
Age (Years)	43.37±12.44	55.79±10.61	<0.0001
BMI	22.14±3.16	27.70±7.44	0.0003
SBP (mmHg)	117.53±13.01	146.90±18.07	<0.0001
DBP (mmHg)	72.50±9.60	87.50±13.90	<0.0001
Cholesterol (mg/dl)	204.77±47.50	203.40±29.83	0.45
TG (mg/dl)	111.10±36.90	160.87±89.40	0.004
HDL (mg/dl)	56.53±17.78	59.37±13.77	0.25
LDL (mg/dl)	114.73±32.57	112.23±28.07	0.38
Glucose (mg/dl)	102.37±38.64	108.73±38.40	0.26

Moreover, the serum ACE levels were significantly different between hypertension group (192.19±104.33 ng/ml) and control

group (146.37±46.65 ng/ml) ($p=0.02$) as showed in Figure 1.

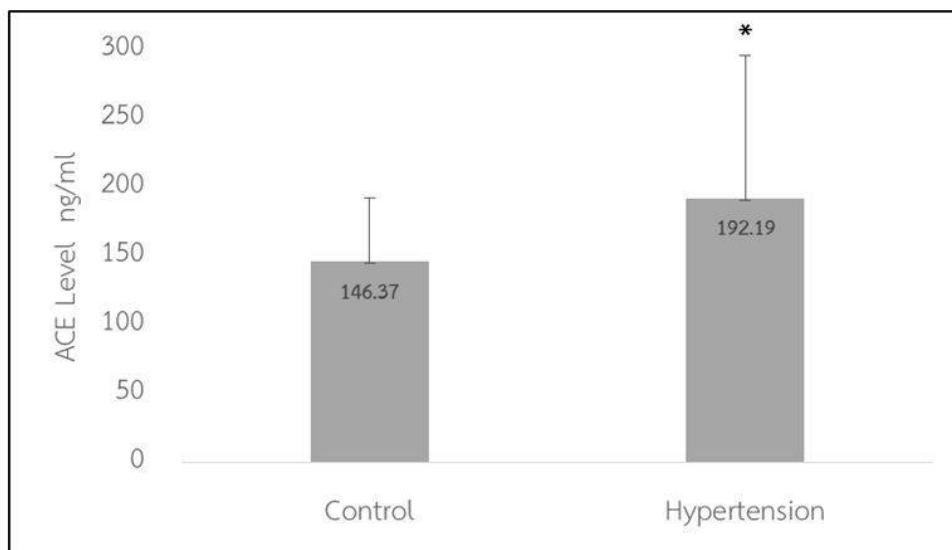


Figure 1 ACE protein levels of normal controls and hypertension patients.

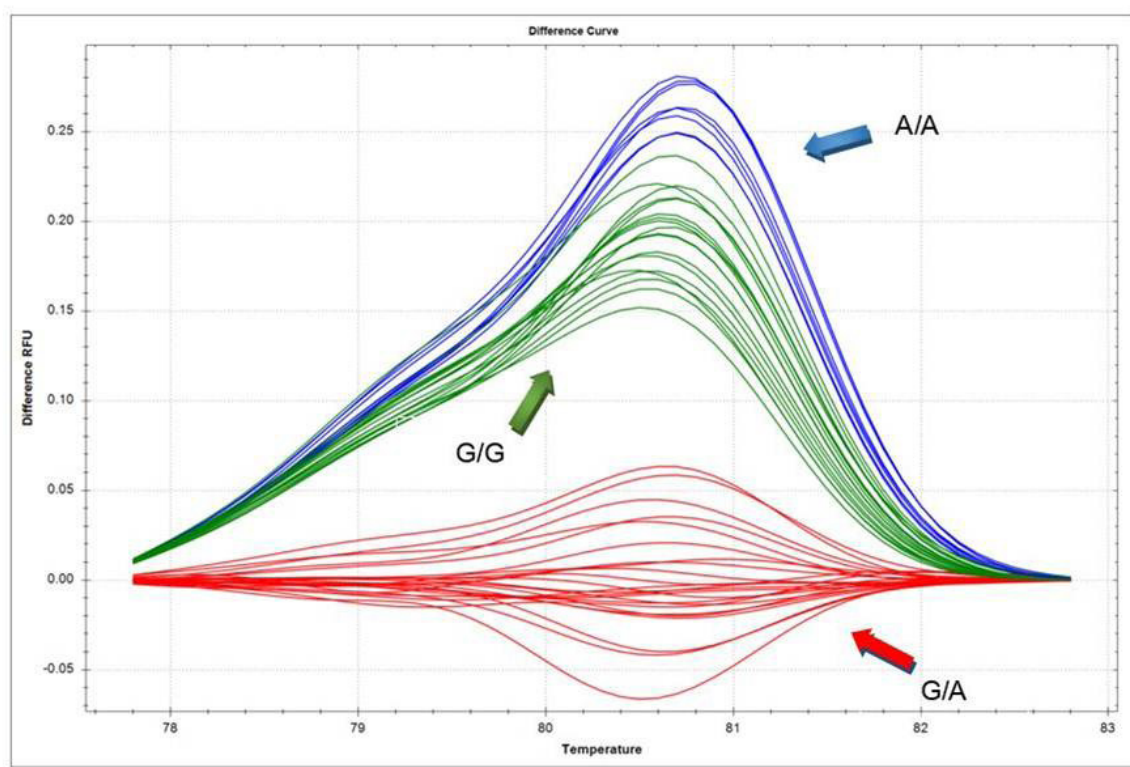


Figure 2 The melting curve of SNP (rs4343; G/A) in *ACE* gene of normal controls and hypertension patients.

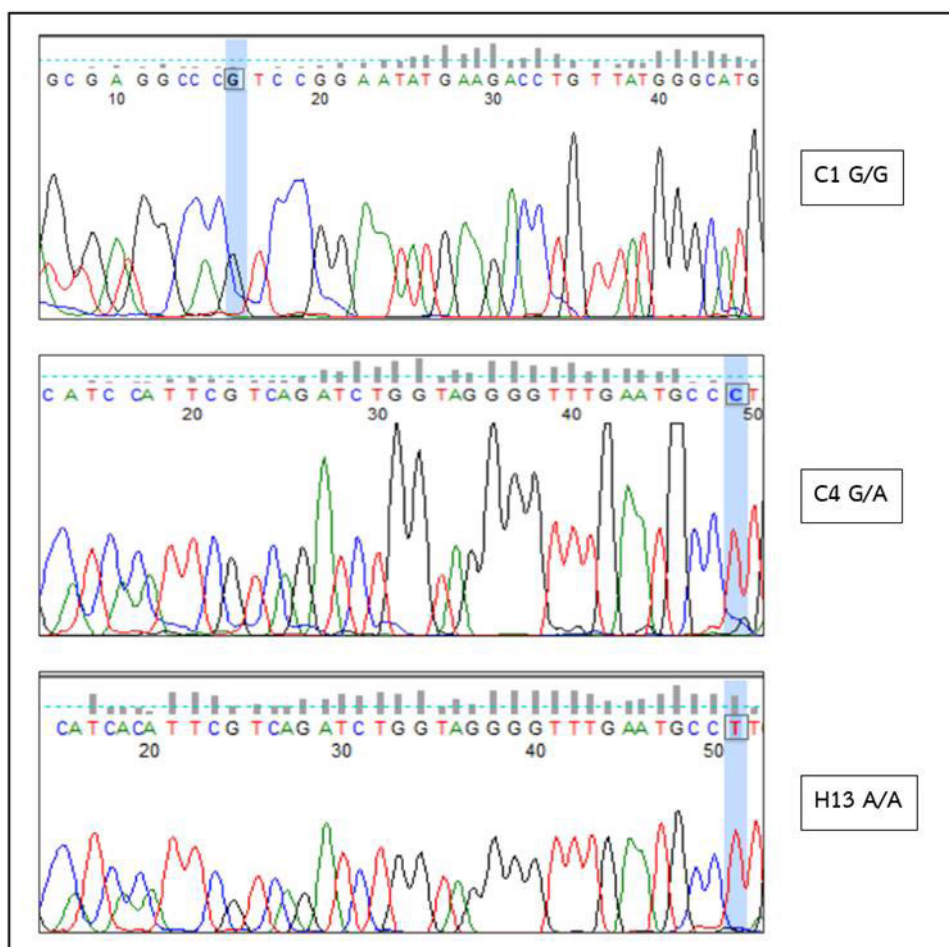


Figure 3 Chromatograms of selected DNA for validation of genotypes.

Table 2 Distribution of genotype and allele frequency of *ACE* gene (rs4343; G/A) in controls and hypertension patients

SNP	Allele	Control		Hypertension		p-value
		n=30		n=30		
rs4343		n	%	n	%	0.629
G/A	GG	10	33	13	43	
	GA	15	50	14	47	
	AA	5	17	3	10	
	Allele G	35	58	40	67	
	Allele A	25	42	20	33	

The melting curve of SNP (rs4343; G/A) in *ACE* gene are shown in Figure 2. Sequencing results of selected DNA are shown in Figure 3. The genotype and allele frequencies of rs4343 of *ACE* gene in controls and hypertension patients and association analysis is summarized in Table 2. The goodness-of-fit test was equal to 1 indicated that both of SNPs tested were in Hardy Weinberg equilibrium. The results indicated that G/A polymorphism in *ACE* gene was not significantly associated with hypertension ($p=0.629$).

Discussion

In this study, we compared the demographic characteristics between two groups, the results showed the average of age in control group was 43.37 years and hypertension group was 55.79 years. In accordance with previous study indicated that hypertension increased with age, from 7.3% in people aged 18-39 to 32.4% in people aged 40-59 and 65.0% in those older than 60 years¹⁷. In addition, hypertension patients had body mass index (BMI) 27.70 indicated obesity. BMI is a risk factor for non-communicable diseases; NCDs such as diabetes mellitus, hypertension, cardiovascular disease and cancer¹⁸. The 6 years period, having body weight over 4.5 kg is a risk factor for hypertension¹⁹. Independent effect of change in SBP and DBP in both women and men, and that people who increase their BMI are at increased risk for hypertension²⁰. Lipid profiles between two groups had shown

triglyceride in hypertension patients higher than control group. Similarly with previous cross-sectional study in a Rural area in Brazil which indicated the age, triglycerides, waist circumference and sex were independent risk factor for hypertension²¹.

In this study, we investigated serum ACE levels with hypertension because it associated with renin and angiotensin enzyme, two of which act synergy with adrenal gland and pituitary gland for control water, minerals, sodium and systolic blood vessels in the body for control blood pressure to renin-angiotensin system²². In addition, the ACE is inactivated bradykinin and vasodilator peptides to inactive metabolites for inhibitor nitric oxide and prostacyclin and induced vasodilation²³. The data from this study showed that the ACE levels were significantly different between hypertension group and control group ($p=0.02$) which consistent to the pervious study in pregnancy²⁴.

Various reports have studied about the genetic variability of *ACE* gene/protein and renin-angiotensin gene (RAS system) in hypertension patient such as the renin angiotensin system (RAS) gene polymorphism, the angiotensin converting enzyme (*ACE*), angiotensinogen (*AGT*) M235T/T174M and angiotensin II type1 receptor (*ATR1*) A1166C were reported in Turkish patients. The frequencies of *ACE* D allele (DD+ID), *AGN* M235T TT genotype and 174M allele and *ATR1* C allele (AC+CC genotypes) were significantly higher in hypertensive group than controls⁵.

The *ACE* 2350 G/A polymorphism (rs4343) was associated with atrial fibrillation and that the A allele showed an increased risk for atrial fibrillation in Han Chinese patients with essential hypertension⁶. The *ACE* gene 2350 G/A polymorphism was associated with diabetic retinopathy (DR) in Han Chinese patients with type 2 diabetes mellitus (DM)⁷. The polymorphic variants of *AGT* gene M235T and T174M showed association with hypertension²⁵. The polymorphisms in *ACE* gene of Trp1197stop resulted in dramatic elevation of serum²⁶. Among genotyped of 13 polymorphisms in the *ACE* gene from Nigeria population showed the two SNPs including G2350A (rs4343) and I/D (rs4291) were strongly linked to ACE concentration and blood pressure⁸. Moreover, the *ACE* gene G2350A polymorphism was significantly and positively associated with the risk of essential hypertension among Han Chinese patients⁹. However, from a Chinese Han population, the *ACE* 2350 G/A polymorphism had no effect on blood pressure in either control or untreated essential hypertension patients but associated with Left Ventricular hypertrophy²⁷. The *ACE* association for SNP in hypertensive population showed AA genotype (rs4343 G/A) in Pakistani hypertensive patients had 23%, A allele increased risk for hypertension¹⁰. On the other hand, the *ACE* gene rs4343 (G/A) from the United Arab Emirates, showed that the frequency of the G allele was statistically significant and indicates a major gene effect

with essential hypertension¹¹. Consistent with the study in Muslims from the Arab Gulf and Pakistan shown A allele (G2350A) was associated with a significantly reduced hypertension risk¹². Analyzed from allele frequency in this study showed allele G higher than allele A in hypertension patients but when we compared between control group and hypertension group showed no significantly ($p=0.629$).

Conclusion

The data from this study indicated that there was a relationship between serum ACE protein levels and hypertension, but no association between SNP (rs4343) in *ACE* gene with risk of hypertension in Thai patient. Further study using a larger sample size and other SNPs in the *ACE* gene will be elucidated.

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