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ORIGINAL ARTICLE

Combined *PCSK9* and *APOE* Polymorphisms are Genetic Risk Factors Associated with Elevated Plasma Lipid Levels in a Thai Population

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Abstract Proprotein convertase subtilisin/kexin type 9 (PCSK9) and apolipoprotein E (ApoE) play a key role in the regulation of lipid metabolism. We aimed to investigate the effects of *PCSK9* (R46L, I474V, and E670G) and *APOE* polymorphisms on lipid levels in a Southern Thai population. A total of 495 participants (307 urban, 188 rural) were recruited for the study. Anthropometric and biochemical variables were evaluated. *PCSK9* and *APOE* polymorphisms were analyzed using PCR–RFLP. The 46L urban male carriers had significantly higher diastolic blood pressure (DBP) and fasting blood sugar compared with non-carriers. In contrast, the 46L urban female carriers had significantly lower total cholesterol (TC) and LDL-C levels compared with non-carriers. The 474V rural female carriers had significantly lower HDL-C levels than non-carriers. The 670G urban female carriers showed significantly higher TC and LDL-C levels compared with non-carriers. *APOE4* carriers had increased TC and LDL-C levels relative to *APOE3* carriers in the urban males. *APOE2* carriers had decreased TC and/or LDL-C levels compared with *APOE3* carriers in urban males and females. A significant trend of increased TC and LDL-C levels was observed in non-*APOE4-PCSK9* 670EE carriers to *APOE4-PCSK9* 670EG carriers in urban subjects. In summary, R46L, I474V, and E670G may be genetic risk factors for cardiovascular disease (CVD) in urban males, rural females, and urban females, respectively. In contrast, R46L had a favorable lipid profiles that may protect against CVD in urban females. The combination of *PCSK9* E670G and *APOE* polymorphisms may represent an independent factor for the determination of lipid levels.

Keywords *APOE* · Lipid levels · *PCSK9* · Polymorphisms

Abbreviations

APOE	Apolipoprotein E
BMI	Body mass index
CARDIA study	Coronary artery risk development in young adults study
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DHS	Dallas heart study
FBS	Fasting blood sugar
GOF	Gain-of-function
HDL-C	High density lipoprotein cholesterol
LCAS	Lipoprotein coronary atherosclerosis study
LDL-C	Low density lipoprotein cholesterol
LDLR	Low density lipoprotein receptor
LOF	Loss-of-function
PCR-RFLP	Polymerase chain reaction and restriction fragment length polymorphism
PCSK9	Proprotein convertase subtilisin/kexin type 9
PLIC study	Progression of lesions in the intima of the carotid study
PROSPER	Prospective study of pravastatin in the elderly at risk

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TC	Total cholesterol
TG	Triglyceride
UK	United Kingdom
UV	Ultraviolet
WC	Waist circumference

Introduction

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a serine protease that plays a key role in the regulation of low-density lipoprotein cholesterol (LDL-C) by promoting the degradation of low-density lipoprotein receptors (LDLR) through a post-transcriptional mechanism [1–4]. The PCSK9 binds directly to the EGF-A domain of LDLR and targets the receptor to the lysosome for degradation, leading to reduced clearance of LDL-C from circulation [5, 6]. Gain-of-function (GOF) mutations in the *PCSK9* gene cause hypercholesterolemia and cardiovascular disease (CVD) [7–10], whereas loss-of-function (LOF) mutations are associated with reduced plasma levels of LDL-C and protection from CVD [11, 12].

PCSK9 is a highly polymorphic gene, with over 40 single nucleotide polymorphisms (SNPs) reported [13]. The relationship of common *PCSK9* polymorphisms, e.g., R46L, I474V, and E670G, with serum lipid levels has been investigated. Nevertheless, the association between *PCSK9* polymorphisms and lipid levels varies across populations. Several studies in European, African American, and white populations, hypobetalipoproteinemia subjects, as well as in the Dallas Heart Study (DHS) and the PROSPER study group have shown that *PCSK9* 46L carriers had decreased LDL-C levels [14–22]. Additionally, Caucasian, African Canadian [23], and Japanese [24] individuals carrying the V allele of *PCSK9* I474V had lower total cholesterol (TC) and/or LDL-C levels than non-carriers. However, *PCSK9* I474V was not associated with lipid levels in healthy UK men [15], Brazilian subjects [26], and the PLIC study [25]. Moreover, in German [27], Brazilian [26], and Tunisian populations [28], as well as in the Belgium Stroke Study [29], the PLIC study [25], and the LCAS [30], *PCSK9* E670G was associated with increased TC and/or LDL-C levels or an increased risk of atherosclerosis. In healthy UK men [15], Taiwanese [31], Guangxi Bai Ku Yao, and Han Chinese populations [32], and in the PROSPER study group [14], *PCSK9* E670G was not associated with increased TC and/or LDL-C levels or increased risk of atherosclerosis.

Apolipoprotein E (ApoE) is a component of plasma chylomicrons, chylomicron remnants, VLDL, IDL, and HDL. ApoE acts as a ligand for LDLR and LDL-related protein (LRP) [33]. There are three common alleles (E2, E3, and E4) in the *APOE* gene, which code for six genotypes of E2E2, E3E3, E4E4, E2E3, E2E4, and E3E4 [33]. The E4 allele is associated with higher levels of LDL-C and total cholesterol, and E2 with lower levels compared with the E3 allele [34]. In addition, E4 allele is associated with the lower levels of HDL-C [35]. It has been reported that the E4 allele is associated with increased risk of CVD [34, 35]. Moreover, the relationship between *PCSK9* and *APOE* polymorphisms on lipid levels and intima media thickness had been reported in a previous study [25].

The effect of *PCSK9* and *APOE* polymorphisms on lipid levels in a Southern Thai population has not been examined. Thus, the present study aimed to investigate the effects of these gene polymorphisms on lipid levels in Southern Thai subjects.

Materials and Methods

Study Population and Data Collection

The study was conducted on 495 unrelated healthy volunteers of a Southern Thai population from urban and rural areas in Pattalung and Nakhon Si Thammarat provinces. Pregnant women, individuals with diabetes mellitus and secondary dyslipidemia due to renal, liver or thyroid disease, and those individuals taking medication to lower lipid levels were not included in the study. Waist circumference (WC) was measured midway between the lower rib margin and the iliac crest. Body mass index (BMI) was calculated as a ratio of weight in kg and square height in meters (kg/m^2). Blood pressure was measured, and data on lifestyle and sociodemographic factors were collected using a questionnaire. The questionnaire included education, occupation, previous diseases, current medication use, cigarette smoking habits, alcohol consumption, and physical activity. Written informed consent was obtained from every subjects. The study protocol was approved by the ethics committee of Walailak University.

Laboratory Examinations

Blood samples were collected from subjects after a 12 h fast. Serum and plasma were separated by centrifugation at 3000 rpm for 10 min and were later used for the analysis of biochemical parameters. Blood was collected in EDTA for DNA extraction. Fasting blood sugar (FBS) and lipid profiles were measured using enzymatic kits. HDL-C was determined after the precipitation of the apolipoprotein (apo) B containing lipoproteins by phosphotungstate/magnesium chloride. LDL-C was calculated using the Friedewald formula.

DNA Extraction

Genomic DNA was extracted from the buffy coat using a GeneAid kit (GeneAid Biotech Ltd., Taiwan). The quality and quantity of DNA were estimated using a NanoDrop 2000 UV–Vis spectrophotometer (Thermo Fisher Scientific Inc., USA). DNA samples were stored at −80 °C until analysis.

Polymorphism Detection

Genotyping for *PCSK9* and *APOE* polymorphisms was conducted using the polymerase chain reaction and restriction fragment length polymorphism (PCR–RFLP) method with slight modifications of the previously described protocol [15, 27, 36]. Each PCR reaction contained 100 ng of genomic DNA, 0.2 μmol/l of each primer (eurofins MWG GmbH, Germany), 200 μmol/l dNTPs, 0.625 U *Taq* polymerase, and PCR buffer [50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 10 mmol/l Tris–HCl (pH 8.3)] [New England BioLabs (NEB), USA], for all polymorphisms, except *PCSK9* R46L and *APOE*, where 10 % (v/v) DMSO was added. The final reaction volume was 25 μL. Enzymes, primers, and PCR conditions used in this study are shown in Table S1. The PCR reactions were performed using GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, USA). PCR products from *PCSK9* R46L, I474V, E670G, and *APOE* assays were digested with *Rsa*I (NEB, USA), *Bcc*I (NEB, USA), *Sau96*I (NEB, USA), and *Afl*III and *Hae*II (NEB, USA), respectively, at 37 °C for overnight except I474V, which was incubated at 37 °C for 1 h. Restriction fragments were identified by 2 and 4 % (w/v) agarose gel electrophoresis for R46L, E670G, and for I474V, *APOE*, respectively. The PCR products and restriction fragments were stained with ethidium bromide and visualized under UV light.

Statistical Analyses

Continuous variables were expressed as the mean ± standard deviation. Categorical variables were presented as percentages. The observed genotype frequencies were compared with the expected frequencies and checked for Hardy–Weinberg equilibrium. Differences in genotypic and allelic distributions between the groups were estimated using the Chi square (χ^2) test. Data normality was determined by the Kolmogorov–Smirnov test. Mean differences between genders and genotypes were assessed by the two-tailed unpaired Student's *t* test for normally distributed parameters and the Mann–Whitney *U* test for nonparametric. For multiple comparisons of means between genotypes, one-way ANOVA followed by Tukey's multiple comparison test was performed. A p value <0.05 was considered statistically significant. The analyses were performed using SPSS version 17 (SPSS, Evanston, IL, USA).

Results

Subjects' Characteristics

Most of rural subjects were farmers, whereas the majority of the urban subjects were government officers. Urban subjects had a higher education level than rural subjects, and the current alcohol consumption was significantly higher in urban subjects than in rural subjects. However, there were no significant differences in the number of current smokers, former smokers, and exercise habits between rural and urban subjects (Table S2). The anthropometric and biochemical variables of the study subjects (307 urban, 188 rural) are shown in Table 1. The urban subjects (total group) showed significantly higher TC and LDL-C levels and lower BMI, HDL-C, and FBS levels compared with the rural subjects (all $p < 0.05$).

Genotypic and Allelic Distributions

The genotypic and allelic frequencies of *PCSK9* R46L, I474V, and E670G and *APOE* polymorphisms in urban and rural subjects are shown in Table 2. All of the observed genotypic frequencies were in Hardy–Weinberg equilibrium. The genotypic and allelic frequencies of *PCSK9* I474V, E670G, and *APOE* polymorphisms, but not *PCSK9* R46L polymorphism were not significantly different between urban and rural subjects (Table 2).

Relationship Between *PCSK9* Genotypes and Anthropometric and Biochemical Variables

The anthropometric and biochemical variables according to *PCSK9* genotypes in males, females, and the total group in urban and rural subjects are shown in Tables 3, 4, and Table S3, respectively.

The 46L urban male carriers showed significantly higher diastolic blood pressure (DBP) and FBS levels ($p < 0.05$), whereas the 46L urban female carriers had significantly lower TC and LDL-C levels ($p < 0.05$). In the rural subjects, the statistical analysis was not determined between 46L carriers and non-carriers because of the lack of the L allele. In males, compared with the urban 46RR genotype, rural 46RR genotype was associated with significantly lower BMI, WC, TC, LDL-C levels, and higher HDL-C levels ($p < 0.05$). In contrast, compared with the urban female 46RR genotype, the rural female 46RR genotype was associated with significantly higher BMI, WC, DBP, triglyceride (TG), HDL-C, FBS levels, and lower TC and LDL-C levels ($p < 0.05$).

The 474V rural female carriers had significantly lower HDL-C levels compared with non-carriers ($p < 0.05$). In comparison with the urban male 474II genotype, the rural male 474II genotype had significantly lower BMI,

Table 1 Anthropometric and biochemical variables of study subjects

Variables	Urban				Rural				Urban vs. rural *p* value[b]
	Total ($n = 307$)	Male ($n = 97$)	Female ($n = 210$)	*p* value[a]	Total ($n = 188$)	Male ($n = 38$)	Female ($n = 150$)	*p* value[a]	
Body mass index (kg/m^2)	23.56 ± 3.16	24.73 ± 2.75	23.03 ± 3.19	0.000	24.40 ± 4.08	22.63 ± 3.51	24.85 ± 4.10	0.003	0.028
Waist circumference (cm)	80.57 ± 10.26	86.62 ± 11.46	77.78 ± 8.31	0.000	82.24 ± 10.69	81.24 ± 11.65	82.50 ± 10.46	0.517	0.058
Systolic blood pressure (mmHg)	129.40 ± 16.71	134.61 ± 15.97	127.00 ± 16.53	0.000	130.13 ± 19.83	132.18 ± 18.18	129.61 ± 20.25	0.262	0.720
Diastolic blood pressure (mmHg)	79.93 ± 11.20	82.53 ± 12.11	78.73 ± 10.58	0.006	81.27 ± 12.97	78.05 ± 9.37	82.09 ± 13.64	0.077	0.386
Total cholesterol (mg/dl)	218.90 ± 51.29	222.23 ± 54.30	217.36 ± 49.90	0.359	207.95 ± 40.88	194.26 ± 34.07	211.41 ± 41.83	0.020	0.000
LDL-C (mg/dl)	145.31 ± 39.93	152.54 ± 43.97	147.81 ± 37.94	0.387	127.04 ± 32.64	114.54 ± 31.91	130.21 ± 32.17	0.008	0.000
Triglyceride (mg/dl)	109.37 ± 65.12	133.08 ± 75.44	98.42 ± 56.69	0.000	119.50 ± 72.43	117.68 ± 71.40	119.96 ± 72.92	0.761	0.083
HDL-C (mg/dl)	52.83 ± 12.94	48.67 ± 12.61	54.76 ± 12.67	0.000	57.53 ± 13.38	56.21 ± 14.21	57.86 ± 13.20	0.647	0.000
Fasting blood sugar (mg/dl)	89.37 ± 20.61	96.64 ± 32.96	86.01 ± 9.33	0.000	91.12 ± 17.21	96.47 ± 26.63	89.75 ± 13.61	0.106	0.039

[a] *p* value obtained in the Student's *t* test or Mann–Whitney *U* test for the comparison between genders

[b] *p* value obtained in the Student's *t* test or Mann–Whitney *U* test for the comparison between residence areas

WC, DBP, TC, LDL-C levels, and higher HDL-C levels ($p < 0.05$). In contrast, the rural female 474II genotype had significantly higher BMI, WC, DBP, TG, HDL-C, FBS levels and lower LDL-C levels compared with the urban female 474II genotype ($p < 0.05$). In addition, the rural female 474IV genotype had significantly higher DBP, and lower HDL-C levels compared with the urban female 474IV genotype ($p < 0.05$).

Moreover, the 670G urban female carriers had significantly higher TC and LDL-C levels than non-carriers ($p < 0.05$). In contrast, no significant differences in lipid levels and other parameters between 670G carriers and non-carriers among the rural subjects were observed. In males, compared with the urban 670EE genotype, the rural 670EE genotype had significantly lower BMI, WC, DBP, TC, LDL-C levels, and higher HDL-C levels ($p < 0.05$). In females, rural 670EE genotype showed significantly higher BMI, WC, DBP, TG, HDL-C, FBS levels, and lower LDL-C levels compared with urban 670EE genotype ($p < 0.05$). Furthermore, significantly higher TC and LDL-C levels were observed in urban females with the EG genotype compared with rural females with the EG genotype ($p < 0.05$).

Relationship Between *APOE* Genotypes and Anthropometric and Biochemical Variables

The anthropometric and biochemical variables according to *APOE* genotypes in males and females, as well as in the total group, in the urban and rural subjects are shown in Table 5 and Table S4, respectively. Subjects with the E2E4 genotype were excluded from this analysis. The *APOE* genotype had a significant effect on TC and LDL levels in both urban males and females ($p < 0.05$). No significant differences were observed in other parameters. In comparison with *APOE3* carriers, *APOE2* carriers showed significantly lower TC and/or LDL-C levels in both urban males and females, whereas *APOE4* carriers showed significantly higher TC and LDL-C levels than *APOE3* carriers in urban males ($p < 0.05$).

PCSK9 E670G Polymorphism and Relation to *APOE* Polymorphism

The analysis of a combination of *PCSK9* E670G and *APOE* polymorphisms in the urban subjects showed an increase in TC and LDL-C levels, with an increase from the lowest level in non-*APOE4-PCSK9* 670EE carriers to the highest level in *APOE4-PCSK9* 670EG carriers (p value for trend = 0.000 for both TC, and LDL-C; Table 6). In addition, non-*APOE4-PCSK9* 670EG carriers had significantly higher TC and LDL-C levels than non-*APOE4-PCSK9* 670EE carriers ($p < 0.05$).

Table 2 Genotypic and allelic frequencies of *PCSK9* (R46L, I474V, and E670G) and *APOE* polymorphisms in urban and rural subjects

Polymorphism	Urban			Rural			Urban vs. rural p value[b]
			p value[a]			p value[a]	
PCSK9 R46L Genotype	RR	293 (95.4 %)	0.683	RR	188 (100 %)	0.971	0.003
	RL	14 (4.6 %)		RL	0 (0 %)		
	LL	0 (0 %)		LL	0 (0 %)		
PCSK9 R46L Allele	R	600 (97.72 %)		R	376 (100 %)		0.003
	L	14 (2.28 %)		L	0 (0 %)		
PCSK9 I474 V Genotype	II	290 (94.5 %)	0.618	II	181 (96.3 %)	0.795	0.362
	IV	17 (5.5 %)		IV	7 (3.7 %)		
	VV	0 (0 %)		VV	0 (0 %)		
PCSK9 I474 V Allele	I	597 (97.23 %)		I	369 (98.14 %)		0.368
	V	17 (2.77 %)		V	7 (1.86 %)		
PCSK9 E670G Genotype	EE	295 (96.10 %)	0.727	EE	184 (97.9 %)	0.883	0.277
	EG	12 (3.90 %)		EG	4 (2.1 %)		
	GG	0		GG	0		
PCSK9 E670G Allele	E	602 (98.05 %)		E	372 (98.94 %)		0.281
	G	12 (1.95 %)		G	4 (1.06 %)		
APOE Genotype	E2E2	5 (1.6 %)	0.625	E2E2	0 (0 %)	0.606	0.346
	E2E3	48 (15.6 %)		E2E3	24 (12.8 %)		
	E3E3	181 (59 %)		E3E3	111 (59 %)		
	E3E4	63 (20.5 %)		E3E4	45 (23.9 %)		
	E4E4	4 (1.3 %)		E4E4	5 (2.7 %)		
	E2E4	6 (2 %)		E2E4	3 (1.6 %)		
APOE Allele	E2	64 (10.42 %)		E2	27 (7.18 %)		0.129
	E3	473 (77.04 %)		E3	292 (77.39 %)		
	E4	77 (12.54 %)		E4	58 (15.43 %)		

[a] Exact p value for Hardy–Weinberg equilibrium

[b] p value was determined using χ^2 test

Discussion

In the present study, we investigated the association of *PCSK9* and *APOE* polymorphisms with lipid levels in a Southern Thai population. Urban subjects had significantly higher TC and LDL-C, and lower BMI, HDL-C, and FBS levels than did rural subjects, results consistent with a previous study [37]. We observed a significantly higher frequency of current alcohol consumption and higher education in urban subjects relative to rural subjects. Most urban subjects were government officers, whereas most rural subjects were farmers, suggesting that the differences in lipid profiles, BMI, and FBS levels between urban and rural subjects could be explained by differences in lifestyle and sociodemographic factors.

In the present study, the frequency of L allele of *PCSK9* R46L polymorphism was 2.28 and 0 %; in the urban and rural subjects, respectively. This allele frequency in urban subjects was similar to the Caucasian individuals (2.40 %) [23], whereas lower frequency of the L allele of R46L was observed in an Italian (1.04 %) population [18], in the CARDIA study (1.64 % in whites) [16], in the Dallas Heart Study (DHS) (1.6 % in whites, 0.75 % in Hispanics, and 0.28 % in black) [22], in the Copenhagen City Heart Study (1.22 %) [19], Copenhagen General Population Study (1.41 %) [19], and Copenhagen Ischemic Heart Disease Study (1.18 %) [19]. In addition, a higher frequency of L allele of R46L was observed in the French Canadian population (4.80 %) [23].

The frequency of the V allele of *PCSK9* I474V polymorphism was 2.77 and 1.86 % in the urban and rural subjects, respectively. This finding in urban subjects was similar to that of a Japanese population (2.48 %) [24]. However, the higher frequency of the V allele of I474V has been observed in many populations: Brazilian subjects (17.59 %) [26], Caucasian Canadian (12.60 %) [23], African Canadians (19.40 %) [23], African Americans (22 %) [23], and healthy UK men (17.68 %) [15], as well as in the Dallas heart study (DHS) (18 % in whites, 9.7 % in Hispanics, and 22 % in blacks) [22] and the PLIC study (17.59 %) [25].

Table 3 Anthropometric and biochemical variables according to *PCSK9* genotypes (R46L, I474V, and E670G) in males in urban and rural subjects

Variables	Urban ($n = 97$)			Rural ($n = 38$)			Urban vs. rural (RR) p value[b]	Urban vs. rural (RL) p value[b]
	R46L genotype			R46L genotype				
	RR ($n = 91$)	RL ($n = 6$)	p value[a]	RR ($n = 38$)	RL ($n = 0$)	p value[a]		
Body mass index (kg/m^2)	24.57 ± 2.57	27.16 ± 4.32	0.204	22.63 ± 3.51	–	ND	0.003	ND
Waist circumference (cm)	86.36 ± 11.41	90.50 ± 12.50	0.616	81.24 ± 11.65	–	ND	0.001	ND
Systolic blood pressure (mmHg)	134.04 ± 16.17	143.17 ± 10.07	0.177	132.18 ± 18.18	–	ND	0.700	ND
Diastolic blood pressure (mmHg)	81.96 ± 12.18	91.17 ± 6.85	0.035	78.05 ± 9.37	–	ND	0.065	ND
Total cholesterol (mg/dl)	222.21 ± 55.94	222.50 ± 16.96	0.990	194.26 ± 34.07	–	ND	0.005	ND
LDL-C (mg/dl)	153.31 ± 44.76	140.88 ± 29.81	0.506	114.54 ± 31.91	–	ND	0.000	ND
Triglyceride (mg/dl)	133.95 ± 77.31	120.00 ± 38.15	0.982	117.68 ± 71.40	–	ND	0.238	ND
HDL-C (mg/dl)	48.08 ± 11.95	57.67 ± 19.46	0.274	56.21 ± 14.21	–	ND	0.001	ND
Fasting blood sugar (mg/dl)	96.03 ± 33.66	105.83 ± 18.97	0.039	96.47 ± 26.63	–	ND	0.365	ND
Variables	I474V genotype			I474V genotype			Urban vs. rural (II) p value[b]	Urban vs. rural (IV) p value[b]
	II ($n = 92$)	IV ($n = 5$)	p value[a]	II ($n = 38$)	IV ($n = 0$)	p value[a]		
Body mass index (kg/m^2)	24.76 ± 2.80	24.14 ± 1.72	0.626	22.63 ± 3.51	–	ND	0.000	ND
Waist circumference (cm)	86.54 ± 11.53	88.00 ± 11.22	0.689	81.24 ± 11.65	–	ND	0.001	ND
Systolic blood pressure (mmHg)	134.79 ± 16.25	131.20 ± 9.98	0.627	132.18 ± 18.18	–	ND	0.423	ND
Diastolic blood pressure (mmHg)	83.03 ± 11.98	73.20 ± 11.65	0.086	78.05 ± 9.37	–	ND	0.024	ND
Total cholesterol (mg/dl)	222.92 ± 53.43	209.40 ± 74.84	0.590	194.26 ± 34.07	–	ND	0.003	ND
LDL-C (mg/dl)	153.13 ± 42.60	141.58 ± 70.40	0.570	114.54 ± 31.91	–	ND	0.000	ND
Triglyceride (mg/dl)	136.57 ± 75.51	69.00 ± 38.34	0.068	117.68 ± 71.40	–	ND	0.150	ND
HDL-C (mg/dl)	48.36 ± 12.69	54.40 ± 10.45	0.173	56.21 ± 14.21	–	ND	0.001	ND
Fasting blood sugar (mg/dl)	96.97 ± 33.67	90.60 ± 15.11	0.624	96.47 ± 26.63	–	ND	0.559	ND
Variables	E670G genotype			E670G genotype			Urban vs. rural (EE) p value[b]	Urban vs. rural (EG) p value[b]
	EE ($n = 95$)	EG ($n = 2$)	p value[a]	EE ($n = 37$)	EG ($n = 1$)	p value[a]		
Body mass index (kg/m^2)	24.74 ± 2.78	24.42 ± 0.28	0.873	22.77 ± 3.46	17.78	ND	0.001	ND
Waist circumference (cm)	87.18 ± 10.10	60.00 ± 39.60	0.253	81.32 ± 11.79	78.00	ND	0.005	ND
Systolic blood pressure (mmHg)	134.88 ± 16.03	121.50 ± 2.12	0.243	132.86 ± 17.94	107.00	ND	0.531	ND
Diastolic blood pressure (mmHg)	82.65 ± 12.19	76.50 ± 4.95	0.388	78.08 ± 9.50	77.00	ND	0.042	ND
Total cholesterol (mg/dl)	221.25 ± 54.07	268.50 ± 62.93	0.225	195.76 ± 33.26	139.00	ND	0.008	ND
LDL-C (mg/dl)	151.63 ± 43.66	195.50 ± 51.62	0.164	115.66 ± 31.59	73.20	ND	0.000	ND
Triglyceride (mg/dl)	132.59 ± 75.66	156.50 ± 84.15	0.431	119.27 ± 71.70	59.00	ND	0.322	ND
HDL-C (mg/dl)	48.81 ± 12.69	42.00 ± 5.66	0.395	56.27 ± 14.40	54.00	ND	0.003	ND
Fasting blood sugar (mg/dl)	96.80 ± 33.29	89.00 ± 2.83	0.980	96.84 ± 26.90	83.00	ND	0.450	ND

ND not determined

[a] p value obtained in the Student's t test or Mann–Whitney U test for the comparison between genotypes for each polymorphism

[b] p value obtained in the Student's t test or Mann–Whitney U test for the comparison between residence areas for each genotype

The frequency of the G allele of *PCSK9* E670G polymorphism was 1.95 and 1.06 %, in urban and rural subjects, respectively. This finding in urban subjects was similar to that in reported in a Guangxi Bai Ku Yao (2 %) population [32], whereas a higher frequency of the G allele was observed in Germany

Table 4 Anthropometric and biochemical variables according to *PCSK9* genotypes (R46L, I474V, and E670G) in females in urban and rural subjects

Variables	Urban ($n = 210$)			Rural ($n = 150$)			Urban vs. rural (RR) p value[b]	Urban vs. rural (RL) p value[b]
	R46L genotype			R46L genotype				
	RR ($n = 201$)	RL ($n = 8$)	p value[a]	RR ($n = 150$)	RL ($n = 0$)	p value[a]		
Body mass index (kg/m^2)	23.03 ± 3.21	23.11 ± 2.92	0.815	24.85 ± 4.10	–	ND	0.000	ND
Waist circumference (cm)	77.81 ± 8.38	77.00 ± 6.72	0.948	82.50 ± 10.46	–	ND	0.000	ND
Systolic blood pressure (mmHg)	127.15 ± 16.67	123.13 ± 12.80	0.510	129.61 ± 20.25	–	ND	0.659	ND
Diastolic blood pressure (mmHg)	78.74 ± 10.70	78.63 ± 7.37	0.977	82.09 ± 13.64	–	ND	0.019	ND
Total cholesterol (mg/dl)	219.15 ± 49.33	172.13 ± 45.14	0.004	211.41 ± 41.83	–	ND	0.018	ND
LDL-C (mg/dl)	148.84 ± 38.10	121.91 ± 22.50	0.020	130.21 ± 32.17	–	ND	0.000	ND
Triglyceride (mg/dl)	99.73 ± 57.30	65.50 ± 19.34	0.076	119.96 ± 72.92	–	ND	0.003	ND
HDL-C (mg/dl)	54.70 ± 12.80	56.13 ± 9.16	0.573	57.86 ± 13.20	–	ND	0.037	ND
Fasting blood sugar (mg/dl)	86.01 ± 9.41	86.13 ± 7.57	0.821	89.75 ± 13.61	–	ND	0.008	ND
Variables	I474V genotype			I474V genotype			Urban vs. rural (II) p value[b]	Urban vs. rural (IV) p value[b]
	II ($n = 198$)	IV ($n = 12$)	p value[a]	II ($n = 143$)	IV ($n = 7$)	p value[a]		
Body mass index (kg/m^2)	22.99 ± 3.17	23.68 ± 3.56	0.626	24.88 ± 4.08	24.26 ± 4.84	0.720	0.000	0.820
Waist circumference (cm)	77.64 ± 8.36	80.00 ± 7.45	0.319	82.40 ± 10.48	84.83 ± 10.61	0.579	0.000	0.437
Systolic blood pressure (mmHg)	127.68 ± 16.86	125.75 ± 10.07	0.994	129.06 ± 20.07	140.71 ± 22.25	0.123	0.817	0.058
Diastolic blood pressure (mmHg)	78.74 ± 10.61	78.67 ± 10.38	0.207	81.64 ± 13.49	91.29 ± 14.48	0.070	0.046	0.041
Total cholesterol (mg/dl)	217.68 ± 49.77	212.08 ± 53.97	0.814	211.95 ± 41.76	200.43 ± 45.14	0.479	0.062	0.592
LDL-C (mg/dl)	147.96 ± 38.54	145.38 ± 27.15	0.941	130.28 ± 32.02	128.63 ± 37.79	0.895	0.000	0.277
Triglyceride (mg/dl)	98.49 ± 57.29	97.33 ± 47.70	0.799	120.22 ± 73.80	114.71 ± 55.72	0.845	0.002	0.481
HDL-C (mg/dl)	54.41 ± 12.74	60.42 ± 10.33	0.069	58.30 ± 13.32	48.86 ± 5.11	0.030	0.010	0.014
Fasting blood sugar (mg/dl)	86.23 ± 9.38	82.42 ± 8.01	0.275	89.70 ± 13.72	90.71 ± 11.90	0.305	0.024	0.086
Variables	E670G genotype			E670G genotype			Urban vs. rural (EE) p value[b]	Urban vs. rural (EG) p value[b]
	EE ($n = 200$)	EG ($n = 10$)	p value[a]	EE ($n = 147$)	EG ($n = 3$)	p value[a]		
Body mass index (kg/m^2)	22.98 ± 3.20	24.06 ± 2.93	0.249	24.84 ± 4.13	25.35 ± 2.30	0.834	0.000	0.504
Waist circumference (cm)	77.64 ± 8.38	80.50 ± 6.55	0.162	82.57 ± 10.52	79.33 ± 7.09	0.598	0.000	0.795
Systolic blood pressure (mmHg)	126.86 ± 16.72	129.80 ± 12.64	0.396	129.75 ± 20.28	122.67 ± 21.08	0.512	0.500	0.472
Diastolic blood pressure (mmHg)	78.57 ± 10.69	81.90 ± 7.68	0.333	82.31 ± 13.69	71.67 ± 3.06	0.057	0.008	0.050
Total cholesterol (mg/dl)	216.03 ± 50.32	244.00 ± 31.46	0.034	211.84 ± 42.11	190.33 ± 15.50	0.380	0.121	0.018
LDL-C (mg/dl)	146.77 ± 38.01	168.66 ± 31.20	0.036	130.51 ± 32.39	115.20 ± 12.47	0.416	0.000	0.016
Triglyceride (mg/dl)	98.90 ± 57.85	88.90 ± 22.92	0.794	120.55 ± 73.24	91.00 ± 57.58	0.368	0.001	0.923
HDL-C (mg/dl)	54.62 ± 12.82	57.50 ± 9.30	0.344	57.88 ± 13.28	57.00 ± 9.54	0.979	0.033	0.937
Fasting blood sugar (mg/dl)	85.92 ± 9.26	87.90 ± 10.94	0.644	89.80 ± 13.73	87.33 ± 6.03	0.822	0.008	0.934

ND not determined

[a] p value obtained in the Student's t test or Mann–Whitney U test for the comparison between genotypes for each polymorphism

[b] p value obtained in the Student's t test or Mann–Whitney U test for the comparison between residence areas for each genotype

(5 %) [27], the UK (3.4 %) [15]), Chinese Taiwanese (11.9 %) [31], and Han Chinese (4.8 %) [32] populations, as well as in the Dallas Heart Study (DHS) (3.6 % in whites, 4.2 % in Hispanics, and 26.0 % in blacks) [22], the TexGen population (4.4 %) [30], the Lipoprotein Coronary Atherosclerosis Study (LCAS) (7.4 %) [30], and the PROSPER study group (6 %) [14]. Overall, we indicate that the frequencies of the *PCSK9* 46L, 474V, and 670G alleles vary across populations.

Table 5 Anthropometric and biochemical variables according to *APOE* genotypes in males and females in urban and rural subjects

Variables	Urban				Rural			
	APOE2 (E2E2, E2E3) ($n = 16$ male, 37 female)	*APOE3* (E3E3) ($n = 59$ male, 122 female)	*APOE4* (E3E4, E4E4) ($n = 19$ male, 48 female)	p value[a]	*APOE2* (E2E2, E2E3) ($n = 6$ male, 18 female)	*APOE3* (E3E3) ($n = 20$ male, 90 female)	*APOE4* (E3E4, E4E4) ($n = 11$ male, 39 female)	p value[a]
Body mass index (kg/m^2)								
Male	24.35 ± 2.31	24.77 ± 2.94	24.90 ± 2.78	0.830	23.52 ± 2.47	21.82 ± 3.64	23.72 ± 3.77	0.304
Female	22.88 ± 3.23	23.09 ± 3.22	22.92 ± 3.18	0.700	25.46 ± 4.18	25.06 ± 4.18	23.99 ± 3.90	0.320
Waist circumference (cm)								
Male	85.25 ± 15.26	87.46 ± 8.99	84.32 ± 15.08	0.832	82.83 ± 8.91	80.55 ± 12.98	81.91 ± 11.76	0.905
Female	77.38 ± 7.86	77.90 ± 8.48	77.83 ± 8.61	0.684	83.78 ± 11.63	83.46 ± 10.16	79.92 ± 10.56	0.188
Systolic blood pressure (mmHg)								
Male	132.63 ± 18.03	135.85 ± 15.87	131.32 ± 11.22	0.483	140.50 ± 19.71	132.30 ± 19.36	129.27 ± 15.02	0.481
Female	125.73 ± 18.28	126.60 ± 15.55	128.77 ± 17.45	0.784	126.94 ± 21.24	129.98 ± 19.78	129.10 ± 20.65	0.663
Diastolic blood pressure (mmHg)								
Male	81.69 ± 10.56	82.14 ± 12.69	83.58 ± 11.22	0.883	84.83 ± 10.13	76.50 ± 7.76	78.55 ± 10.20	0.148
Female	77.68 ± 12.21	78.45 ± 10.17	80.25 ± 10.58	0.492	77.67 ± 8.80	82.77 ± 14.76	82.51 ± 12.97	0.407
Total cholesterol (mg/dl)								
Male	192.13 ± 38.57	218.83 ± 51.43	259.53 ± 58.50[c]	0.001	166.67 ± 18.75	201.90 ± 33.57	198.27 ± 35.90	0.076
Female	201.51 ± 52.01[d]	215.16 ± 47.70	230.75 ± 42.66	0.010	195.78 ± 31.71	214.71 ± 40.77	215.05 ± 40.92	0.168
LDL-C (mg/dl)								
Male	122.01 ± 30.53[b]	150.99 ± 35.36	182.93 ± 59.61[c]	0.000	90.10 ± 20.47	119.10 ± 30.73	121.27 ± 35.76	0.113
Female	129.05 ± 44.58[d]	148.79 ± 30.01	156.48 ± 37.97	0.002	114.68 ± 30.50	132.99 ± 30.86	133.50 ± 33.21	0.069
Triglyceride (mg/dl)								
Male	113.00 ± 64.55	137.56 ± 78.73	142.63 ± 77.96	0.497	130.00 ± 86.64	106.65 ± 59.91	137.00 ± 84.34	0.499
Female	98.57 ± 50.16	96.61 ± 57.91	96.21 ± 51.14	0.823	96.89 ± 63.33	116.60 ± 68.54	130.05 ± 74.05	0.060
HDL-C (mg/dl)								
Male	47.50 ± 17.86	49.53 ± 11.57	48.05 ± 11.53	0.652	50.67 ± 12.88	61.45 ± 14.13	49.64 ± 12.78	0.050
Female	55.92 ± 11.61	54.52 ± 12.97	55.02 ± 13.03	0.709	61.72 ± 17.08	58.36 ± 13.03	55.54 ± 11.30	0.422
Fasting blood sugar (mg/dl)								
Male	105.56 ± 36.57	97.37 ± 37.05	88.32 ± 9.77	0.159	105.00 ± 29.03	94.10 ± 32.77	95.27 ± 9.79	0.077
Female	86.30 ± 9.54	85.13 ± 9.27	87.81 ± 9.34	0.231	86.06 ± 6.69	89.33 ± 15.33	91.24 ± 10.38	0.131

[a] p value obtained in the ANOVA test for the comparison between genotypes

[b, c] p value obtained in the Tukey post hoc test, [b] *APOE2* vs. *APOE3*, p value <0.05, [c] *APOE4* vs. *APOE3*, p value <0.05

[d] p value obtained in Mann–Whitney U test, *APOE2* vs. *APOE3* p value <0.05

Table 6 Anthropometric and biochemical variables of carriers of the *PCSK9* E670G according to *APOE* alleles in urban subjects

Variables	Non-*APOE4*			*APOE4*			ANOVA *p* value[b]	*p* for trend[c]
	EE ($n = 225$)	EG ($n = 9$)	*p* value[a]	EE ($n = 64$)	EG ($n = 3$)	*p* value[a]		
Body mass index (kg/m^2)	23.51 ± 3.20	24.79 ± 2.46	0.237	22.55 ± 3.21	22.11 ± 2.55	0.505	0.570	0.930
Waist circumference (cm)	80.99 ± 9.67	74.11 ± 16.32	0.381	79.38 ± 11.14	86.00 ± 10.15	0.268	0.446	0.288
Systolic blood pressure (mmHg)	129.06 ± 16.89	132.78 ± 9.87	0.329	130.16 ± 16.03	115.33 ± 6.81	0.118	0.241	1.000
Diastolic blood pressure (mmHg)	79.38 ± 11.40	82.11 ± 7.98	0.347	81.36 ± 10.98	77.67 ± 5.03	0.617	0.675	0.279
Total cholesterol (mg/dl)	211.15 ± 49.34	242.44 ± 33.67	0.040	237.69 ± 42.22	265.00 ± 52.76	0.349	0.001	0.000
LDL-C (mg/dl)	143.37 ± 34.97	169.98 ± 32.79	0.026	163.10 ± 46.55	182.60 ± 42.81	0.480	0.002	0.000
Triglyceride (mg/dl)	109.07 ± 66.21	90.89 ± 23.75	0.950	108.50 ± 62.84	128.00 ± 76.86	0.606	0.932	0.910
HDL-C (mg/dl)	52.96 ± 13.19	54.22 ± 8.42	0.569	52.86 ± 12.82	57.00 ± 17.69	0.591	0.892	0.784
Fasting blood sugar (mg/dl)	89.84 ± 23.41	88.67 ± 11.24	0.829	88.03 ± 9.56	86.33 ± 5.69	0.976	0.992	0.508

[a] *p* value obtained in the Student's *t* test or Mann–Whitney *U* test for the comparison between genotypes

[b] *p* value obtained in the ANOVA test for the comparison between genotypes

[c] *p* value for trend obtained in the ANOVA test with polynomial contrasts for linear trend

In the present study, we found that the 46L urban male carriers had significantly higher DBP and FBS compared with non-carriers. In contrast, the 46L urban female carriers had significantly lower TC and LDL-C levels compared with non-carriers. Our results in urban females were consistent with previous studies. Several populations in the UK [15], Sweden [17], Italy [18], Denmark [19], white populations [21], and hypobetalipoproteinemia subjects, as well as in the PROSPER study group [14], the CARDIA Study [16], and the Bogalusa Heart Study [20] have shown that 46L carriers are associated with lower LDL-C levels.

In contrast to 46L urban carriers, the 474V rural female carriers had significantly lower HDL-C levels than non-carriers ($p < 0.05$). It has been shown that 474V carriers are associated with lower LDL-C and PCSK9 levels in Caucasian, African Canadian [23], and Japanese [24] populations, but the association of 474V carriers with lipid levels was not observed in healthy UK men [15], Brazilian subjects [26], or in the PLIC study [25]. This suggests that differences in study population characteristics, study design, and the frequency of the 474V allele may explain the inconsistent results.

Furthermore, the 670G urban female carriers had significantly higher TC and LDL-C levels than did non-carriers. Our findings were inconsistent with the previous studies in Germany [27], which showed that the association of E670G with increased LDL-C in men, but not in women. However, our results were consistent with previous studies conducted in Brazilian [26] and Tunisian populations [28], as well as in the Belgium stroke study [29], the PLIC study [25], and the LCAS [30], all which showed the association of E670G with an increased risk of CVD. In the present study, the association of *PCSK9* E670G with lipid parameters was not observed in the rural subjects. This finding was compatible with the healthy UK men [15] and the PROSPER study group [14], which showed no association of E670G with any significant effects on lipid levels or CVD risk. Moreover, some studies demonstrated the preventive effect on CVD risk by E670G. In Han populations, 670G carriers had significantly higher serum HDL-C and APOAI levels in males, and lower ApoB levels and ApoAI/ApoB ratio in females than the non-carriers [32]. In a Chinese population in Taiwan, E670G was found to be associated with low LDL-C levels are not a risk variant for CVD [31]. Altogether, the discrepancies of the association of *PCSK9* R46L, I474V, and E670G polymorphisms with lipid levels among various populations may be due to ethnicity and/or environmental factors, as well as gene–environment interactions.

In the present study, the various effects on anthropometric data and lipid levels between urban and rural carriers of the 46RR, 474II, 474IV, 670EE and 670EG genotypes in both males and females were observed. Our results suggest that the differences in lifestyle and sociodemographic factors between urban and rural subjects may be an influential factor on the anthropometric data and lipid profiles according to each genotype. In a previous study, Aung et al. [38] reported that alcohol consumption can modify the effects of the *PCSK9* E670G polymorphism on serum TC and LDL-C levels. Subjects with the AA genotype of *PCSK9* E670G benefit more from alcohol consumption than do the subjects with the AG genotype.

Additionally, we found that *APOE4* carriers had increased TC and LDL-C levels relative to *APOE3* carriers in the urban males. *APOE2* carriers had decreased TC and/or LDL-C levels compared with *APOE3* carriers in both urban males and females. Our results are similar to other studies [39, 40]. Finally, we also observed the combined

effect of *APOE4* and *PCSK9* E670G polymorphisms on TC and LDL-C levels in the urban subjects. A significant trend of increased TC and LDL-C was observed in non-*APOE4-PCSK9* 670EE carriers to *APOE4–PCSK9* 670EG carriers in urban subjects. Our finding was similar to the PLIC study, which showed that *APOE2-PCSK9*-670EE carriers had a more favorable lipid profile and decreased intima media thickness compared with *APOE4–PCSK9*-670G carriers [25].

In conclusion, we demonstrated the association of *PCSK9* and *APOE* polymorphisms with serum lipid levels in a Southern Thai population. *PCSK9* R46L, I474V, and E670G polymorphisms may represent genetic risk factors for CVD in urban males, rural females and urban females, respectively. However, females with *PCSK9* R46L showed a favorable lipid profile that may protect against CVD in urban subjects. The combination of *APOE* and *PCSK9* E670G polymorphisms may be suggestive of an independent factor for the determination of lipid levels. Our study had several limitations, including a small sample size, the low frequencies of the minor alleles of *PCSK9* polymorphisms, and a lack of data on nutritional status. Further study using a larger sample is needed to confirm our findings. Additionally, the interaction between *PCSK9* polymorphisms and environmental factors on lipid profiles is required.

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