Analysis of Polymorphisms in the 5'-Flanking Region of the Apolipoprotein(a) [Apo(a)] Gene in Thai Subjects with Coronary Artery Diseases

Nednapis Tirawanchai PhD*, Wattana Leowattana MD, PhD**,
Anchaleekorn Somkasetrin MSc*, Pushdee Thongsaen MSc*

** Department of Biochemistry, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok*
*** Faculty of Tropical Medicine, Mahidol University, Bangkok*

Lipoprotein(a) [Lp(a)] is a complex lipoprotein particle in human plasma. It is composed of apolipoprotein B (Apo B)-100 and apolipoprotein(a) which are linked by a disulfide bond. Plasma levels of the Lp(a) vary greatly (over 1,000 folds) among individuals. Elevated plasma levels of the Lp(a) have been shown to be an independent risk factor for coronary artery diseases (CAD). The level of Lp(a) is controlled by a single gene, the Apo(a) gene, with multiple alleles; each encodes different concentrations of the Lp(a). Previous studies revealed the presence of polymorphisms in the 5'-flanking region (FL) of the Apo(a) gene at 3 positions: G or A (-914), C or T (-49), and G or A (-21), which can be detected by cleavage of PCR-amplified DNA products with TaqI, MaeII and HhaI, respectively. The 5'-FL genotypes of the Apo(a) gene can be classified by the combination of the presence (+) or absence (-) of these restriction sites into 5 types; type A, +++, type B, -++, type C, -+-, type D, --+ and type E, +-+. In the present study, the authors analyzed the 5'FL types of the Apo(a) gene by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) in 100 healthy control subjects, 26 CAD patients with [Lp(a)] ≤ 30 mg/dL, and 94 CAD patients with [Lp(a)] > 30 mg/dL. The authors found that the genotype frequencies of the Apo(a) gene were 53, 16, 27 and 4%, for types A, B, C and D respectively in normal healthy controls. In CAD patients with [Lp(a)] ≤ 30 mg/dL, the distribution of the genotype frequencies were 53.8, 11.5, 30.8 and 3.9% for types A, B, C and D, respectively. Additionally in CAD patients with [Lp(a)] > 30 mg/dL, the genotype frequencies were 60.6, 11.7, 21.3 and 6.4% for types A, B, C and D, respectively. The present study might shed some light to understand CAD at the molecular level.

Keywords: *Coronary artery diseases, Lipoprotein(a) [Lp(a)], Polymorphism*

J Med Assoc Thai 2008; 91 (1): 86-92
Full text. e-Journal: *http://www.medassocthai.org/journal*

Coronary artery disease (CAD) is a major cause of morbidity and mortality in industrialised and developing countries including Thailand. The etiology of CAD is multifactorial since several genes and environmental factors are involved. Plasma levels of Lp(a) have been considered to be an independent and most prevalent inherited risk factor for atherosclerosis and CAD[1-3]. Lp(a) is a complex lipoprotein in human plasma. It is composed of apolipoprotein B-100 (apo B-100) and apolipoprotein(a) [Apo(a)] which shares at least 75% homology with plasminogen[4,5].

Concentrations of Lp(a) in plasma vary greatly among individuals from < 0.1 to > 200 mg/dL[6,7]. Many studies have found an independent association between high Lp(a) levels (> 30 mg/dL) and a high incidence of CAD, acute myocardial infarction and cerebral infarction[6-11]. Today, it is certain that plasma Lp(a) levels are strongly genetically determined by Apo(a) gene[12]. The Apo(a) gene consists of multiple tandem copies of a sequence resembling the plasminogen kringle IV domain, plasminogen-like kringle V and protease domains.

Correspondence to : Tirawanchai N, Department of Biochemistry, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand. Phone: 0-2419-9142, Fax: 0-2411-1428, E-mail: sintr@mahidol.ac.th

It is demonstrated that the 5'-flanking (5'-FL) region of the Apo(a) gene plays an important role in determining Lp(a) levels through transcriptional regulation and there are sequence polymorphisms in this region which can be analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)[13]. The presence of three nucleotide differences are at three positions: G or A (-914), C or T (-49) and G or A (-21) can be detected by cleavage of the PCR-amplified DNA products with *Taq*I, *Mae*II and *Hha*I restriction endonucleases, respectively. Thus, polymorphisms at the 5'-FL region which are determined by combination of the presence (+) or absence (-) of *Taq*I, *Mae*II and *Hha*I restriction sites, respectively, can be classified into 5 types: type A, +++, type B, -++, type C, -+-, type D, --+ and type E, +-+.

Since there is no report about the relation between polymorphisms of the Apo(a) gene and the plasma level of Lp(a) in Thailand, the authors, therefore, investigated the genotypes at the three polymorphic sites (nucleotides -914, -49 and -21) of the Apo(a) gene in the present study. In addition, the relationship between polymorphisms at the 5'-FL region of the Apo(a) gene and plasma Lp(a) levels in Thai normal healthy controls and patients with CAD were identified and this might be used as a genetic risk factor for CAD in Thai populations.

Material and Method

Study subjects

Two hundred and twenty subjects were divided into 100 normal healthy control subjects and 120 CAD patients. One hundred normal healthy subjects (45 males and 55 females; mean age = 30.6 $\pm$ 4.4 years) were recruited with informed consent. These were individuals without the history of CAD who have their health checked up annually at Siriraj Hospital. Their cholesterol, triglycerides and Lp(a) levels were 193.8 $\pm$ 29.3, 80.3 $\pm$ 33.3 and 13.9 $\pm$ 7.0 mg/dL respectively.

One hundred and twenty CAD patients who visited Her Majesty's Cardiac Centre of Siriraj Hospital were recruited with informed consent. They were divided into two groups. The first group was 26 CAD patients (21 males and 5 females; mean age = 58.6 $\pm$ 9.7 years) with plasma Lp(a) levels $\leq$ 30 mg/dL. Their cholesterol, triglycerides and Lp(a) levels were 189.9 $\pm$ 40.4, 165.4 $\pm$ 84.6 and 17.3 $\pm$ 6.4 mg/dL respectively. The second group was 94 CAD patients (68 males and 26 females; mean age = 60.1 $\pm$ 11.0 years) with plasma Lp(a) levels > 30 mg/dL. Their cholesterol, triglycerides and Lp(a) levels were 206.3 $\pm$ 40.1, 144.5 $\pm$ 94.1 and 100.2 $\pm$ 105.1 mg/dL respectively.

Lipid profiles and leucocyte DNA extraction

Blood samples were collected after fasting for at least 12 h. Serum lipid profiles were determined by Hitachi 917 auto-analyzer. Plasma Lp(a) levels were measured using COBAS INTEGRA 700 auto-analyzer. Genomic DNA was extracted from peripheral leucocyte by Guanidine-HCl method[14] then dissolved in 10 mM Tris-HCl, 1 mM EDTA pH 7.6 buffer.

Oligonucleotide primers

Amplicon I and II were amplified using oligonucleotide primers A1/1 & A1/2 and A2/1 & A2/2, respectively[13].

A1/1; 5'-CTTGAATTCCCAAAGTGCTGGGATTACAGAG-3'

A1/2; 5'-TAAGGATCCGGCATATGTATTTTTACTACATTGTGGGAG-3'

A2/1; 5'-CCAGGATCCAGCATCTATTGACATTGCACT-3'

A2/2; 5'-TTAGAATTCATTTTGGGACTGGCCAGCAGCG-3'

PCR-RFLP analysis

Polymorphisms at nucleotide -914, -49 and -21 which resided in the 5'-FL region of the Apo(a) gene were analysed by PCR-RFLP using the modified method of Ichinose and Kuriyama[13]. To study polymorphism at the nucleotide -914, PCR-amplified amplicon I (1,284 bp) was digested with *Taq*I into 1041 and 243 bp. To study polymorphisms at the nucleotides -49 and -21, PCR-amplified amplicon II (228 bp) was digested with *HpyCH*IV (an isochizomer of *Mae*II) or *Hha*I, respectively. Upon digestion with *HpyCH*IV, amplicon II was cleaved into 168 and 60 bp. In addition, following digestion with *Hha*I, amplicon II was cleaved into 199 and 29 bp. The restriction enzyme-digested products were analysed by agarose gel electrophoresis.

Statistical analyses

Distribution of the 5'-FL genotypes of the Apo(a) gene were calculated. Chi-square (χ^2) analysis was used to determine the statistical significance of the genotypes between the normal healthy controls and CAD subjects. To investigate the relationship between polymorphism of the Apo(a) gene and Lp(a) concentrations, either Kruskall-Wallis or Mann-Whitney U tests were applied. All calculations were completed using Statview release 5.0 and SPSS 11.0.

Results

Polymorphisms of the Apo(a) gene at the 5'-FL region were observed using PCR-RFLP. Polymorphisms at nucleotides -914, -49 and -21 of the Apo(a) gene are shown in Fig. 1, 2 and 3, respectively.

Following PCR-RFLP, polymorphisms at the 5'-FL region of the Apo(a) gene in normal healthy controls and CAD patients were classified into 4 types (Table 1). It is observed that type A was the most abundant 5'-FL type of the Apo(a) gene in normal healthy controls and CAD patients. The hierachical orders of the genotype distribution in normal healthy control subjects and CAD patients were type A > C > B > D. However, the frequencies of the 5'-FL types of the Apo(a) gene between the normal healthy controls and 2 groups of CAD patients were not significantly different ($p > 0.05$).

Relation between polymorphisms at the 5'-FL region of the Apo(a) gene and plasma Lp(a) levels in normal healthy controls and CAD patients were shown in Table 2. It was observed that normal healthy controls and CAD patients with [Lp(a)] > 30 mg/dL who had type C of the 5'-FL region of the Apo(a) gene showed the highest concentration of plasma Lp(a). However, in CAD patients with Lp(a) ≤ 30 mg/dL, type A had slightly higher Lp(a) level than type C. Interestingly, although type A was the most abundant 5'-FL type of the Apo(a) gene (Table 1), type C was likely to have the highest level of the Lp(a) (Table 2).

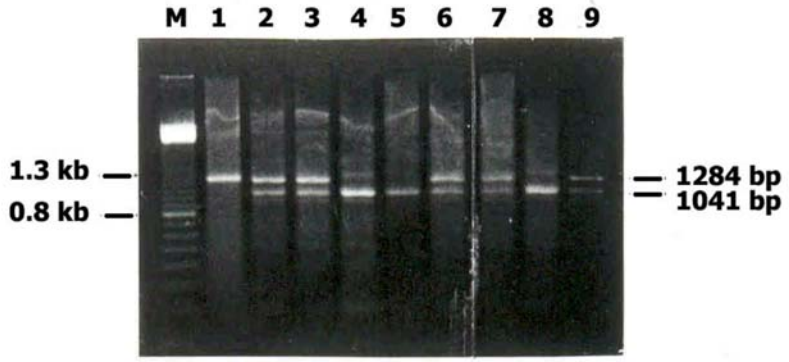

Fig. 1 2% Agarose gel electrophoretic pattern of *Taq*I-digested the PCR-amplified 1284 bp DNA from 5 normal healthy individuals (lanes 2-6) and 3 CAD patients (lanes 7-9), lane M was a 100 bp DNA ladder marker, lane 1 was an undigested PCR-amplified DNA, lanes 2-9 were *Taq*I-digested the PCR-amplified DNA, lanes 2, 3, 6, 7 and 9 were heterozygotes, lanes 4, 5 and 8 were homozygotes

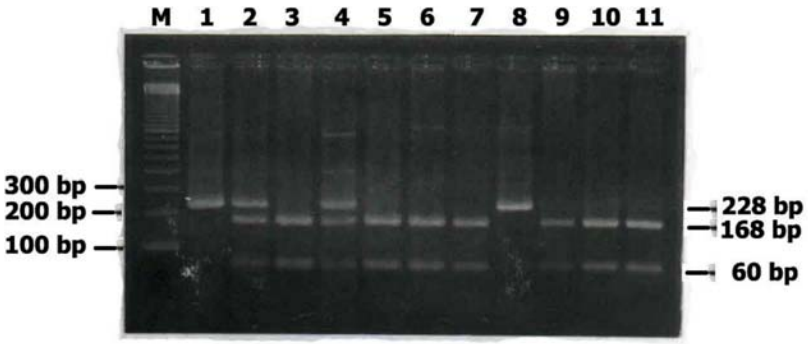

Fig. 2 2.5% Agarose gel electrophoretic pattern of *HpyCH*IV-digested the PCR-amplified 228 bp DNA from 5 normal healthy individuals (lanes 2-6) and 5 CAD patients (lanes 7-11), lane M was a 100 bp DNA ladder marker, lane 1 was an undigested PCR-amplified DNA, lanes 2-11 were *HpyCH*IV-digested the PCR-amplified DNA, lanes 2 and 4 were heterozygotes, lanes 3, 5-7 and 9-11 were homozygotes, lane 8 was the PCR-amplified DNA which was not cleaved by the enzyme

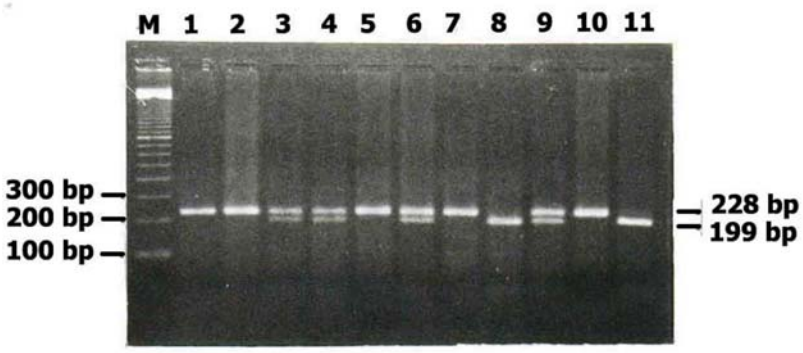

Fig. 3 3% Agarose gel electrophoretic pattern of *Hha*I-digested the PCR-amplified 228 bp DNA from 5 normal healthy individuals (lanes 2-6) and 5 CAD patients (lanes 7-11), lane M was a 100 bp DNA ladder marker, lane 1 was an undigested PCR-amplified DNA, lanes 2-11 were *Hha*I-digested the PCR-amplified DNA, lanes 3, 4, 6 and 9 were heterozygotes, lanes 8 and 11 were homozygotes, lane 2, 5, 7 and 10 were the PCR-amplified DNA which were not cleaved by the enzyme

Kruskall-Wallis and Mann-Whitney U tests were used to analyse the difference of plasma Lp(a) levels in each 5'-FL type within the same subject group. It was found that Lp(a) levels between different 5'-FL types within the same subject group were not significantly different ($p > 0.05$).

Table 1. Distribution of the 5'-FL types of the apo(a) gene in healthy control subjects and CAD patients

5'-FL types	Healthy control subjects (n = 100) n (%)	CAD patients with Lp(a) ≤ 30 mg/dL (n = 26) n (%)	CAD patients with Lp(a) > 30 mg/dL (n = 94) n (%)
A	53 (53.0)	14 (53.8)	57 (60.6)
B	16 (16.0)	3 (11.5)	11 (11.7)
C	27 (27.0)	8 (30.8)	20 (21.3)
D	4 (4.0)	1 (3.9)	6 (6.4)

Table 2. Distribution of plasma Lp(a) levels in each 5'-FL type of the normal healthy control subjects and CAD patients (mean ± SD)

5'-FL types	[Lp(a)] (mg/dL) in normal healthy control subjects (n = 100)	[Lp(a)] (mg/dL) in CAD patients with Lp(a) ≤ 30 mg/dL (n = 26)	[Lp(a)] (mg/dL) in CAD patients with Lp(a) > 30 mg/dL (n = 94)
A	13.25 ± 6.83 (n = 53)	17.72 ± 6.80 (n = 14)	95.53 ± 100.32 (n = 57)
B	14.33 ± 5.74 (n = 16)	14.73 ± 2.37 (n = 3)	80.76 ± 77.3 (n = 11))
C	14.93 ± 8.17 (n = 27)	17.68 ± 7.42 (n = 8)	121.58 ± 137.5 (n = 20)
D	14.8 ± 8.0 (n = 4)	16.8 (n = 1)	108.57 ± 77.42 (n = 6)

Discussion

In the present study, the authors analyzed the polymorphisms of the 5'-FL region of the Apo(a) gene in normal control subjects and CAD patients using PCR-RFLP. The authors found 4 genotypes of the Apo(a) gene; A > C > B > D. The present results were compared with the results from Ichinose & Kuriyama[13] (Table 3). Interestingly, type A of the Apo(a) gene was also the most abundant genotype found in Caucasian Americans. There was no significant difference between Caucasian Americans and Thais ($\chi^2 = 7.326$, $p = 0.062$).

On the contrary, genotype distributions at the 5'-FL region of the Apo(a) gene in Japanese individuals were different from Thai and Caucasian Americans[13] (Table 3). The 5'-FL types of the Apo(a) gene between the Thai and Japanese were significantly different ($\chi^2 = 36.329$, $p = 0.000$). In addition, the 5'-FL types of the apo(a) gene between the Caucasian Americans and Japanese were significantly different ($\chi^2 = 21.939$, $p = 0.000$).

From the present results, the genotype distributions of the 5'-FL region of the Apo(a) gene were also compared with Japanese myocardial infarction (MI) patients[13] (Table 4). The authors demonstrated that type A > C > B > D. The present results were significantly different from the Japanese MI patients which revealed that type C > A > D > B > E ($\chi^2 = 66.843$, $p = 0.000$). Type E was found in only one Japanese MI patient. Interestingly, the authors did not observe type E in the presented subjects.

The 5'-FL types of the Apo(a) gene in Thai CAD patients were also compared with Japanese subjects with cerebral infarction[13] (Table 4). It was observed that distribution of the 5'-FL genotypes of the Apo(a) gene in Japanese CI subjects was type C > D > A > B and E. Their results were significantly different from the present results ($\chi^2 = 32.511$, $p = 0.000$).

Furthermore, Thai CAD patients recruited in the present study could be subdivided into patients with stable angina (SA), unstable angina (UA) and myocardial infarction (MI). Genotype distributions of

Table 3. Comparison of the 5'-FL types of the apo(a) gene among normal healthy individuals of Caucasian American, Japanese[13] and Thai

5'-FL types	Caucasian American* (n = 66)	Japanese* (n = 58)	Thai** (n = 100)
A	53%	27%	53%
B	14%	-	16%
C	17%	42%	27%
D	16%	31%	4%

* The results from the study of Ichinose and Kuriyama[13]
** The results from our study

Table 4. Genotype distribution of the 5'-FL types of the apo(a) gene in Thai CAD and Japanese patients with myocardial infarction (MI) or cerebral infarction (CI)

5'-FL genotypes of the Apo(a) gene	Thai CAD patients (n = 120)[1]	Japanese MI patients (n = 100)[2]	Japanese CI patients (n = 136)[2]
A	59.2%	34%	24%
B	11.7%	2%	-
C	23.3%	41%	59%
D	5.8%	22%	45%
E	-	1%	-

[1] The results were from this study
[2] The results were from Ichinose & Kuriyama[13]

Table 5. Distribution of the 5'-FL region of the apo(a) gene in Thai CAD patients with stable angina (SA), unstable angina (UA) and myocardial infarction (MI)

5'-FL genotypes	CAD patients with SA (n = 84)	CAD patients with UA (n = 18)	Myocardial infarction MI (n = 18)
A	57.14%	50.0%	77.78%
B	14.29%	11.11%	-
C	21.43%	33.33%	22.22%
D	7.14%	5.56%	-

the 5'-FL region of the apo(a) gene were distinguished upon their clinical characteristics (Table 5). It was shown that distribution of the 5'-FL types of the Apo(a) gene was A > C > B >D in CAD patients with SA or UA. In MI patients, the authors found type A more than C but interestingly, the authors did not find types B and D.

In conclusion, the authors found a relationship between the 5'-FL genotypes of the Apo(a) gene and plasma levels of Lp(a). Subjects with type C showed a trend to have the highest level of Lp(a) which might be a genetic risk for CAD. In addition, distributions of the 5'-FL genotype of the Apo(a) gene in different populations depended on ethnic background.

Acknowledgement

This research work was supported by Mahidol University Research Fund.

References

1. Moon JY, Kwon HM, Kwon SW, Yoon SJ, Kim JS, Lee SJ, et al. Lipoprotein(a) and LDL particle size are related to the severity of coronary artery disease. Cardiology 2007; 108: 282-9.
2. Morita Y, Himeno H, Yakuwa H, Usui T. Serum lipoprotein(a) level and clinical coronary stenosis progression in patients with myocardial infarction: re-revascularization rate is high in patients with high-Lp(a). Circ J 2006; 70: 156-62.
3. Homma Y. Predictors of atherosclerosis. J Atheroscler Thromb 2004; 11: 265-70.
4. Berg K. A new serum type system in man - the Lp system. Acta Pathol Microbiol Scand 1963; 59: 369-82.
5. Fless GM, ZumMallen ME, Scanu AM. Physicochemical properties of apolipoprotein(a) and lipoprotein(a-) derived from the dissociation of human plasma lipoprotein(a). J Biol Chem 1986; 261: 8712-8.
6. Utermann G. The mysteries of lipoprotein(a). Science 1989; 246: 904-10.
7. Scanu AM. Lipoprotein(a): looking ahead. Nutr Metab Cardiovasc Dis 2005; 15: 331-3.
8. Rigal M, Ruidavets JB, Viguier A, Petit R, Perret B, Ferrieres J, et al. Lipoprotein(a) and risk of ischemic stroke in young adults. J Neurol Sci 2007; 252: 39-44.
9. Tsuchida K, Hori T, Tanabe N, Makiyama Y, Ozawa T, Saigawa T, et al. Relationship between serum lipoprotein(a) concentrations and coronary vasomotion in coronary spastic angina. Circ J 2005; 69: 521-5.
10. Ariyo AA, Thach C, Tracy R. Lp(a) lipoprotein, vascular disease, and mortality in the elderly. N Engl J Med 2003; 349: 2108-15.
11. Lentini A, Fornengo P, Bosco G, Caprioli M, Destefanis E, Cerrato P. Cryptogenic cerebral infarction in a young patient with very high lipoprotein(a) serum level as the only risk factor. Neurol Sci 2007; 28: 42-4.
12. Boerwinkle E, Leffert CC, Lin J, Lackner C, Chiesa G, Hobbs HH. Apolipoprotein(a) gene accounts for greater than 90% of the variation in plasma lipoprotein(a) concentrations. J Clin Invest 1992; 90: 52-60.
13. Ichinose A, Kuriyama M. Detection of polymorphisms in the 5'-flanking region of the gene for apolipoprotein(a). Biochem Biophys Res Commun 1995; 209: 372-8.
14. Jeanpierre M. A rapid method for the purification of DNA from blood. Nucleic Acids Res 1987; 15: 9611.

การศึกษาความหลากหลายของจีนอะโปไลโปโปรตีน(เอ) ที่ปลายทางด้าน 5' ในผู้ป่วยโรคหลอดเลือดหัวใจของไทย

เนตรนภิส ธีระวัลย์ชัย, วัฒนา เลี้ยววัฒนา, อัญชลีกร โสมเกษตริน, ผุสดี ทองแสน

Lipoprotein(a) [Lp(a)] เป็นไลโปโปรตีนในพลาสมาที่ประกอบด้วย apolipoprotein B (Apo B)-100 จับอยู่กับ apolipoprotein(a) ด้วยพันธะไดซัลไฟด์ ระดับของ Lp(a) ในพลาสมาของคนแต่ละคน จะมีความแตกต่างกันได้มากถึง 1000 เท่า จากการศึกษาพบว่า ระดับของ Lp(a) ในพลาสมา จะเป็นปัจจัยเสี่ยงอิสระในการทำให้เกิดโรคหลอดเลือดหัวใจ พบว่าความหลากหลายของจีน Apo(a) ที่ใช้ในการสร้าง Lp(a) จะมีความสัมพันธ์กับระดับของ Lp(a) ในพลาสมา โดยที่ปลายทางด้าน 5'-flanking region (FL) ของจีน Apo(a) จะมีความหลากหลายอยู่ 3 ตำแหน่ง คือ G หรือ A ที่นิวคลิโอไทด์ตำแหน่ง -914, C หรือ T ที่นิวคลิโอไทด์ตำแหน่ง -49 และ G หรือ A ที่นิวคลิโอไทด์ตำแหน่ง -21 ความหลากหลายของจีนดังกล่าว จะศึกษาได้โดยใช้วิธี PCR-RFLP ซึ่งจะมีการเพิ่มจำนวนจีนตรงตำแหน่งที่ต้องการแล้วตัดย่อยด้วยเอนไซม์ TaqI, MaeII และ HhaI สำหรับนิวคลิโอไทด์ตำแหน่งที่ -914, -49 และ -21 ตามลำดับ จากนั้นจึงทำการจำแนกจีโนไทป์ของจีน Apo(a) ตามลักษณะของการตัดย่อยด้วยเอนไซม์ทั้ง 3 ว่าตัดได้ (+) หรือตัดไม่ได้ (-) ออกเป็น 5 ชนิด คือ type A, +++, type B, -++, type C, -+-, type D, --+ และ type E, +-+ การศึกษานี้ จะทำการจำแนกจีโนไทป์ของจีน Apo(a) ในคนปกติ 100 คน เปรียบเทียบกับผู้ป่วยโรคหัวใจของไทยที่มีระดับของ Lp(a) ใน พลาสมา น้อยกว่าหรือเท่ากับ 30 มก./ดล. จำนวน 26 คน และผู้ป่วยโรคหัวใจที่มีระดับของ Lp(a) ในพลาสมามากกว่า 30 มก./ดล. อีก 94 คน พบว่า ในคนปกติ จะมีความถี่จีโนไทป์ของจีน Apo(a) แบบ A, B, C และ D ร้อยละ 53, 16, 27 และ 4 ตามลำดับ สำหรับผู้ป่วยโรคหัวใจของไทยที่มีระดับของ Lp(a) ในพลาสมาน้อยกว่าหรือเท่ากับ 30 มก./ดล. จะมีความถี่จีโนไทป์ของจีน Apo(a) แบบ A, B, C และ D ร้อยละ 53.8, 11.5, 30.8 และ 3.9 ตามลำดับ ส่วนผู้ป่วยโรคหัวใจที่มีระดับของ Lp(a) ในพลาสมามากกว่า 30 มก./ดล. จะมีความถี่จีโนไทป์ของจีน Apo(a) แบบ A, B, C และ D ร้อยละ 60.6, 11.7, 21.3 และ 6.4 ตามลำดับ