

The finding of new genetic polymorphism of UCP-1 A-1766G and its effects on body fat accumulation

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Abstract

A-1766G polymorphism, for the first time, has been found in the sequencing of pooled and individual genomic DNA of Korean subjects at the 5' flanking region of the UCP-1 gene. The effects of new polymorphism on body fat were elucidated among 387 Korean female subjects. It was shown that the genotypes AA, AG, and GG were consisted of 57.4%, 37.7%, and 4.9%, respectively, which was in agreement with Hardy-Weinberg equilibrium ($P=0.327$). The frequency of major A allele was 0.762 and that of minor G allele was 0.238. It is found that the waist-hip ratio (WHR) ($P=0.008$), body fat mass ($P=0.023$), and percent body fat ($P=0.014$) are significantly higher in the AG/GG type compared to the AA type. When the subjects were analyzed using computerized tomography, there were significant increases in the AG/GG type compared to the AA type in the abdominal subcutaneous fat ($P=0.015$) and the abdominal visceral fat ($P=0.013$), respectively. A-1766G is approximately 2 kb downstream from the well-known A-3826G polymorphism, and no linkage between them was found ($D'=0.929$, $R^2=0.283$). Three haplotypes (frequency >0.05) were examined from two polymorphisms and studied for their physiological effects. It was found that haplotype [GG] was significantly associated with increased body fat, while haplotype [GA] was associated with decreased body fat.

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1. Introduction

UCP-1 is a proton transporter which uncouples oxidative metabolism from ATP synthesis and dissipates energy through the heat [1]. UCP-1 had been known to be mainly expressed in brown adipose tissue, which was reported to

play important roles for energy homeostasis in rodents and neonate of larger mammals including human. Although the amount of brown adipose tissue is reported to be decreased in human adult, it is still responsible for 1–2% of the energy expenditure, preventing a weight gain of 1–2 kg/year [2–4]. This small portion of energy expenditure can increase the risk for obesity and related metabolic disorders when accumulated for decades [5]. UCP-1 had been thought to be expressed only in brown adipose tissue before; however, it was recently reported that UCP-1 mRNA and protein were detected in the white adipose tissue of mouse and humans [6,7]. The presence of UCP-1 was also reported in the perirenal fat of adult patients with pheochromocytoma [8], and around the neck arteries and

Abbreviations: AI, atherogenic index; BMI, body mass index; CT, computerized tomography; DBP, diastolic blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; RFLP, restriction fragment length polymorphism; SBP, systolic blood pressure; UCP-1, uncoupling protein-1; WHR, waist-hip ratio

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Table 1
General characteristics of the subjects in this study

		Total subjects (n=387)
Age	(year)	27.02±0.40 ^a
Weight	(kg)	68.54±0.64
BMI	(kg/m ²)	26.41±0.22
WHR		0.881±0.004
SBP	(mm Hg)	115.48±0.70
DBP	(mm Hg)	71.92±0.56

^a Mean±S.E.

in the pericardium of outdoor workers [9], as well as long-term alcohol consumers [10]. Oberkofler et al. directly measured UCP-1 mRNA level in adipose tissues obtained from fat biopsy of morbid obese subjects and found that UCP-1 mRNA expression levels in the adipose tissues were significantly lower in morbidly obese subjects than in lean subjects [11].

The human UCP-1 gene has been located on a long arm of chromosome 4 [12], and the first genetic polymorphism of UCP-1, a Bcl I restriction fragment length polymorphism, was found from a Quebec Family Study in 1994 [13], and its molecular nature was elucidated to be an A→G point mutation in the –3826 position from the TATA box in the 5'-flanking region of the UCP-1 gene (A-3826G) [14]. Until now, most association studies about the physiological effects of UCP-1 genetic polymorphism were focused on A-3826G, and many studies were conducted in various populations to elucidate the association of this polymorphism with obesity and biochemical parameters. Oppert et al. [13] reported that the G allele of A-3826G polymorphism was associated with higher body fat gain among 261 Canadians. Heilbronn et al. [15] suggested that the –3826G allele of UCP-1 was associated with increased BMI among 526 overweight Australian women. According to the report of Nagai et al. [16], A-3826G polymorphism was significantly associated with reduced postprandial thermogenesis and could have adverse effects on the regulation of body weight. Matsushita et al. [17] reported that A-3826G polymorphism was associated with an increase in body weight in premenopausal Japanese women. Fumeron et al. [18] also showed that A-3826G polymorphism was associated with lower body weight loss by low calorie diet in French overweight subjects. Even though the above studies have suggested the associations of A-3826G polymorphism with obesity and related metabolic disorders, controversies remain because other reports do not support them. Urhammer et al. [19] reported that A-3826G polymorphism was not associated with obesity phenotype in 380 Danes. In the report of Gagnon et al. [20], A-3826G polymorphism was not related to obesity indices in 985 Swedish subjects. Schaffler et al. [21] showed that A-3826G polymorphism does not play a major role in the pathogenesis of obesity and diabetes in 1020 Caucasian cohort. Kiec-Wilk et al. [22] also reported that A-3826G polymorphism does not play a major role in

the development of obesity among 180 Polish overweight subjects.

These controversies about the effects of genetic polymorphism in the 5' flanking region of UCP-1 suggest that more studies are needed in this genomic region. In this study, a new polymorphism, A-1766G, was found in the same genomic region about 2 kb apart from the previously found polymorphism, and the associations with obesity phenotypes, such as body fat mass and fat tissue areas, were analyzed among the Korean female subjects.

2. Materials and methods

2.1. Subjects

387 Korean female subjects were recruited at Kirin Oriental Medical Hospital (Seoul, Korea). The general characteristics of the subjects are listed in Table 1. Genomic DNA was obtained with informed consent. Body compositions were measured by bio-impedance analysis using a commercial device (Inbody 2.0, Biospace Co., Korea). The areas of abdominal subcutaneous and visceral fat of all subjects were measured from computerized tomography (CT) cross sectional pictures of the abdominal region as described [23]. The subcutaneous fat areas on the thigh were also measured using CT (Hispeed CT/e, GE, USA).

2.2. DNA preparation and pooling

Genomic DNA from each subject was extracted from the blood using the Accuprep™ Genomic DNA Extraction Kit

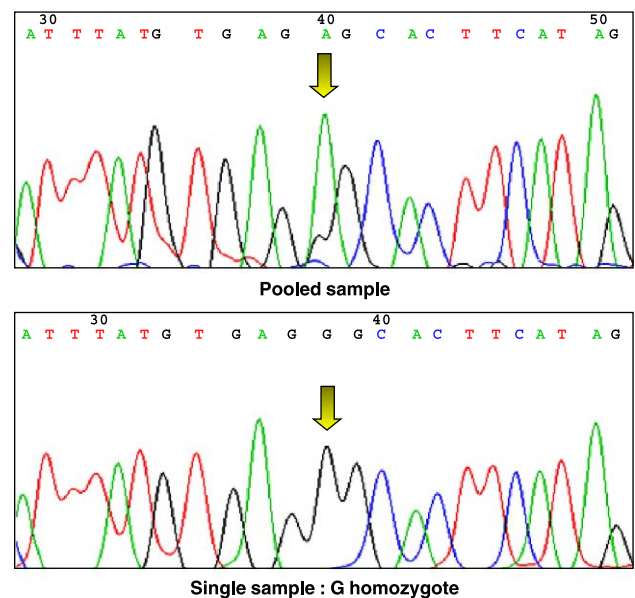


Fig. 1. Finding of A-1766G polymorphism in the Korean subjects. Sequence trace views for positions from –1777 to –1755 within the UCP-1 5'-flanking region for pooled and individual DNA. The vertical arrow indicates nucleotide position –1766.

Table 2
Comparisons of physical characteristics and body compositions by A-1766G polymorphism

Genotype	AA type (n=222)	AG/GG type (n=165)	P
<i>Physical characteristics</i>			
Weight (kg)	67.55±0.81 ^a	69.89±1.09	0.079 ^b
BMI (kg/m ²)	26.06±0.29	26.90±0.37	0.078
WHR	0.870±0.004	0.897±0.006	0.008
SBP (mm Hg)	114.66±0.95	116.68±1.08	0.318
DBP (mm Hg)	72.26±0.74	71.27±0.90	0.160
<i>Body composition</i>			
Water (kg)	30.43±0.22	30.93±0.35	0.299
Fat mass (kg)	23.67±0.55	25.64±0.72	0.023
Lean body mass (kg)	43.88±0.35	44.25±0.48	0.597
Percent body fat (%)	34.24±0.41	35.76±0.51	0.014

^a Mean±S.E.

^b P-values were obtained by general linear model analysis adjusted for age.

(Bioneer Co., Korea) according to the manufacturer's protocol. For the preparation of pooled DNA, the concentration of randomly selected 200 DNAs was measured using Picogreen (Molecular Probes, OR, USA). The DNAs were diluted in a standard concentration, and individual aliquots of DNA were pooled into a single tube. The pooled DNA was then mixed gently and re-quantitated before further dilution, to a working concentration of 2.5 ng/μl.

2.3. Polymorphism discovery

A working draft of the UCP-1 genomic sequence (NT_034706.2) was obtained through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>) and assembled manually for the primer design. Screening for genetic variation was performed by PCR amplification and direct sequencing. Forward and reverse

Table 3
Comparison of CT-measured fat areas by A-1766G polymorphism

Genotype	AA type (n=222)	AG/GG type (n=165)	P
Abdominal subcutaneous fat (mm ²)	27,822±806 ^a	31,119±1018	0.015 ^b
Abdominal visceral fat (mm ²)	5565±211	6551±278	0.013
Total abdominal fat ^c (mm ²)	33,231±960	37,638±1230	0.009
V/S ratio ^d (mm ²)	0.200±0.004	0.210±0.006	0.565
Thigh subcutaneous fat (mm ²)	14,970±244	15,530±339	0.086

^a Mean±S.E.

^b P-values were obtained by general linear model analysis adjusted for age.

^c Total abdominal fat is the sum of abdominal subcutaneous fat and abdominal visceral fat.

^d V/S ratio is the ratio of abdominal visceral fat to abdominal subcutaneous fat.

sequences of pooled and individual DNA were analyzed and compared with the working draft of the UCP-1 genomic sequence. Sequencing was carried out using a Dynamic™ ET Dye Terminator Kit (Amersham Biosciences, UK) and a MegaBACE 500 Genetic Analyzer (Amersham Biosciences, UK) according to the manufacturer's instructions. Base calling of the sample files was performed using Cimarron Base Caller software (Amersham Biosciences, UK), version 3.12. ClustalX alignment software version 1.8 [24] was used to mark potential heterozygote positions and to display them for evaluation.

2.4. Genotyping assay

The genotyping of the A-1766G polymorphism was based upon the analysis of primer extension products generated from previously amplified genomic DNA using a chip-based matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry MassARRAY system (SEQUENOM, Inc., CA, USA). The general procedures were followed by the manufacturer's standard protocol. PCR and extension primers were designed using the Primer3 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The PCR primers were

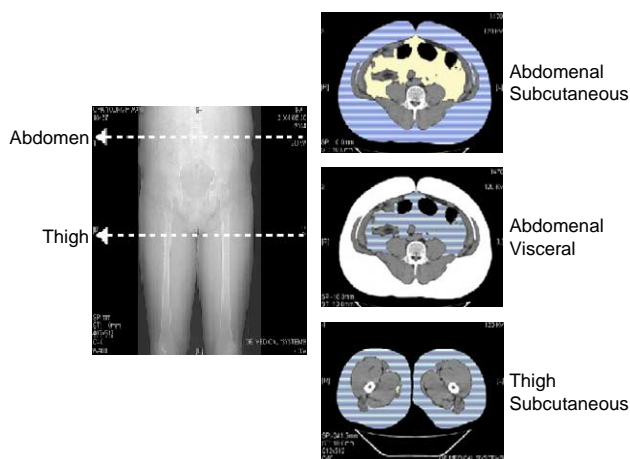


Fig. 2. Measurement of body fat distribution using computerized tomography. Positions of abdomen and thigh used for cross-sectional fat area measurements were indicated in the left panel. Examples of abdominal subcutaneous fat, abdominal visceral fat, and thigh subcutaneous fat were shown in the right panels.

Table 4
Comparison of serum cholesterol level by A-1766G polymorphism

Genotype	AA type (n=222)	AG/GG type (n=165)	P
Total cholesterol (mg/dL)	217.74±4.71 ^a	227.82±5.91	0.279 ^b
LDL cholesterol (mg/dL)	156.49±4.16	163.67±4.79	0.420
HDL cholesterol (mg/dL)	48.94±1.02	48.40±1.54	0.779
Atherogenic index ^c	2.83±0.09	3.14±0.16	0.079

^a Mean±S.E.

^b P-values were obtained by general linear model analysis adjusted for age.

^c Atherogenic Index=(Total cholesterol–HDL cholesterol)/HDL cholesterol.

Table 5
Estimated haplotypes form A-3826G and A-1766G

Haplotype ^a	Genotype		Frequency	HWE ^b
	-3826	-1766		
[AA]	A	A	0.497	0.505
[GA]	G	A	0.269	0.912
[GG]	G	G	0.227	0.441
[AG]	A	G	0.007	0.897

^a Haplotypes with frequency >0.05 will be considered for analysis.

^b *P*-values of deviation from Hardy-Weinberg equilibrium.

5'ACGTTGGATGATTGGTACTGTCACTGAGCTTG3' and 5'ACGTTGGATGGAGCCTGC AAATCCATTATGA3'. The extension primer was 5'ATTAATCTGACATTTA-TGTGAG3'. Each allele specific primer extension product was analyzed in the fully automated mode with the MALDI-TOF MassARRAY system. After the overall automatic measurement, assays with bad peaks were checked and tested again manually. To perform the quality control of the genotyping method used in this study, 21 randomly selected samples were analyzed by direct sequencing to determine the precise nature of the sequence variation, and no discrepancies were found.

The genotyping of the A-3826G polymorphism was done by the RFLP method described by Valve et al. [25]. PCR reactions were conducted to amplify a genomic DNA fragment containing the A-3826G position of the UCP-1 gene. Upstream primer (5'CCAGTGGTGGCTAATGAGAGAA3'), downstream primer (5'GCACAAAGAAGAAGCAGAGAGG3'), 3 µl dNTP mix (1 mM), 0.2 µl Taq DNA polymerase (1 unit), and 3 µl PCR buffer (10 ×) were added and adjusted to a total volume of 30 µl with distilled water. The amplification protocol consisted of 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. The amplified PCR products were checked for a correct size of 279 bp by electrophoresis in a 3% agarose gel. The PCR products were subsequently digested with a restriction enzyme Bcl I for 1 h at 50 °C and were subjected to electrophoresis in a 3% agarose gel. The resulting band patterns were GG type; single band of 279 bp, AG type; three bands of 279, 157, 122 bp, and AA type; two bands of 157 and 122 bp.

Table 6
Comparisons of body fat percent by haplotype constructed from A-3826G and A-1766G

Haplotype	Body fat percent (%)			<i>P</i>
[AA]	-/- (<i>n</i> =93) 34.82±0.67 ^b	-/[AA] (<i>n</i> =197) 34.80±0.45	[AA]/[AA] (<i>n</i> =91) 35.30±0.64	0.862 ^a
[GA]	-/- (<i>n</i> =204) 35.63±0.44	[GA] (<i>n</i> =177) 34.11±0.47		0.022
[GG]	-/- (<i>n</i> =225) 34.41±0.41	[GG]+ (<i>n</i> =156) 35.67±0.51		0.045

^a *P*-values were obtained by general linear model analysis adjusted for age.

^b Mean±S.E.

2.5. Serum cholesterol analysis

Blood samples were obtained from each subject, after fasting overnight for more than 12 h, and were centrifuged at 2000 rpm for 10 min. Serum concentrations of total cholesterol, HDL cholesterol, and LDL cholesterol were measured by auto-biochemical analyzer (SP-4410, Arkray Co., Japan). The atherogenic index (AI) was calculated as (total cholesterol–HDL cholesterol)/HDL cholesterol.

2.6. Statistical analyses

All values were presented as a mean±standard error (S.E.). Age-adjusted univariate analysis of variance was performed by general linear model procedure to examine the independent effect of A-1766G polymorphism on the dependent variables. Statistical significance was established at the level of *P*<0.05. All analyses were performed by using SPSS ver. 10.0 (SPSS Inc., IL, USA).

3. Results

The variations of genomic DNA sequences were screened within the 5' flanking region of the UCP-1 gene. The simultaneous sequencing of pooled and individual DNAs revealed A-1766G polymorphism (Fig. 1). This polymorphism is a newly identified SNP, which has not been reported in the NCBI dbSNP database (<http://www.ncbi.nlm.nih.gov/SNP/>). The A-1766G polymorphism was analyzed by MALDI-TOF for the 387 Korean female subjects. It was shown that the genotypes AA, AG, and GG were consisted of 57.4% (*n*=222), 37.7% (*n*=146), and 4.9% (*n*=19), respectively, which followed the Hardy-Weinberg equilibrium (*P*=0.327). The frequency of major A allele was 0.762 and of minor G allele was 0.238.

Table 2 shows the comparison of the physical characteristics and body compositions of the subjects by A-1766G polymorphism of UCP-1. For the comparison of G allele carriers with non-carriers, GG type was combined with AG type into AG/GG type and compared with the AA type. WHR (*P*=0.008), body fat mass (*P*=0.023), and percent body fat (*P*=0.014) were significantly higher in the AG/GG type

Table 7

Comparisons of abdominal visceral fat area by haplotype constructed from A-3826G and A-1766G

Haplotype	Abdominal visceral fat area (mm ²)			P
[AA]	–/– (n=90) 6296±386 ^b	–/[AA] (n=193) 6018±238	[AA]/[AA] (n=89) 5787±373	0.94 ^a
[GA]	–/– (n=200) 6213±245	[GA]+(n=172) 5818±260		0.069
[GG]	–/– (n=219) 5702±228	[GG]+(n=153) 6500±283		0.066

^a P-values were obtained by general linear model analysis adjusted for age.^b Mean±S.E.

compared with the AA type. Body weight and BMI also showed tendencies to be higher in the AG/GG type ($P=0.079$ and $P=0.078$, respectively). However, lean body mass and body water content were almost the same between the groups.

For a more accurate evaluation of the effects of A-1766G polymorphism on body fat accumulation, all subjects were tested using CT to measure the cross sectional fat areas at the abdominal and distal parts of the body (Fig. 2). Mean abdominal subcutaneous fat area and visceral fat area were 11.8% and 17.7%, respectively, higher in the AG/GG type compared with the AA type ($P=0.015$ and $P=0.013$, respectively) (Table 3). Total abdominal fat area, which combines abdominal subcutaneous fat area and visceral fat areas, was 13.3% higher in the AG/GG type ($P=0.009$). Subcutaneous fat area in the distal part of the body was measured at a thigh, and it also showed a tendency to be increased in the AG/GG type ($P=0.086$).

Table 4 shows the comparison of the serum cholesterol level of the subjects by A-1766G polymorphism. Mean total cholesterol and LDL cholesterol levels were higher in the AG/GG type compared to the AA type, even though statistical significance was not found. Atherogenic index, a metabolic syndrome indicator, showed a tendency to be higher in the AG/GG type compared to the AA type ($P=0.079$).

When the physical characteristics, body composition, and body fat areas of the subjects were compared by A-3826G polymorphism, as described by Valve et al. [25], no significant associations were found ($P>0.05$) (data not shown). Linkage disequilibrium (LD) testing demonstrated that there was no linkage between newly found A-766G and A-3826G polymorphisms ($D'=0.929$, $R^2=0.283$) [26]. Four haplotypes were estimated from these two polymorphisms

by using Phase software (Table 5) [27,28]. Since the frequency of haplotype [AG] was very low as 0.007, it was not considered in further analysis. The G allele of A-3826G polymorphism can be divided into haplotypes [GG] and [GA]. The G allele of A-1766G polymorphism is identical to haplotype [GG], because haplotype [AG] can be ignored for its low frequency. Subjects with [GG]/[GG] type ($n=17$) were combined with the –/[GG] type into [GG]+ type for the comparison between carriers and non-carriers of [GG] haplotype. The [GA]/[GA] type ($n=28$) was also similarly combined with the –/[GA] type into [GA]+ type.

The body fat percent of the subjects were compared according to three haplotypes considered, and it was found that haplotype [GG] was significantly connected to higher body fat percent ($P=0.045$) (Table 6). On the contrary, haplotype [GA] was significantly associated with lower body fat percent ($P=0.022$). Haplotype [AA] showed no significant associations. Similar tendencies were also found when CT-measured visceral fat areas were compared (Table 7). Carriers of haplotype [GG] showed a tendency to have more visceral fat accumulation than do non-carriers ($P=0.066$), but [GA] haplotype carriers were shown to have less visceral fat accumulation ($P=0.069$). Haplotype [GG] was also significantly associated with larger abdominal subcutaneous fat area ($P=0.016$) (Table 8).

4. Discussion

Recently, numerous candidate genes were searched to find out the genetic factors implicated in the pathogenesis of obesity and body fat accumulation. UCP-1, which plays a major role in thermogenesis, is suggested to be one of the

Table 8

Comparisons of abdominal subcutaneous fat area by haplotype constructed from A-3826G and A-1766G

Haplotype	Abdominal subcutaneous fat area (mm ²)			P
[AA]	–/– (n=90) 30,508±1477 ^b	–/[AA] (n=193) 29,654±936	[AA]/[AA] (n=89) 27,915±1082	0.463 ^a
[GA]	–/– (n=200) 29,846±861	[GA]+(n=172) 28,977±1007		0.435
[GG]	–/– (n=219) 28,059±796	[GG]+(n=153) 31,427±1101		0.016

^a P-values were obtained by general linear model analysis adjusted for age.^b Mean±S.E.

candidates. A genetic polymorphism of UCP-1, a Bcl I restriction fragment length polymorphism, was found from a Quebec Family Study [13], and its molecular nature was elucidated to be an A→G point mutation in the –3826 position in the 5'-flanking region of the UCP-1 gene (A-3826G) [14]. Until now, most studies about the genetic variation of UCP-1 were focused on A-3826G polymorphism, and many association studies were conducted in various populations to elucidate the association of this polymorphism with obesity phenotypes [13–22]. Even though some studies suggest associations of A-3826G polymorphism with obesity phenotypes, other reports do not support them. These controversies on the physiological effects of the UCP-1 5'-flanking sequence polymorphism may suggest that more study is needed in this genomic region.

Through this study, a new polymorphism of UCP-1 gene, A-1766G, was discovered about 2 kb downstream from A-3826G, and there was no linkage between them (D' =0.929, R^2 =0.283). A-1766G was an A→G point mutation at –1766 bp upstream from the transcription start site of the UCP-1 gene, and it may possibly be located in or near the genomic region which is involved in the transcriptional regulation, even though more study is needed to prove it. The G allele of A-1766G polymorphism displayed its significant association with higher WHR, body fat mass, percent body fat, and CT-measured abdominal fat area ($P<0.05$). It also showed tendencies to be associated with higher body weight, BMI, CT-measured thigh fat area, and atherogenic index ($P<0.1$) (Tables 2–4).

Among the subjects of this study, A-3826G polymorphism showed no statistically significant association with body fat (data not shown), suggesting that single polymorphism is not enough and that combination of polymorphisms, haplotype, should be analyzed. Three haplotypes (frequency >0.05) were analyzed from the A-3826G and A-1766G polymorphisms (Table 5). Haplotype [GG] was shown to be significantly associated with higher body fat percent and the CT-measured fat area, and haplotype [GA] was associated with lower body fat. Haplotype [AA] showed no significant effects on body fat accumulation (Tables 6–8). It was shown that the physiological effects of the G allele of A-1766G were similar to those of haplotype [GG]. In this case, however, the P -value for haplotype [GG] was larger than that for A-1766G, even though a smaller P -value is usually expected for haplotype than individual SNP. One of the reasons may be that we have only 2 loci for the haplotype and, therefore, the improvement of the genetic information may be not significant. The G allele of A-1766G polymorphism was divided into haplotype [GG] and [AG], but is almost identical to haplotype [GG] because [AG] can be ignored for its low frequency. On the other hand, the G allele of A-3826G polymorphism was divided into haplotypes [GG] and [GA]. The results of this study identified two subgroups among the G allele carriers of A-3826G polymorphism, haplotype [GA] with lower fat accumulation and haplotype [GG] with higher fat accumulation, and it may provide some explanation for the

controversies about the physiological effects of A-3826G polymorphism reported in previous association studies.

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