Identification of Leptin Gene Variants in School Children with Early Onset Obesity

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Objective: *To investigate leptin gene variations in Thai primary school children.*
Material and Method: *Direct DNA sequencing was performed following polymerase chain reaction (PCR) amplification of the leptin gene in 30 obese children aged 10-12 years old.*
Results: *A heterozygous variant 19 (G > A), located in the non-coding region of exon 1 was detected in 13 subjects (43.3%, A: 0.22). Only 2 subjects (6.7%, G: 0.03) harbored a heterozygous (CAA > CAG) polymorphism in codon 25 of exon 2.*
Conclusion: *The 25 (CAA > CAG) polymorphism appeared to be a new leptin gene variant in Thai people. This study provides basic information concerning the prevalence of leptin gene polymorphisms in Thai children with early onset obesity. It might be useful as genetic marker for screening for obesity potential in large populations.*

Keywords: *Leptin, Gene variation, Polymorphism, Obesity, School children*

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Childhood obesity is one of the most serious public health problems, and many of its health and social consequences may continue into adulthood[1] leading to a global burden of disability and chronic diseases including diabetes, hypertension, cardio-vascular problems and dyslipidemia[2,3]. In the United States, the prevalence of overweight children and adolescents increased significantly from 13.9% in 2000 to 17% in 2004[4]. In Thailand, the national prevalence of obesity in children of 5 to 12 years rose rapidly from 12.2% in 1991 to 15.6% in 1993[5]. Causes of obesity are multi-factorial and influenced by genetic, behavioral, environmental and cultural factors[6]. Genetic factors can contribute from 30 to as much as 70%, to the etiology of obesity[7].

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Leptin is an adipocyte-secreted hormone that plays a key role in regulating energy intake and energy expenditure via appetite and metabolism[8]. It is controlled by the leptin gene (Ob) which is located on human chromosome 7q31.3. In humans, it consists of three exons separated by two introns[9]. Leptin gene mutations can cause phenotypes of abnormal eating behavior followed by the development of severe, early onset obesity[10]. Various studies on polymorphisms in leptin gene have been reported. A study in North-America reported that 19 (G > A) polymorphism was detected in non-coding region of exon 1 and associated with obesity among women[11]. This same variant was also identified in Japanese subjects including its association with sweet preference[12].

Knowledge of genes related to obesity in the Thai population is very limited. This study aimed to identify single nucleotide polymorphisms (SNP) or mutations in the leptin gene of school children with early onset obesity using PCR and DNA sequencing methods.

Material and Method

A cross-sectional study was conducted from July 2008 to January 2009. Because of ethical considerations regarding research in human subjects, it was not possible to do random sampling. Inclusion of subjects was on a voluntary basis. Thirty voluntary, obese school children who participated in the study were from a public primary school that joined the Bright and Healthy Thai Kid Project in Bangkok. They were 10 to 12 years old, with early onset obesity (*i.e.*, under the age of 10). Informed consent was obtained from all participants and their parents. All protocols for the study were reviewed and approved by the Institutional Review Board, Faculty of Public Health, Mahidol University, Thailand (No. MUPH2008-043).

The nutritional status of all participants was assessed by anthropometric measurements. Children's weight was determined using an electronically calibrated scale (Seca, Germany). Their height was measured using a calibrated stadiometer. Child nutritional status was assessed by criteria listed in the INMU Thai Growth program[13]. Obesity was defined as a weight for height ratio greater than 2 SD from the median. Completed interview questionnaires were collected from participants and their parents. Responses included details regarding family history of obesity, child eating behavior and physical activities. Six milliliters of venous blood was collected from each participant (in an EDTA treated tube) by a registered nurse.

Laboratory methods

Genomic DNA was extracted from white blood cells in blood samples by using the Flexi Gene DNA kit (Qiagen, Hilden, Germany). Genotyping of leptin gene variants was achieved by amplification by the polymerase chain reaction (PCR) method. The sequence of the target gene was retrieved from the Nickerson Lab Master Gene List (LEP: LEP Color FASTA, University of Washington record). The gene coding number of leptin gene (NCBI) is NM_000230.2, Gene ID: 169790920 of chromosome 7. The program Primer 3 was used to design the necessary primers[15]. Forward and reverse primers were designed to cover all the exons of the leptin gene as shown in Table 1.

Individual PCR reactions were performed in a total volume of 50 µl using 50-100 ng of genomic DNA, 0.3 µM of each oligonucleotide primer specific for the leptin gene (Table 1), 250 µM of dNTPs (dATP, dCTP, dGTP, dTTP), 1.0 U of Taq DNA polymerase and PCR buffer [10 mM Tris-HCl pH 8.3, 50 mM of KCl, 1.5 mM of $MgCl_2$] to make up the volume. DNA amplification was carried out using a Biometra T personal Thermal Cycler (Biometra, Germany). The amplification protocol was as follows: 35 cycles of denaturation at 94°C for 5 min, annealing at 65°C for exon 1, 58°C for exon 2 and 3 for 30 seconds and extension at 72°C for 1 min. The amplified products were purified and sequenced by automatic sequencer 3730X (Macrogen, Korea). The NCBI BLAST program was used to compare deduced protein and nucleotide sequences. BLAST was also used for local alignments and identification of regions of similarity between paired sequences.

Statistical analysis

SPSS for windows, version 11.5 (License No.: 3040357211276308256096011184 7) was used. Descriptive statistics such as mean, standard deviation and percentages were used to describe general characteristics of the children, their physical activities, eating behavior and allele frequency. The Fisher's exact test and odds ratios were used to explain the relationship between parental obesity, gender and occurrence of variants.

Results

General characteristics of subjects

A total of 30 obese children participated in the study, 73.3% were male, 26.7% were female and they were aged 10-12 years (Mean 11 $\pm$ 0.8 years). Of these, 86.7% had a family history of obesity.

Eating and physical activity behavior

The unhealthy eating behavior of the study group included regular consumption (more than 5 times/ week) of high sugar foods (22.4%), high sugar and high fat foods (12.1%), and high fat foods (8.4%). Nearly half of the 30 subjects exerted a low level of physical activity and exercised less than 30 min/day. By contrast, sedentary behavior such as watching

Table 1. The primer sequences for the leptin gene

Exon	Oligonucleotide sequence	Size (bp)
1	F: 5'-CCC GCG AGG TGC ACA CTG-3'	221
	R: 5'-AGG AGG AAG GAG CGC GCC-3'	
2	F: 5'-CTT CTG TTT TCA GGC CCA AG-3'	368
	R: 5'-GGC CAA AAG AAA CAA CCA GA-3'	
3	F: 5'-TAG AGG CTT GGC AGT CAC CT-3'	598
	R: 5'-ACC TGG AAG CCA GAG TTC CT-3'	

television and playing computer games of more than 2 hours a day was reported by 75.9% on weekdays, which increased to 100% on weekends.

Genetic variations and allele frequency

A heterozygous variant at base 19, consisting of a *G* to *A* transition [19 (G > A)] polymorphism was detected in the non-coding region of exon 1 (Fig. 1). The AG heterozygous condition was found in 13 subjects (43.3%) and GG homozygous in 17 subjects (56.7%). No AA homozygotes were detected. The frequency of A allele was 22% and the G allele was 78%. Another variation was found at codon 25 of exon 2 (Fig. 2). The heterozygous variant exhibited a silent change from CAA (glutamine) to CAG (glutamine) [25 (CAA > CAG)]. This codon 25 CAG variant was detected in 2 out of 30 subjects (6.7%) and was found only in subjects with parental obesity. The G allele frequency for the 25 (CAA > CAG) polymorphism in the study population was 3%. That is, 2 AG individuals, 0 GG individuals, and 28 AA individuals gave (2 x 1) + (0 x 2) = 2 G alleles, (2 x 1) + (28 x 2) = 58 A alleles, and a G allele frequency of 2/(58 + 2) = 2/60 = 0.03 respectively.

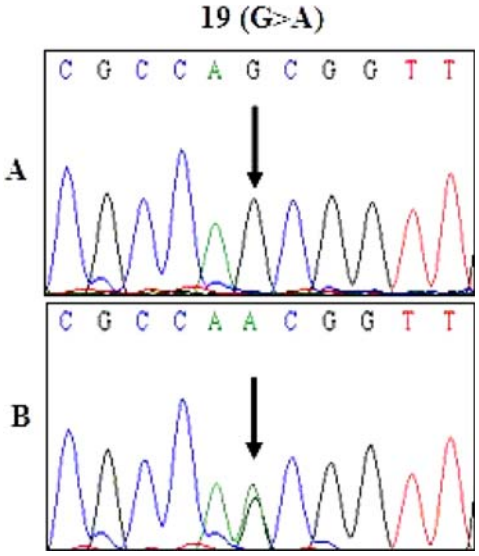
Parental obesity and genotype variations

The relationship between leptin genotype variation and parental obesity was analyzed (Table 2). For children with the 19 (G > A) variation, 40% of the parents were obese. The odds ratio for parental obesity in children with the 19 (G > A) variation was 2.6 (95% CI; 0.24-28.09, p = 0.613). On the other hand, for children with the 25 CAG variation, only 7% had obese parents.

Table 2. The relationship between genotype variations and parental obesity

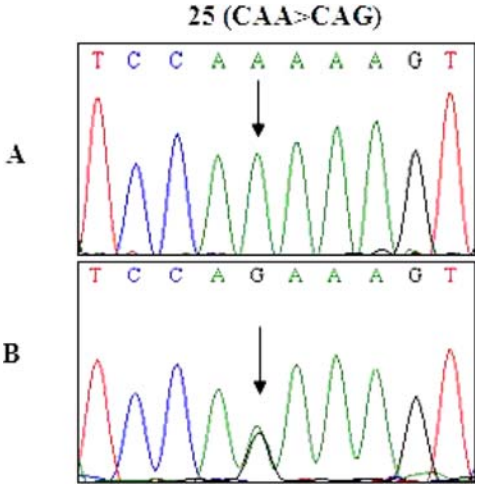
Variants	Parental nutritional status		p-value*	OR	95% CI
	Obese n (%)	Normal n (%)			
19 (G > A)					
GA	12 (40)	1 (3)	0.613	2.6	0.24-28.09
GG	14 (47)	3 (10)			
25 (CAA > CAG)					
AG	2 (7)	0 (0)	-	-	-
AA	24 (80)	4 (13)			

* Fisher's exact test, p > 0.05



A; Wild type and B; heterozygous variant (Comparison with NCBI database)

Fig. 1 Sequencing output showing the 19 (G > A) variation of the nucleotide sequence of exon 1



A; Wild type and B; heterozygous variant (Comparison with NCBI database)

Fig. 2 Sequencing output showing the 25 (CAA > CAG) variation of the nucleotide sequence of exon 2

Gender and genotype variations

The proportion of children with the variation at 19 (G > A) was 9: 22 for boys and 1:1 for girls, while that for codon 25 (CAA > CAG) was 1:21 for boys and 1:7 for girls (Table 3). The odds ratios of having 19

(G > A) or 25 CAG polymorphism in obese girls in this study were 1.4 (95% CI; 0.28-7.34, p = 0.698) and 3.0 (95% CI; 0.17-54.57, p = 0.469), respectively. In other words, obese girls were 1.4 and 3.0 times more likely to show AG heterozygous variations [19 (G > A) and 25 CAG] than boys.

Table 3. The association between genetic variations and gender

Variants	Gender (%)		p-value*	OR	95% CI
	Girl	Boy			
19 (G > A)					
GA	4 (13.3)	9 (30)	0.698	1.4	0.28-7.34
GG	4 (13.3)	13 (43.4)			
25 (CAA > CAG)					
AG	1 (3.3)	1 (3.3)	0.469	3.0	0.17-54.57
AA	7 (23.4)	21 (70)			

* Fisher's exact test, p > 0.05

Behavior and genotype variation

There was an association between 19 (G > A) polymorphism and eating high sugar foods such as sweet chocolate (p = 0.002) and drinking yoghurt (p = 0.006), but this association was not seen in children who had the 25 *CAG* variants (Table 4). Children who had the 19 (*G > A*) polymorphism did less daily exercise than children who had the wild type (*GG* homozygous) constitution.

Discussion

Our study group exhibited unhealthy eating behavior associated with consumption of high sugar and high fat foods as reported in a previous study[3]. That study also reported that Thai obese children ate fried foods at high frequencies. Nearly half of our study group exhibited a low level of physical activity (*i.e.*, daily exercise less than 30 minutes) which is less than the standard recommended by the American Heart Association[16]. They also spent more than 2 hours a day for watching television and playing computer games. The American Academy of Pediatrics recommended

Table 4. The association between eating behavior and genetic variants in obese subjects

Food frequency (per week)	A19G (%)		p-value	25 CAG (%)		p-value
	GG	AG		AA	AG	
Carbonated drink			0.895			0.469
< 1 time	1 (3.5)	1 (3.5)		2 (6.9)	0 (0)	
1-4 times	10 (34.5)	7 (24.1)		15 (51.7)	2 (6.9)	
> 5 times	5 (17.2)	5 (17.2)		10 (34.5)	0 (0)	
Sweet Chocolate			0.002*			0.754
< 1 time	5 (17.2)	6 (20.7)		10 (34.6)	1 (3.4)	
1-4 times	10 (34.6)	2 (6.9)		11 (37.9)	1 (3.4)	
> 5 times	1 (3.4)	5 (17.2)		6 (20.7)	0 (0)	
Drinking yoghurt			0.006*			0.664
< 1 time	2 (6.9)	0 (0)		2 (6.9)	0 (0)	
1-4 times	14 (48.3)	7 (24.1)		19 (65.5)	2 (6.9)	
> 5 times	0 (0)	6 (20.7)		6 (20.7)	0 (0)	
Cake			0.466			1.000
< 1 time	5 (17.2)	6 (20.8)		10 (34.6)	1 (3.4)	
1-4 times	11 (37.9)	7 (24.1)		17 (58.6)	1 (3.4)	
> 5 times	0 (0)	0 (0)		0 (0)	0 (0)	
Fried chicken			0.471			0.617
< 1 time	2 (6.9)	1 (3.4)		3 (10.3)	0 (0)	
1-4 times	12 (41.4)	8 (27.6)		18 (62.1)	2 (6.9)	
> 5 times	2 (6.9)	4 (13.8)		6 (20.7)	0 (0)	
French fries			0.266			0.910
< 1 time	9 (31)	6 (20.7)		14 (48.4)	1 (3.4)	
1-4 times	7 (24.2)	5 (17.2)		11 (37.9)	1 (3.4)	
> 5 times	0 (0)	2 (6.9)		2 (6.9)	0 (0)	

limit is no more than 1 to 2 hours of quality TV and video a day for older children[17]. Thus unhealthy eating and sedentary behavior in our study group played important roles in causing obesity.

The association between parental obesity and leptin gene polymorphism in our study was not statistically significant, but this may have been due to the small sample size. However, a recent study indicated that parental obesity might present an increased susceptibility to other adipose-associated traits[18]. In addition, parental obesity significantly enhanced the risk of school age obesity in children[19]. Our study reported that obese children of obese parents were 2.6 times more likely to possess the 19 (G > A) variation than children of non-obese parents. In other words, parental obesity might be related to the 19 (G > A) variation in the leptin gene of their children.

Our finding was similar to a recent study reported that *AG* heterozygosity was detected in Japanese subjects (29.2%) as was the association between the 19 (G > A) polymorphism and sweet preference. Altogether it suggests that polymorphism of the leptin gene might be used as a marker for a propensity to eat high sugar foods[12]. In genetics, non-coding DNA in eukaryocytes is a large percentage and still has no known function[20]. It is possible that these allelic variants in non-coding region may play role in the regulation of the leptin gene and influence in high sugar eating habit. However, for the time being, there is no study as yet to confirm this postulation.

We discovered another polymorphism in the third base of codon 25 (CAA > CAG) of the leptin gene (*i.e.* also an AG heterozygous polymorphism) at 6.7%. This polymorphism was only in children of obese parents. A study on mutations in the leptin gene in 53 morbidly obese Japanese subjects identified a similar silent mutation at codon 25 (CAA > CAG) in eight subjects. They reported a significantly higher prevalence of the 25 CAG leptin variant among obese subjects (0.085) than among non-obese control subjects (0.011, $p < 0.001$). They suggested that the leptin 25 CAG allele might have been linked to their morbidly obese subjects[21].

The polymorphic variants of leptin at 19 (G > A) and 25 (CAA > CAG) might serve as new genetic markers for Thai children who are susceptible to obesity. To our knowledge, this is the first report of leptin 25 CAG variants in obese Thai children. No homozygous AA variants were detected in our study group. It may be a rare variant or it may have simply gone undetected due to the small sample size. Regarding gender, the odds ratio indicated that obese girls were 1.4 and 3 times more likely, respectively, to possess the 19 (G > A) and the 25 CAG polymorphisms than boys, although the difference was not statistically significant. If the sample size were increased a statistically significant difference might be revealed.

In conclusion, the results of this study tend to provide basic information on the prevalence of leptin gene variations in Thai children with early onset obesity. Future research on leptin gene variants with larger sample size in both normal weight and obese Thai children are required in order to be used as guidelines for developing genetic markers for obesity screening in a large population. Since genetic predispositions are mostly irrevocable, the improvement of environmental factors combined with a healthy lifestyle could be effective strategies for obesity control.

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References

1. Venn AJ, Thomson RJ, Schmidt MD, Cleland VJ, Curry BA, Gennat HC, et al. Overweight and obesity from childhood to adulthood: a follow-up of participants in the 1985 Australian Schools Health and Fitness Survey. Med J Aust 2007; 186: 458-60.
2. Lobstein T, Baur L, Uauy R. Obesity in children and young people: a crisis in public health. Obes Rev 2004; 5(Suppl 1): 4-104.
3. Sirikulchayanonta C, Pavadhgul P, Chongsuwat R, Srisorrachata S. A preliminary study of hyperlipidemia in Bangkok school children. Asia Pac J Public Health 2006; 18: 15-9.
4. Ogden CL, Carroll MD, Curtin LR, McDowell MA, Tabak CJ, Flegal KM. Prevalence of overweight and obesity in the United States, 1999-2004. JAMA

2006; 295: 1549-55.
5. World Health Organization [homepage on the Internet]. Global strategy on diet, physical activity and health. Childhood overweight and obesity. 2009 [cited 2009 May 6]; Available from: http://www.who.int/dietphysicalactivity/childhood/en/
6. Krassas GE, Tzotzas T. Do obese children become obese adults: childhood predictors of adult disease. Pediatr Endocrinol Rev 2004; (1 Suppl 3): 455-9.
7. Herbert A, Gerry NP, McQueen MB, Heid IM, Pfeufer A, Illig T, et al. A common genetic variant is associated with adult and childhood obesity. Science 2006; 312: 279-83.
8. Brennan AM, Mantzoros CS. Drug Insight: the role of leptin in human physiology and pathophysiology-emerging clinical applications. Nat Clin Pract Endocrinol Metab 2006; 2: 318-27.
9. Isse N, Ogawa Y, Tamura N, Masuzaki H, Mori K, Okazaki T, et al. Structural organization and chromosomal assignment of the human obese gene. J Biol Chem 1995; 270: 27728-33.
10. Baratta M. Leptin-from a signal of adiposity to a hormonal mediator in peripheral tissues. Med Sci Monit 2002; 8: RA282-92.
11. Li WD, Reed DR, Lee JH, Xu W, Kilker RL, Sodam BR, et al. Sequence variants in the 5' flanking region of the leptin gene are associated with obesity in women. Ann Hum Genet 1999; 63: 227-34.
12. Mizuta E, Kokubo Y, Yamanaka I, Miyamoto Y, Okayama A, Yoshimasa Y, et al. Leptin gene and leptin receptor gene polymorphisms are associated with sweet preference and obesity. Hypertens Res 2008; 31: 1069-77.
13. Institute of Nutrition Research, Mahidol University. INMU Thai growth program for nutritional assessment (using weight for height references from national survey, Department of Health, Ministry of Public Health). Bangkok: INMU; 2002.
14. Nickerson Group, Department of Genome Sciences, University of Washington [homepage on the Internet]. Nickerson Lab Master Gene List; LEP: LEP Color FASTA. 2008 [cited 2008 Sep 10] Available from: http://pga.gs.washington.edu/data/lep/lep.ColorFasta.html
15. Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 2000; 132: 365-86.
16. Centers for Disease Control and Prevention [homepage on the Internet]. Obesity prevalence: trends in childhood obesity. 2009 [cited 2009 May 20]. Available from: http://www.cdc.gov/nccdphp/dnpa/obesity/childhood/prevalence.htm
17. American Academy of Pediatrics. Children, adolescents, and television. Pediatrics [serial on the Internet]. 2001 Feb [cited 2008 Feb 20]; 107(2): 423-6. Available from: http://aappolicy.aappublications.org/cgi/reprint/pediatrics;107/2/423.pdf
18. Lieb W, Pencina MJ, Lanier KJ, Tofler GH, Levy D, Fox CS, et al. Association of parental obesity with concentrations of select systemic biomarkers in nonobese offspring: the Framingham Heart Study. Diabetes 2009; 58: 134-7.
19. Lee K, Kwon ER, Park TJ, Park MS, Lenders CM. Parental overweight as an indicator of childhood overweight: how sensitive? Asia Pac J Clin Nutr 2006; 15: 196-200.
20. Carroll SB, Prud'homme B, Gompel N. Regulating evolution. Sci Am 2008; 298: 60-7.
21. Ohshiro Y, Ueda K, Nishi M, Ishigame M, Wakasaki H, Kawashima H, et al. A polymorphic marker in the leptin gene associated with Japanese morbid obesity. J Mol Med 2000; 78: 516-20.

การวิเคราะห์หาการเปลี่ยนแปลงของยีนเลปตินในเด็กวัยเรียนที่มีภาวะอ้วนก่อนวัย

ปาลีรัฐ คงมาชีพ, ชุติมา ศิริกุลชยานนท์, รังสรรค์ ตั้งตรงจิตร, กิติพงษ์ หาญเจริญ

วัตถุประสงค์: *เพื่อตรวจหาการเปลี่ยนแปลงของยีนเลปตินในเด็กอ้วนวัยเรียนระดับประถมศึกษา*
วัสดุและวิธีการ: *เพิ่มปริมาณของดีเอ็นเอโดยใช้เทคนิคพีซีอาร์ แล้วค้นหาการเรียงตัวของลำดับเบส บนยีนเลปติน ในนักเรียนอ้วนจำนวน 30 คน อายุ 10-12 ปี*
ผลการศึกษา: *พบการเปลี่ยนแปลงของยีนเลปตินเป็นแบบเฮทเทอโรไซกัส ในตำแหน่งที่ 19 (G > A) ของเอกซอนที่ 1 บริเวณ ที่ไม่สามารถถอดรหัสเป็นโปรตีนได้ จำนวน 13 ราย (ร้อยละ 43.3, A :0.22) และ พบจำนวน 2 ราย (ร้อยละ 6.7, G: 0.03) มีการเปลี่ยนแปลงของยีนเลปตินแบบเฮทเทอโรไซกัส ใน โคดอนที่ 25 ของเอกซอนที่ 2 (CAA > CAG) ซึ่งเป็นตำแหน่งใหม่ที่ตรวจพบในกลุ่มตัวอย่างคนไทย*
สรุป: *การศึกษานี้เป็นรายงานการพบข้อมูลเบื้องต้น เกี่ยวกับความชุกของการเปลี่ยนแปลงของยีนเลปตินในเด็กไทย ที่มีภาวะอ้วนก่อนวัย และ จะเป็นประโยชน์ต่อการนำไปใช้เป็นเครื่องหมายยีน (genetic marker) ในการคัดกรองปัจจัย ทางพันธุกรรมของโรคอ้วนในประชากรกลุ่มใหญ่ต่อไป*