

Study of leptin levels and gene polymorphisms in patients with central precocious puberty

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INTRODUCTION: Three single-nucleotide polymorphisms (SNPs) in the leptin (*LEP*) or leptin receptor (*LEPR*) genes were assessed for their association with central precocious puberty (CPP).

RESULTS: The control group with the A/G SNP at *LEPR* 223 or A/G SNP at *LEPR* 109 exhibited significantly higher peak luteinizing hormone (LH) levels. The leptin level in the CPP group was significantly higher than that in the control group, but SNPs in either *LEP* or *LEPR* gene could not explain this observation.

DISCUSSION: In conclusion, SNPs at *LEPR* 223 and *LEPR* 109 were significantly associated with higher levels of LH in girls without CPP, but none of the genotypes at these SNPs were significantly associated with CPP.

METHODS: The SNP genotypes of *LEP* (polymorphism at promoter at nt-2548) and *LEPR* (223A/G, 109A/G) of 219 healthy girls and 249 girls diagnosed with CPP were compared. Allele frequencies in SNPs were compared with anthropometric measures, circulating leptin, hormones (estradiol, follicle-stimulating hormone, and LH), and lipid concentrations for CPP risk.

Puberty is a complex process involving the activation and maturation of the hypothalamic–pituitary–gonadal axis. The mean age of pubertal onset has declined in recent decades; currently, it is 10 y in girls and 11.5–12 y in boys (1). Normal puberty onset ranges from 8 to 13 y in girls and 9 to 14 y in boys (2). Precocious puberty is defined as the appearance of the initial signs of sexual maturation—breast buds in girls and testicular volume greater than 3–4 ml in boys—at an age that is more than 2–2.5 SDs below the mean of the population. During the past few decades, the age limit for precocious puberty has been <8 y for girls and 9 y for boys (3). This definition is somewhat arbitrary because of the marked variation in the age at which puberty begins in healthy children, particularly in different ethnic groups. Precocious puberty is classified as central (gonadotropin-releasing hormone–dependent; GnRH dependent) or peripheral (GnRH independent). Central precocious puberty (CPP) accounts for 80% of patients with precocious puberty and is more frequent in girls than in boys. Incidence of CPP ranges from <0.05 to 0.4% (4) to approximately 1% in China (5). Early onset of puberty may be caused by a lesion in

the central nervous system (6), so patients with early pubertal onset should be evaluated for the possibility of a central nervous system lesion. Early menarche may increase the risk of breast cancer (7). On the basis of worldwide studies, pubertal onset seems to be influenced by race, ethnicity, environmental conditions, geographic location, and nutrition (8). Kaprio *et al.* conclude that onset age of puberty is affected by environmental agents, which contribute to 26% of variation, and by genetic differences (74% of variation) (9). Polymorphisms of genes that modulate pubertal onset are hotly pursued topics.

Puberty and infancy are two main periods of accelerated linear growth. Pubertal growth spurt results mainly from the synergistic effects of significantly higher levels of gonadal sex steroids, growth hormone (GH), and insulin growth factor-1 (IGF-1) during this period (2). A polymorphism in the IGF-1 receptor gene (GAA1013 to GAG) is significantly associated with early pubarche (10). Furthermore, a potential effect of SNP3 (rs6214) in the *IGF-1* gene on the age at menarche in white women has been reported (11).

Pubertal growth is also dependent on the quantity and distribution of body fat. Obesity is associated with early onset of puberty (1). Leptin is a 146-amino-acid protein that plays a major role in fat metabolism. The essential role of leptin in onset of puberty was elucidated by a study of patients with a homozygous mutation in the leptin receptor (12). They did not undergo puberty and had reduced secretion of GH and thyrotropin (12). Furthermore, administration of recombinant leptin to patients with human congenital leptin deficiency induced the onset of puberty (13). Leptin also acts as a skeletal bone growth factor. Leptin levels increase just before the onset of puberty and may induce augmented proliferation and differentiation of chondrocytes in the epiphyseal growth plate. Leptin exerts its effect by stimulating IGF-1 receptor gene expression and abundance in the growth center. Although leptin may act on bone independently of GH, it possibly exerts a synergistic effect with the GH/IGF-1 axis (14). Because leptin concentrations are associated with body weight and body mass index (BMI) in a CPP population (15), we hypothesized that differences in leptin responsiveness may affect the age of pubertal onset. Previous groups have shown that single-nucleotide polymorphisms (SNPs) of the leptin

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receptor at K109R and Q223R were associated with bone mineral density in a Korean population (16). An SNP (G>A variant) in the promoter region of the leptin gene at nt-2548 is associated with higher leptin levels in obese girls (17) and extreme obesity in overweight Europeans and Taiwanese aborigines (18). On the basis of these associations, we hypothesized that the frequency of the G>A variant in the leptin promoter at nt-2548 (rs7799039) or the SNPs in the leptin receptor Q223R (rs1137101) or K109R (rs1137100) may be more frequent in girls with CPP. To test this hypothesis, we conducted a case-control study of 249 CPP cases and 219 controls and examined the association of three common genetic variants in leptin and leptin receptor genes with anthropometric measures, precocious puberty risk, and concentrations of circulating leptin, hormones (estradiol (E₂), follicle-stimulating hormone (FSH), luteinizing hormone (LH)), and fat (cholesterol and triglyceride (TG)).

RESULTS

The demographics of 249 girls who had early breast development (<8 y) and were diagnosed with CPP were compared with 219 girls in the control group (peak LH levels <7 U/l for all controls). Among the 249 girls with CPP, there were 134 girls in Tanner stage II and 115 girls in Tanner stage III; whereas among the 219 control girls, 90 girls were in Tanner stage I and 129 girls were in Tanner stage II. As shown in **Table 1**, the CPP group exhibited significantly higher bone age (BA), BA/chronological age (CA), height, weight, BMI, and levels of several sex hormones (E₂, FSH, and LH) than the control group, but the CA showed no significant differences between the two groups. In addition, leptin expression in the CPP group was 7.6 ng/ml (SD = 5.7), whereas leptin expression in the control group was 6.2 ng/ml (SD = 5.4) ($P < 0.01$; **Table 1**). Thus, leptin expres-

sion was significantly higher in the CPP group as compared with the control group.

We next analyzed whether a significantly higher level of leptin in patients with CPP correlated with the early onset of puberty. Because BMI and leptin levels are higher in the CPP group and BMI may act as a confounding factor, the correlation of leptin with relative demographics and clinical pathologic features by group and the overall population were adjusted for BMI as a covariate. These represented partial correlations (**Table 2**). In the overall population, height, weight, BMI, and serum level of TG were significantly and directly correlated with leptin levels. Similarly, height, weight, and BMI, but not TG levels, were correlated with leptin in the control group. The CPP group showed no significant correlation between leptin and demographic features (height, weight, BMI, and sex hormones) but showed a positive correlation between serum levels of TGs and leptin levels.

Table 3 shows the distribution of genotypes of SNPs at *LEP* -2548, *LEPR* 109, and *LEPR* 203 in the CPP and control groups. The distribution of A/A, A/G, or G/G for the SNPs at *LEP* -2548, *LEPR* 109, and *LEPR* 203 in the CPP group was not significantly different from that in the control group. No significant differences were detected in the distribution between A/A and non-A/A (A/G or G/G) in the two groups. The distribution of the A and G alleles at leptin -2548 in the CPP group was not significantly different from that in the control group.

We subsequently investigated whether various combinations of *LEP* -2548 plus *LEPR* 223 polymorphisms and combinations of *LEP* -2548 plus *LEPR* 109 polymorphisms were associated with CPP phenotype. We compared the demographics and pathologic features with the prevalence of three combinations of *LEP* -2548 and *LEPR* 223 SNPs, and three combinations of *LEP* -2548 and *LEPR* 109 SNPs, in the CPP and control groups separately, which would avoid confounding effects of the significantly different values (**Table 4**). No significant difference was found in demographics and pathologic features among the girls diagnosed with CPP with different combinations of SNPs at *LEP* promoter -2548 plus *LEPR* 223 or *LEP* promoter -2548 plus *LEPR* 109 (**Table 4**). However, significant differences in mean LH levels were observed among control girls with different combinations of SNPs: *LEP* promoter -2548 plus *LEPR* 223 ($P = 0.002$) and *LEP* promoter -2548 plus *LEPR* 109 ($P = 0.003$) (**Table 4**). Furthermore, control girls with *LEP* -2548A/A and *LEPR* 223 A/G alleles had significantly higher LH levels ($P = 0.002$) than those with *LEP* -2548A/G and *LEPR* 223 G/G (**Table 4**).

Demographics and pathologic features of CPP were compared in both CPP and control groups among three genotypes of *LEPR* 223 SNP and three genotypes of *LEPR* 109 SNP (**Table 5**). Demographics and pathologic features of the CPP group did not significantly associate with any specific genotype of *LEPR* 223 SNP or *LEPR* 109 SNP (**Table 5**). Analysis of the control group suggested significant associations between specific genotypes at *LEPR* SNPs and mean FSH levels or mean LH levels. Significant differences were found in mean FSH levels among the control girls with

Table 1. Demographics and clinical pathological features of subjects in CPP and control groups

Variable ^a	Control group (n = 219)	CPP group (n = 249)	P value
Chronological age (y)	8.3 ± 1.4	8.5 ± 1.4	0.16
Bone age (y)	8.9 ± 2.1	10.4 ± 1.5	<0.001
BA/CA	1.1 ± 0.2	1.2 ± 0.1	<0.001
Height (cm)	130.5 ± 8.8	140.4 ± 10.5	<0.001
Weight (kg)	30.4 ± 8.4	37.3 ± 9.3	<0.001
BMI (kg/m ²)	17.6 ± 3.5	18.7 ± 2.9	<0.001
Estradiol (pmol/l)	28.4 ± 11.5	33.8 ± 18.7	<0.001
FSH (U/l)	8.8 ± 8.0	18.1 ± 9.0	<0.001
LH (U/l) ^b	2.8 ± 2.9	38.2 ± 31.1	<0.001
Cholesterol (mg/dl)	171.4 ± 28.9	170.0 ± 28.7	0.64
Triglyceride (mg/dl)	110.3 ± 65.7	107.5 ± 62.2	0.68
Leptin (ng/ml)	6.2 ± 5.4	7.6 ± 5.7	<0.01

BA/CA, bone age/chronological age; CPP, central precocious puberty; FSH, follicle-stimulating hormone; LH, luteinizing hormone.

^aData were presented as mean ± SD with two-sample t test. ^bStimulated luteinizing hormone.

Table 2. Correlation of leptin with relative demographics and clinical pathological features after adjustment for BMI^a

Variable	Overall (n = 468)		Control group (n = 219)		CPP group (n = 249)	
	r ^a	P value	r	P value	r	P value
Chronological age (y)	0.006	0.89	0.073	0.28	-0.040	0.54
Bone age (y)	0.057	0.23	0.125	0.07	-0.005	0.93
BA/CA	0.057	0.23	0.090	0.19	0.022	0.73
Height (cm)	0.101	0.03	0.135	0.05	0.080	0.21
Weight (kg)	0.112	0.02	0.142	0.04	0.095	0.14
BMI (kg/m ²)	0.797	<.001	0.826	<.001	0.748	<0.001
Estradiol (pmol/l)	-0.042	0.37	-0.044	0.52	-0.041	0.52
FSH (U/l)	-0.036	0.44	-0.068	0.32	-0.016	0.80
Luteinizing hormone (U/l)	0.006	0.89	-0.017	0.09	0.016	0.81
Cholesterol (mg/dl)	0.009	0.18	0.020	0.77	0.011	0.13
Triglyceride (mg/dl)	0.187	<.001	0.129	0.07	0.239	0.001

BA/CA, bone age/chronological age; CPP, central precocious puberty; FSH, follicle-stimulating hormone.

^aCorrelation was presented as coefficient of correlation (*r*) with respective *P* value by using the Pearson correlation analysis for BMI, and *r* with respective *P* value by using the partial Pearson correlation analysis for other variables with adjustment for BMI as a covariate.

Table 3. The distribution of genotypes of *LEP* -2548, *LEPR* 109, and *LEPR* 223 of subjects in the CPP and control groups

Variable ^a	Control group (n = 219)	CPP group (n = 249)	P value
<i>LEP</i> -2548			
A/A	108 (49.3%)	141 (56.6%)	0.26
A/G	91 (41.6%)	86 (34.5%)	
G/G	20 (9.1%)	22 (8.9%)	
A/A	108 (49.3%)	141 (56.6%)	0.07
A/G or G/G	111 (50.7%)	108 (43.4%)	
A	307 (70.1%)	368 (73.9%)	0.11
G	131 (29.9%)	130 (26.1%)	
<i>LEPR</i> 109			
G/G	161 (73.5%)	179 (71.9%)	0.70
A/G	55 (25.1%)	64 (25.7%)	
A/A	3 (1.4%)	6 (2.4%)	
G/G	161 (73.5%)	179 (71.9%)	0.39
A/G or A/A	58 (26.5%)	70 (28.1%)	
G	377 (86.1%)	422 (84.7%)	0.32
A	61 (13.9%)	76 (15.3%)	
<i>LEPR</i> 223			
G/G	171 (78.1%)	187 (75.1%)	0.74
A/G	46 (21%)	59 (23.7%)	
A/A	2 (0.9%)	3 (1.2%)	
G/G	171 (78.1%)	187 (75.1%)	0.26
A/G or A/A	48 (21.9%)	62 (24.9%)	
G	388 (88.6%)	433 (86.9%)	0.26
A	50 (11.4%)	65 (13.1%)	

CPP, central precocious puberty.

^aData were presented as numbers (%) with Pearson correlation or Fisher's exact test.

different genotypes at *LEPR* 223 (*P* = 0.03). In addition, significant differences were detected in mean LH levels among the control girls with different genotypes at *LEPR* 223 (*P* = 0.01) and *LEPR* 109 (*P* = 0.004).

DISCUSSION

A recent meta-analysis of genome-wide association studies identified 30 new loci associated with age of menarche (19). Four genes were associated with BMI, three with energy homeostasis, and three with regulation of hormones (19). Su *et al.* (15) proposed that leptin concentrations are associated with body weight and BMI in a CPP population. In addition, genetic regulation of pubertal (GRP) onset most likely arises from multiple genes that are critical for the pubertal activation of the hypothalamic-pituitary axis. Genes involved in regulating puberty are being identified by their association with abnormal genetic pubertal development and include the GnRH receptor gene (20), GRP54 receptor gene (21), estrogen gene (2), FSH gene (22), LH gene (23), *LEP* gene (24), and *LEPR* gene (12). Thus, our study hypothesizes that the polymorphisms in *LEP* and *LEPR* genes may correlate with the variant phenotypes of patients with CPP. However, our thorough analyses suggest that specific alleles at these SNPs of *LEP* or *LEPR* 223 and 109 were not significantly associated with CPP.

Our results indicated that leptin levels in the CPP group positively correlated with both BMI and TG. It is reasonable because it has been well documented that BMI has a positive association with the serum TG levels (25,26). In the control group, leptin levels significantly correlated with height, weight, and BMI but did not correlate with TG levels. Our study found that leptin levels were significantly higher in the CPP group than in the control group, which may partially explain the higher BMI in girls with CPP.

Previous studies observed a significant association of higher leptin levels with the leptin promoter polymorphism (G-2548A)

Table 4. Comparison of demographic and pathological features of CPP in both the CPP group and the control group among three combinations of *LEP* –2548 and *LEPR* 223 SNPs, and three combination of *LEP* –2548 and *LEPR* 109 SNPs

Variable	<i>LEP</i> –2548 and <i>LEPR</i> 223				Leptin –2548 and <i>LEPR</i> 109			
	A/A and G/G	A/G and G/G	A/A and A/G	P value	A/A and G/G	A/G and G/G	A/A and A/G	P value
CPP group	(n = 109)	(n = 62)	(n = 31)		(n = 103)	(n = 59)	(n = 35)	
Chronological age (y)	8.30 ± 1.48	8.71 ± 1.14	8.48 ± 1.11	0.16	8.31 ± 1.48	8.69 ± 1.15	8.41 ± 1.20	0.21
Bone Age (y)	10.23 ± 1.6	10.77 ± 1.00	10.61 ± 1.28	0.04*	10.27 ± 1.60	10.75 ± 0.98	10.40 ± 1.40	0.12
BA/CA	1.23 ± 0.09	1.24 ± 0.1	1.24 ± 0.07	0.62	1.23 ± 0.09	1.24 ± 0.10	1.23 ± 0.06	0.77
Height (cm)	139.82 ± 10.91	142.12 ± 8.97	139.97 ± 7.13	0.32	139.74 ± 10.67	142.12 ± 8.99	139.80 ± 8.82	0.31
Weight (kg)	37.08 ± 9.72	38.58 ± 8.73	35.93 ± 7.96	0.38	36.87 ± 9.38	38.82 ± 8.51	36.29 ± 9.17	0.32
BMI (kg/m ²)	18.67 ± 3.11	18.89 ± 2.68	18.18 ± 2.79	0.55	18.62 ± 3.06	19.02 ± 2.58	18.31 ± 2.90	0.49
E2 (pmol/l)	33.33 ± 18.2	34.74 ± 25.01	34.53 ± 12.47	0.89	33.49 ± 16.60	34.65 ± 25.56	36.04 ± 17.83	0.61
FSH (U/l)	18.76 ± 11.6	16.69 ± 5.68	17.75 ± 5.21	0.37	18.79 ± 11.67	17.16 ± 5.72	17.73 ± 6.52	0.55
Luteinizing hormone (U/l)	36.81 ± 29.95	36.69 ± 30.95	40.58 ± 35.13	0.82	36.18 ± 28.33	37.29 ± 31.51	41.27 ± 38.86	0.71
Cholesterol (mg/dl)	169.54 ± 28.64	174.56 ± 30.85	173.88 ± 26.49	0.60	172.92 ± 29.29	170.33 ± 26.15	164.04 ± 24.92	0.35
Triglyceride (mg/dl)	102.71 ± 59.88	104.84 ± 45.51	112.96 ± 70.63	0.74	106.82 ± 65.82	98.30 ± 40.12	103.67 ± 56.76	0.77
Leptin (ng/ml)	7.81 ± 6.02	7.67 ± 4.54	6.77 ± 5.47	0.65	7.69 ± 5.69	7.91 ± 4.68	7.50 ± 6.62	0.94
Control group	(n = 78)	(n = 81)	(n = 30)		(n = 78)	(n = 71)	(n = 29)	
Chronological age (y)	8.19 ± 1.17	8.47 ± 1.51	8.37 ± 1.07	0.39	8.21 ± 1.15	8.53 ± 1.56	8.37 ± 1.13	0.31
Bone Age (y)	8.79 ± 2.01	8.98 ± 2.13	8.77 ± 1.94	0.81	8.69 ± 2.08	9.00 ± 2.23	9.31 ± 1.63	0.29
BA/CA	1.07 ± 0.24	1.06 ± 0.20	1.05 ± 0.21	0.85	1.06 ± 0.24	1.06 ± 0.21	1.11 ± 0.16	0.44
Height (cm)	130.14 ± 8.77	130.76 ± 8.61	131.91 ± 7.37	0.62	130.14 ± 8.54	130.68 ± 9.01	132.58 ± 7.83	0.39
Weight (kg)	29.65 ± 7.63	31.03 ± 9.37	30.58 ± 7.97	0.59	29.85 ± 7.92	31.10 ± 9.71	30.59 ± 7.18	0.63
BMI (kg/m ²)	17.28 ± 2.89	17.90 ± 4.09	17.40 ± 3.59	0.53	17.40 ± 3.19	17.94 ± 4.18	17.23 ± 2.86	0.54
E2 (pmol/l)	27.72 ± 12.27	29.30 ± 12.21	26.70 ± 8.43	0.52	27.03 ± 11.82	29.83 ± 12.87	28.80 ± 10.27	0.34
FSH (U/l)	8.37 ± 6.81	7.78 ± 8.29	10.93 ± 8.23	0.16	8.66 ± 7.11	7.39 ± 8.38	10.16 ± 7.71	0.24
LH (U/l)	2.67 ± 2.67	2.31 ± 2.59	4.43 ± 3.65**	0.002*	2.79 ± 2.81	2.06 ± 2.41	4.20 ± 3.53	0.003*
Cholesterol (mg/dl)	173.73 ± 27.13	169.93 ± 31.36	172.96 ± 30.56	0.73	176.08 ± 27.68	169.38 ± 29.55	165.88 ± 29.16	0.21
Triglyceride (mg/dl)	106.23 ± 54.28	112.12 ± 68.86	110.61 ± 81.74	0.86	107.52 ± 64.79	114.45 ± 70.84	106.44 ± 60.48	0.78
Leptin (ng/ml)	5.67 ± 4.74	6.46 ± 5.60	5.81 ± 5.23	0.61	5.92 ± 5.17	6.71 ± 5.81	5.33 ± 3.85	0.44

Data were presented as mean ± SD, and compared using one-way ANOVA with Bonferroni multiple comparisons. BA/CA, bone age/chronological age; CPP, central precocious puberty; E2, estradiol; FSH, follicle-stimulating hormone; LH, luteinizing hormone.

**P* < 0.05, significantly different among three types of genotypes from one-way ANOVA.

***P* < adjusted significance level 0.001 as compared with genotype A/G+G/G.

and *LEPR* polymorphism Q223R (17,27). Higher leptin levels in the CPP group might also associate with SNPs in the *LEP* promoter (G-2548A) and/or the *LEPR* genes. SNP analysis of the CPP and control groups showed that the distribution of the A/A, A/G, and G/G alleles at *LEP* –2548, *LEPR* 109, and *LEPR* 223 were not significantly different. The two groups showed no distinct disparities in the frequencies of A/A and non-A/A (A/G, G/G) genotypes. The distribution of As and Gs at *LEP* –2548 also did not differ significantly between the two groups. These data suggest that disparities at each individual SNP were not sufficient to explain the higher leptin levels among girls with CPP.

Specific combinations of *LEP* and *LEPR* polymorphisms may associate with a certain abnormality of the human body. For example, the combination of *LEP* (G-2548A) and *LEPR* polymorphism Q223R significantly increased the risk for oral cancer (28). We subsequently investigated whether

various combinations of *LEP* –2548 plus *LEPR* 223 polymorphisms and combinations of *LEP* –2548 plus *LEPR* 109 polymorphisms were associated with CPP phenotype. We found no significant difference in demographics and pathologic features among the girls with CPP with different combinations of SNPs at *LEP* promoter –2548 plus *LEPR* 223 or *LEP* promoter –2548 plus *LEPR* 109. We then compared the demographics and pathologic features of CPP in both the CPP and control groups among three genotypes of *LEPR* 223 and three genotypes of *LEPR* 109. The results also showed that demographics and pathologic features among the girls with CPP were not significantly associated with any specific genotype of *LEPR* 223 or *LEPR* 109. These observations failed to support our original hypothesis. However, our findings were consistent with the reports of genome-wide association studies that there was a lack of association of age of menarche with SNPs in the *LEP* promoter or *LEPR* (19).

Table 5. Comparison of demographics and pathological features of CPP in both the CPP group and the control group among three genotypes of *LEPR 223* and three genotypes of *LEPR 109*

Variable	<i>LEPR 223</i>				<i>LEPR 109</i>			
	G/G	A/G	A/A	P value	G/G	A/G	A/A	P value
CPP group	(n = 187)	(n = 59)	(n = 3)		(n = 179)	(n = 64)	(n = 6)	
Chronological age (y)	8.46 ± 1.43	8.55 ± 1.10	8.71 ± 1.00	0.89	8.46 ± 1.43	8.52 ± 1.16	7.73 ± 3.24	0.44
Bone age (y)	10.41 ± 1.44	10.61 ± 1.30	11.50 ± 0.71	0.36	10.42 ± 1.43	10.51 ± 1.39	9.66 ± 3.77	0.42
BA/CA	1.23 ± 0.09	1.23 ± 0.07	1.32 ± 0.07	0.28	1.23 ± 0.1	1.22 ± 0.06	1.30 ± 0.16	0.14
Height (cm)	140.84 ± 10.37	140.44 ± 7.50	132.95 ± 17.61	0.52	140.78 ± 10.22	140.3 ± 8.36	131.8 ± 27.97	0.12
Weight (kg)	37.59 ± 9.48	37.01 ± 7.97	32.50 ± 13.44	0.68	37.51 ± 9.2	37.24 ± 8.85	31.88 ± 14.88	0.35
BMI (kg/m ²)	18.68 ± 2.95	18.61 ± 2.77	17.85 ± 2.84	0.92	18.67 ± 2.89	18.7 ± 2.87	17.43 ± 3.52	0.58
E2 (pmol/l)	33.65 ± 20.14	33.60 ± 12.85	47.35 ± 35.71	0.59	33.18 ± 19.49	35.08 ± 16.49	36.77 ± 18.4	0.73
FSH (U/l)	18.03 ± 9.64	17.77 ± 5.52	21.30 ± 10.47	0.85	18.18 ± 9.64	17.51 ± 6.17	22.69 ± 13.87	0.40
LH (U/l)	37.45 ± 29.95	40.99 ± 35.10	31.75 ± 27.08	0.72	37.39 ± 29.14	41.09 ± 36.97	29.28 ± 18.83	0.56
Cholesterol (mg/dl)	170.21 ± 29.78	169.66 ± 25.83	168.00 ± 19.80	0.99	171.09 ± 28.63	166.77 ± 28.16	177.6 ± 34.22	0.55
Triglyceride (mg/dl)	104.69 ± 57.54	112.12 ± 71.50	165.0 ± 114.55	0.32	105.1 ± 60	113.38 ± 68.44	100.6 ± 47.65	0.70
Leptin (ng/ml)	7.64 ± 5.57	7.68 ± 6.00	5.26 ± 4.06	0.84	7.62 ± 5.41	8.02 ± 6.41	2.92 ± 0.98	0.11
Control group	(n = 171)	(n = 46)	(n = 2)		(n = 161)	(n = 55)	(n = 3)	
Chronological age (y)	8.31 ± 1.35	8.13 ± 1.41	8.21 ± 0.53	0.73	8.28 ± 1.42	8.28 ± 1.20	7.75 ± 0.47	0.80
Bone age (y)	8.94 ± 2.09	8.67 ± 2.11	9.00 ± 1.41	0.74	8.83 ± 2.17	9.14 ± 1.80	7.00 ± 1.73	0.18
BA/CA	1.08 ± 0.22	1.07 ± 0.22	1.09 ± 0.10	0.99	1.07 ± 0.23	1.10 ± 0.16	0.91 ± 0.26	0.28
Height (cm)	130.66 ± 8.70	130.07 ± 9.54	131.2 ± 5.94	0.92	130.2 ± 8.97	131.6 ± 8.59	129.8 ± 5.7	0.61
Weight (kg)	30.51 ± 8.45	30.06 ± 8.61	32.50 ± 1.41	0.89	30.42 ± 8.68	30.39 ± 7.75	32.17 ± 9.57	0.94
BMI (kg/m ²)	17.65 ± 3.54	17.53 ± 3.67	18.90 ± 0.89	0.86	17.71 ± 3.66	17.34 ± 3.12	18.95 ± 5.01	0.66
E2 (pmol/l)	28.35 ± 11.94	28.20 ± 9.77	35.15 ± 17.89	0.71	1032.93 ± 441.29	1061.05 ± 364.98	1158.81 ± 531.40	0.81
FSH (U/l)	8.04 ± 7.47	11.46 ± 9.27	11.11 ± 14.14	0.03*	8.25 ± 7.79	10.55 ± 8.56	5.00 ± 6.07	0.13
LH (U/l)	2.45 ± 2.58	3.98 ± 3.47	2.61 ± 3.50	0.01*	2.45 ± 2.59	3.84 ± 3.35	1.07 ± 1.59	0.004*
Cholesterol (mg/dl)	171.05 ± 29.08	172.93 ± 28.50	149.50 ± 6.36	0.53	172.24 ± 28.34	168.94 ± 30.89	161.33 ± 18.88	0.66
Triglyceride (mg/dl)	108.45 ± 61.99	114.61 ± 78.28	140.00 ± 11.31	0.70	109.46 ± 5.75	111.62 ± 63.78	117.67 ± 43.32	0.96
Leptin (ng/ml)	6.18 ± 5.50	5.87 ± 4.96	10.38 ± 3.89	0.51	6.41 ± 5.75	5.36 ± 4.09	6.57 ± 4.61	0.47

Data were presented as mean ± SD, and compared using one-way ANOVA with Bonferroni multiple comparisons. BA/CA, bone age/chronological age; CPP, central precocious puberty; E2, estradiol; FSH, follicle-stimulating hormone; LH, luteinizing hormone.

* $P < 0.05$, significantly different among three types of genotypes from one-way ANOVA.

However, for the control girls, there were significant differences in mean LH levels among girls with different combinations of SNPs at the *LEP* promoter -2548 plus *LEPR 223* and the *LEP* promoter -2548 plus *LEPR 109*. Furthermore, there were significant differences in mean FSH and mean LH levels among the control girls with different genotypes at *LEPR 223*, and in mean LH levels among the control girls with different genotypes at *LEPR 109*. Because there were no differences in the leptin levels between the three genotypes, leptin might not directly affect the hormone levels in control girls. Instead, polymorphisms in the leptin receptor gene seem to affect critical elements in the FSH and LH synthesis pathway. These potential mechanisms warrant further study.

In conclusion, none of the alleles at the three SNPs in this study or combinations of two SNPs were significantly associated with demographics and pathologic features in girls with CPP. However, control group individuals with the genotype

LEP promoter -2548 A/A and *LEPR 223* A/G combination exhibited higher LH levels than individuals with *LEP* promoter -2548 A/A and *LEPR 223* G/G combination. Comparative analysis showed that the control group individuals with A/G at *LEPR 223* exhibited significantly higher peak LH and FSH levels and control group individuals with A/G at *LEPR 109* exhibited significantly higher peak LH levels. The associations between FSH and LH levels and SNPs at both *LEPR 223* and *LEPR 109* warrant further investigation in the general population.

METHODS

Subjects

A group of 249 girls with progressive breast development and early puberty (onset before 8 y of age) and a control group of 219 girls with mild breast development and pubertal onset after 8 y of age were recruited and closely followed up every 3 mo for up to 1 y. Physical examination was performed for all the girls. They had no genetic or systemic diseases, and thyroid and adrenal function was normal. CPP

and health status were defined on the basis of LH responses to GnRH stimulation. Girls were grouped with the healthy controls if peak LH levels were <10 mIU/ml. They were diagnosed with CPP if LH levels were >10 mIU/ml and the ratio of BA to chronological age (CA) (BA/CA) was >1.2, as previously described (29). The study protocol was approved by the Human Investigations Committee of the Hospital of Chung Shan Medical University, and written parental consent was obtained for each subject.

Blood and Data Collection

Eight-hour fasting blood specimens were collected for measurement of basal levels of FSH, LH, E₂, cholesterol, and leptin and for quantitation of stimulated levels of FSH, LH, and E₂. Because cholesterol is a precursor of estrogen and other hormones, its levels were compared between groups. Left-hand X-rays were performed, and BA was determined using the method of Greulich and Pyle (30). Three replicate measurements of standing height were made using a wall-mounted stadiometer. BMI was calculated by dividing body weight (kg) by the square of height in meters (m²). Tanner stage standards were used to assess breast and pubic hair development (31).

Immunoassays

Serum leptin levels were measured after acid extraction by using commercial radioimmunoassay kits (Diagnostic Systems Laboratories, Webster, Texas). Sensitivity for leptin was 0.01 ng/ml. Intra- and interassay coefficients of variation for leptin were 5.5% and 6.3%, respectively.

Serum E₂ levels were measured using a commercial radioimmunoassay kit (Diagnostic Systems Laboratories). Sensitivity was 2.2 pg/ml, and intra- and interassay coefficients of variations were 7.5% and 9.3%, respectively. Serum FSH and LH levels were measured by enzyme immunoassay (FSH: Abbott Laboratories, Rome, Italy; LH: Dade Behring, Milan, Italy). Sensitivity for both assays was 0.2 mIU/ml. Intra- and interassay coefficients of variations were 4.7% and 8.9%, respectively, for FSH, and 3.1% and 4.0%, respectively, for LH.

Cholesterol and TG levels in serum and plasma were quantitated using enzymatic color test and Olympus analyzers (AU600, Clare, Ireland). Sensitivity for cholesterol and TG levels were 3.0 mg/dl (0.07 mmol/l) and 2.44 mg/dl (0.057 mmol/l), respectively. Intra- and interassay coefficients of variations for cholesterol and TG levels were 0.91% and 1.06%, respectively.

Genomic DNA Extraction

Venous blood from each subject was drawn into Vacutainer tubes containing EDTA and stored at 4 °C. Genomic DNA was extracted by QIAamp DNA Mini Kits (Qiagen, Valencia, CA) according to the manufacturer's instructions. DNA was dissolved in Tris-EDTA buffer (10 mM Tris (pH 7.8) and 1 mM EDTA) and then quantitated by a measurement of optical density at 260 nm. The final preparation was stored at -20 °C and used as a template for PCR.

PCR-Restriction Fragment-Length Polymorphism

Gene polymorphisms at *LEP* G-2548A (rs7799039) and *LEPR* K109R (rs1137100) and Q223R (rs1137101) were determined by PCR-restriction fragment-length polymorphism assay as described previously (13,17). Primer sequences for analysis of leptin G-2548A genotype were 5'-CACAGACGGACTGGAAAG-3' (forward) and 5'-AAGCAAAGACAGGCATAA-3' (reverse) and yielded a product of 478 bp. PCR products were digested with *Clostridium formicoaceticum* into fragments of 298 and 180 bp (-2548GG homozygote) and 472 bp, 298 bp, and 180 bp (-2548GA heterozygote) and 472 bp (-2548AA homozygote). Primer sequences for analysis of leptin receptor K109R genotype were 5'-TTTCCACTGTTGCTTTTCGGA-3' (forward) and 5'-AAACTAAAGAATTTACTGTTGAAACAAATGGC-3' (reverse); they yielded a product of 101 bps. Primer sequences for analysis of leptin receptor Q223R genotype were 5'-ACCTTTAAGCTGGGTGTCCCAAATAG-3' (forward) and 5'-AGCTAGCAAATATTTTTGTAAGCAATT-3' (reverse); they yielded a product of 416 bp. PCR was performed in a 10- μ l volume containing 100 ng DNA template, 1.0 μ l of 10 \times PCR buffer (Invitrogen, Carlsbad, CA), 0.25 U of Taq DNA polymerase (Invitrogen), 0.2 mM

deoxyribonucleotide triphosphates (Promega, Madison, Wisconsin), and 200 nM of each primer (MDBio, Taipei, Taiwan). PCR cycling conditions were 5 min at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 57 °C, and 2 min at 72 °C, with a final step at 72 °C for 20 min to allow a complete extension of all PCR fragments. PCR products were digested using the restriction endonucleases HaeIII and MspI (New England Biolabs, Beverly, Massachusetts) for the K109R and Q223R polymorphisms, respectively. DNA fragments were electrophoresed on 3% agarose gels and genotyped.

Statistical Analysis

Statistical analyses were performed using SPSS 15.0 statistics software (SPSS, Chicago, Illinois). Demographic characteristics and clinical pathologic features were summarized as mean and SD for subjects grouped in the control and CPP groups with a two-sample *t* test. In addition, a Pearson correlation coefficient was used to assess the correlation of leptin levels with demographic characteristics, adjusting for BMI as a covariate. Genotype frequencies were tabulated as numbers of subjects grouped in control and CPP groups with χ^2 test. Fisher's exact test was used for cells with less than five cases. Odds ratios with 95% confidence intervals from multiple logistic regression models were calculated to identify the association of CPP with the combination of genotype frequencies of leptin -2548 and leptin receptor 223, or with the combination of genotype frequencies of leptin -2548 and leptin receptor 109. Demographics and clinical pathologic features were compared among different selections of genotypes from leptin -2548, and leptin receptors 223 and 109, by using one-way ANOVA. Statistical assessments were considered significant at a *P* value of <0.05. The Bonferroni method was performed for a *post hoc* paired-groups comparison. Because the high number of analyses (48 analyses) can increase the study-wide type I error rate, the Bonferroni method indicated that statistical significance be adjusted to 0.001 (0.05/48).

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