KISS1 gene polymorphism in Korean girls with central precocious puberty

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Directed by Professor Ho-Seong Kim

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This certifies that the Doctoral Dissertation of Young Jun Rhie is approved.

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To my father and mother who are always in my heart.

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Kisspeptin and G-protein coupled receptor-54 (GPR54) system is the essential gatekeeper of the reproductive system, playing a key role in the activation of the gonadotropic axis at puberty. Central precocious puberty (CPP) is caused by the premature activation of hypothalamic gonadotropin-releasing hormone secretion. Recently, an activationg mutation of *GPR54* gene was identified in a girl with CPP, implicating the kisspeptin system in the pathogenesis of sexual precocity. Because of the important function of kisspeptin in the regulation of puberty onset, alterations in *KISS1* gene might contribute to the pathogenesis of CPP, as well as *GPR54* gene. This study was aimed to evaluate the occurrence of sequence variations, including mutations and single-nucleotide polymorphisms (SNP) of *KISS1* gene and attempted to clarify the effect of each sequence variation that differed in frequencies between Korean girls with CPP and their controls by investigating serum kisspeptin levels. All coding exons of *KISS1* gene were sequenced in 143 Korean girls with CPP and 101 their controls. Serum kisspeptin levels of CPP (n=40) and control (n=40) groups were assayed with a competitive enzyme immunoassay. Nine polymorphisms

were identified in *KISS1* gene. A novel SNP, 55648176 *T/G* was identified for the first time. SNP 55648184 *C/G* and 55648186 *-/T* were detected more frequently in CPP group compared to control group. Moreover, subjects with these polymorphisms had higher serum kisspeptin levels than subjects lacking them. SNP 55648176 *T/G* was detected less frequently in CPP group compared to control group and subjects with these polymorphisms had lower serum kisspeptin levels than subjects lacking them. In total, 21 haplotypes were constructed based on the typing results. Haplotype *GGGC-ACCC* was detected less frequently in CPP group and seems to exert a protective effect on CPP. This study has found a novel SNP and identified three SNPs with significant differences in allele frequencies with the clinical significance of these polymorphisms. The association between SNPs and CPP should be validated by further evidence obtained from large-scaled studies. Efforts to identify other ligands activating GPR54 instead of kisspeptin are also needed.

Key words: *KISS1* gene, kisspeptin, G-protein coupled receptor-54, central precocious puberty, single nucleotide polymorphism

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I. INTRODUCTION

Puberty is a complex and coordinated biologic process of sexual development that lead to complete gonadal maturation and function, and attainment of reproductive capacity. Puberty can be influenced by genetic, nutritional, environmental and socioeconomic factors[1]. The activation of pulsatile gonadotropin-releasing hormone (GnRH) secretion from specialized hypothalamic neurons to stimulate hormonal cascades and gonadal activation is a key event in the onset of puberty[2]. But, the ultimate mechanisms underlying the increase in pulsatile GnRH secretion at puberty are yet to be fully elucidated[3].

Kisspeptin, the peptide product of *KISS1* gene and its putative receptor G protein-54 (GPR54) signaling complex has recently emerged as essential gatekeepers of pubertal activation of GnRH neurons and the reproductive axis and it has been the focus of intense study by investigators[4-8]. An increase in kisspeptin signaling which is caused by enhanced expression of *KISS1* and

GPR54 gene at the time of puberty contributes to, or even drives, the activation of the gonadotropic axis during pubertal development[9]. Kisspeptin is a powerful stimulus for GnRH-induced gonadotropin secretion and intermittent kisspeptin administration to immature animals was able to induce precocious activation of the gonadotropic axis and pubertal development[10]. Loss-of-function point mutations and deletions in *GPR54* gene have been identified in patients with familial or sporadic idiopathic hypogonadotropic hypogonadism (IHH)[4, 7, 8]. In addition, *GPR54*-knockout mice had a similar failure of sexual maturation[7, 11].

Precocious puberty is defined as the development of secondary sexual characteristics before the age of 8 years for girls and 9 years for boys[12]. Central precocious puberty (CPP) is defined if the process is driven by premature activation of hypothalamic GnRH secretion. Unlike IHH, CPP is the other extreme of pubertal development. As CPP may cause early epiphyseal maturation with compromised final height as well as psychological stress, early initiation of treatment is important to improve final height[13]. CPP has remarkable female gender predominance and most of CPP cases are idiopathic[1, 12]. However, it is known that genetic factors play a fundamental role in the timing of pubertal onset[14, 15]. Segregation analysis suggested autosomal dominant transmission with incomplete, sex-dependent penetrance[14]. Recently, an activating mutation of *GPR54* gene was identified in a girl with CPP, implicating the kisspeptin system in the pathogenesis of sexual precocity[16]. Because of the important function of kisspeptin in the regulation of puberty onset, alterations in *KISS1* gene might contribute to the pathogenesis of CPP, as well as *GPR54* gene. So far, few studies, especially in Korean population have previously described mutations or polymorphisms of *KISS1* gene[17-19]. Moreover, further evidence will be necessary to confirm the pathogenicity of these variations because no functional analyses were conducted in these studies.

KISS1 gene was first discovered in 1996, and has been mapped to the long arm of chromosome 1q32. This gene consists of three exons, of which only part of the second and third exons are finally translated into a 145-amino acid precursor peptide[20]. This precursor is then cleaved into three forms of kisspeptins containing 54, 14, and 13 amino acids. The three peptides exhibit the same affinity for the receptor since they share a common C-terminal decapeptide designated kisspeptin-10[5, 6].

In this study, I evaluated the occurrence of sequence variations, including mutations and single-nucleotide polymorphisms (SNPs) of *KISS1* gene and attempted to clarify the effect of each sequence variation that differed in frequencies between Korean girls with CPP and their controls by investigating serum kisspeptin levels.

II. MATERIALS AND METHODS

1. Subjects

Two groups of subjects were involved in this study. Korean girls with CPP (n=143) were recruited from Korea University Ansan Hospital in Gyeonggi-Do, Korea. CPP was diagnosed in accordance with the following criteria: (1) objective breast budding appeared before the age of 8 years, (2) advanced bone age at least 1 year ahead of their chronological age, (3) significantly higher peak luteinizing hormone (LH) values comparing with the cut-off value of 5 mIU/ml under the GnRH stimulation test conducted prior to the age of 9 years. CPP patients with identified etiology, such as brain tumor or cranial irradiation, were excluded. The pubertal stage of each participant was determined by a pediatric endocrinologist and rated according to the Tanner criteria. Bone age was determined by a single observer according to Greulich and Pyle method, expressed in years.

Control group (n=101) consists of healthy Korean girls who showed the breast development after the age of 9 years. They were recruited as volunteers.

The Institutional Review Board at Korea University Ansan Hospital approved the study. Written informed consents were obtained from all subjects or their parents.

2. Methods

(1) *KISS1* gene analysis

Genomic DNA was isolated from the peripheral blood leukocytes of the study subjects using a DNA isolation kit (QIAamp DNA Blood Maxi prep kit; Qiagne, Valencia, CA). All coding exons (exon 2 and 3) and the intronic flanking regions of *KISS1* gene were PCR amplified with four pairs of specific primers (Table 1). Amplifications were conducted over 35 cycles, and each cycle consisted of denaturation at 95˚C for 30 sec (exon 2) or 50 sec (exon 3), annealing at 50˚C (exon 2) or 63˚C (exon 3) for 30 sec (exon 2) or 50 sec (exon 3), and extension at 72˚C for 30 sec (exon 2) or 50 sec (exon 3). Additional extension at 72˚C for 10 min after last amplification cycle. PCR was performed in a reaction volume of 20 μl containing 100 ng of genomic DNA template, 1 μM of each primer, 10 mM of each dNTP, 25 mM MgCl2, 100 mM KCl, 20 mM Tris-HCl (pH 8.3), and 1 U of Taq DNA polymerase (Takara Bio Inc.; Shiga, Japan). After amplification, PCR mixtures were separated on 1.5% agarose gels with ethidium bromide to confirm the size and purity of the PCR products. Subsequently, DNA sequencing reactions were conducted using the same primer pairs and a BigDye Terminator V3.1 Cycle Sequencing kit (Applied Biosystems; Foster City, CA) in accordance with the manufacturer's instructions. The sequencing reaction mixtures were electrophoresed and analyzed using an ABI3130xl Genetic Analyzer (Applied Biosystems) and Sequencing Analysis v.5.2 software.

Table 1. Primers used in the analysis of *KISS1* gene

Primer	Forward (5' to 3')	Reverse (3' to 5')
Exon 2	CTCTACCAGGAGCCTCCAAAG	TGATCTTTCCTGTTTACCAGCC
Exon 3	ATGGGATGACAGGAGGTGTTG	ACCATCCATTGAGGATGGAAG

(2) GnRH stimulation test

The GnRH stimulation test was conducted to evaluate the pubertal status in all patients. Basal serum samples were obtained prior to GnRH injection, and poststimulation samples were acquired 30, 60, and 90 min after injection for measurements of LH and FSH (follicle-stimulating hormone) levels.

(3) Kisspeptin assay

Serum kisspeptin levels of CPP (n=40) and control (n=40) groups were assayed with a competitive enzyme immunoassay. Blood was collected into a lavender vacutainer tube containing EDTA at 4℃ (2.5 ml of blood for each subject). The blood was transferred from the lavender vacutainer tube to the centrifuge tube. The blood was centrifuged for 15 minutes at 4℃ and the plasma was collected. The plasma was kept at -70℃ until all samples were collected. The peptide was extracted using SEP-COLUMN (Strata, Phenomenex, Torrance, CA, USA) containing 200 mg of C18. The concentration of kisspeptin was measured using Kisspeptin-10 (Metastin (45-54)-Amide) and competitive enzyme immunoassay (Kit# EK-048-56, Phoenix Pharmaceuticals, Inc. Burlingame, CA, USA). The method was described in the product insert. Fifty-five microliters of plasma with 25 μl of primary antibody and 25 μl of biotinylated peptide were incubated at room temperature for 2 hours. Then, 0.1 ml of streptavidin-horseradish peroxidase solution was added. After incubation for 1 hour at room temperature, 0.1 ml of TMB substrate solution was added. One hour later for incubation at room temperature, the reaction was terminated with 0.1 ml of 2N HCl. After loading the immunoplate onto a microtiter plate reader, the absorbance optical density was read at 450 nm and the results were calculated. All measurements were in duplicates and average was calculated.

(4) Haplotype construction and statistical analysis

Haplotypes were estimated from genotype data for individual participants using PHASE program version 2.0. Deviations from Hardy-Weinberg equilibrium were tested by comparison of observed and expected genotype frequencies with permutation test. The SPSS 16.0 software package (SPSS Inc.; Chicago, IL, USA) was used to perform statistical analyses. Data are expressed as mean ± standard deviation (SD). Fisher's exact test and independent t- test were used for data analysis, and p-values of < 0.05 were considered statistically significant. The allele frequencies were compared between patient and control groups. When the significant difference of allele frequencies between patient and control groups was noted for each polymorphism, serum kisspeptin levels were compared between subjects harboring a certain polymorphism (homozygote or heterozygote) and those lacking that polymorphism. The odds ratio for SNP was also calculated with respective 95% confidence intervals.

III. RESULTS

1. Clinical characteristics and results of GnRH stimulation test in CPP patients

In patient group, the mean age was 8.30 ± 0.81 years. The mean bone age was 10.28 ± 1.12 years, and the mean discrepancy with the chronological age was 1.84 ± 1.48 years. The mean height and weight standard deviation score (SDS) were 1.11 ± 0.96 and 1.33 ± 1.30 respectively. The mean serum kisspeptin level was 4.61 ± 1.86 pmol/l. According to the results of the GnRH stimulation test, the basal and the peak LH values were 0.43 ± 0.73 mIU/l and 12.24 ± 10.50 mIU/ml, respectively. The basal and peak FSH values were 2.63 ± 1.11 mIU/ml and 13.77 ± 3.91 mIU/ml respectively, and the peak LH/FSH ratio was 0.93 ± 0.71. The baseline clinical characteristics and results of the GnRH stimulation tests in the patient group are summarized in Table 2.

Table 2. Clinical characteristics and the results of GnRH stimulation test in patient group

Parameters	Patients (n=143)
Age (year)	8.30 ± 0.81
Height (SDS)	1.11 ± 0.96
Weight (SDS)	1.33 ± 1.30
BMI (kg/m^2)	18.29 ± 2.50
BA (year)	10.28 ± 1.12
BA-CA (year)	1.84 ± 1.48
Kisspeptin (pmol/l)	4.61 ± 1.86
Basal LH (mIU/ml)	0.43 ± 0.73
Peak LH (mIU/ml)	12.24 ± 10.50
Basal FSH (mIU/ml)	2.63 ± 1.11
Peak FSH (mIU/ml)	13.77 ± 3.91
Peak LH/FSH ratio	0.93 ± 0.71

BA: bone age, CA: chronological age, BA-CA: bone advancement, SDS: standard deviation score, BMI: body mass index, LH: luteinizing hormone, FSH: follicle-stimulating hormone.

2. Identified polymorphisms in the *KISS1* gene analysis

Direct sequencing of *KISS1* gene revealed nine SNPs as listed in Table 3. Among the 9 polymorphisms detected in this study, 55648176 *T/G* was a novel polymorphism whereas the other eight have been previously reported. 55648343 *C/A* was previously identified in Chinese CPP patients in 2007[18] and Korean CPP patients in 2010[17], which was found to be nonsynonymous, leading to substitution of P110T. Additionally, there was another nonsynonymous SNP (55648429 *C/G*) leading to substitution of P81R. 55648386 *C/T* was initially identified Korean CPP patients in 2010[17], which was found to be synonymous. The novel SNP in this study, 55648196 *T/G* was located in untranslated region. SNP 55648184 *C/G*, 55648180 *G/A* and 55648186 *-/T* were also located in untranslated region.

Table 3. The *KISS1* gene polymorphisms identified by sequencing

No	Position[1]	Allele	Location	dbSNP ID	Frequency in sample	Note
1	55648429	*C/G*	Exon 3	rs4889	0.545/0.455	p. P81R
2	55648254	*A/-*	Exon 3	rs71745629	0.553/0.447	p.X139fx
3	55648386	*C/T*	Exon 3	-	0.988/0.012	Synonymous
4	55648343	*C/A*	Exon 3	-	0.941/0.059	p.P110T
5	55648184	*C/G*	Exon 3	rs1132506	0.494/0.506	Untranslated
6	55648181	*G/C/A*	Exon 3	rs1132514	0.969/0.031	Untranslated
7	55648176	*T/G*	Exon 3	-	0.963/0.037	Novel, Untranslated
8	55648180	*G/A*	Exon 3	rs112442813	0.969/0.031	Untranslated
9	55648186	*-/T*	Exon 3	rs35128240	0.514/0.486	Untranslated

[1]The positions of polymorphisms are defined according to contig NT_004487.19.

3. Comparison of allele and genotype frequencies between CPP and control groups

Allele and genotype counts and frequencies in the two groups are shown in Table 4. Using Fisher's exact test, the associations between the polymorphisms and the two phenotypes were evaluated. 55648184 *C/G*, 55648176 *T/G* and 55648186 *-/T* had showed different allele frequencies between CPP and control groups. No significant differences in the frequencies of the other six polymorphisms were noted to exist between the two groups.

55648184 *C/G* was detected more frequently in CPP group compared to control group (p=0.017). Using *C* allele as the reference, the odds ratio of *G* allele for CPP was 1.567 (1.091-2.252) with respective 95% confidence intervals. 55648186 *-/T* was also detected more frequently in CPP group compared to control group (p=0.044). Using - allele as the reference, the odds ratio of Ins *T* allele for CPP was 1.458 (1.015-2.095) with respective 95% confidence intervals. 55648176 *T/G* was detected less frequently in CPP group compared to control group (p=0.030). Using *T* allele as the reference, the odds ratio of *G* allele for CPP was 0.339 (0.125-0.920) with respective 95% confidence intervals.

Table 4. Allele and genotype frequencies of *KISS1* polymorphisms

Polymorphism	Group	Allele counts (frequency)				Genotype counts (frequency)						P-value*
		1		2		11		12		22		
55648429 *C/G* *C*=1, *G*=2	Patient	146	0.510	140	0.490	36	0.252	74	0.517	33	0.231	0.079
	Control	120	0.594	82	0.406	39	0.386	42	0.416	20	0.198	
55648254 *A/-* *A*=1, Del *A*=2	Patient	150	0.524	136	0.476	36	0.252	78	0.545	29	0.203	0.140
	Control	120	0.594	82	0.406	39	0.386	42	0.416	20	0.198	
55648386 *C/T* *C*=1, *T*=2	Patient	282	0.986	4	0.014	139	0.972	4	0.028	0	0.000	0.999
	Control	200	0.990	2	0.010	99	0.980	2	0.020	0	0.000	
55648343 *C/A* *C*=1, *A*=2	Patient	266	0.930	20	0.070	123	0.860	20	0.140	0	0.000	0.331
	Control	193	0.955	9	0.045	92	0.911	9	0.089	0	0.000	
55648184 *C/G* C=1, *G*=2	Patient	128	0.448	158	0.552	28	0.196	72	0.503	43	0.301	0.017
	Control	113	0.559	89	0.441	35	0.347	43	0.426	23	0.228	
55648181 *G/C/A* *G*=1, *C* or *A*=2	Patient	277	0.969	9	0.031	134	0.937	9	0.063	0	0.000	0.999
	Control	196	0.970	6	0.030	95	0.941	6	0.059	0	0.000	
55648176 *T/G* *T*=1, *G*=2	Patient	280	0.979	6	0.021	137	0.958	6	0.042	0	0.000	0.030
	Control	190	0.941	12	0.059	89	0.881	12	0.119	0	0.000	
55648180 *G/A* *G*=1, *A*=2	Patient	277	0.969	9	0.031	134	0.937	9	0.063	0	0.000	0.999
	Control	196	0.970	6	0.030	95	0.941	6	0.059	0	0.000	
55648186 *-/T* Wild=1, Ins *T*=2	Patient	136	0.476	150	0.524	36	0.252	64	0.448	43	0.301	0.044
	Control	115	0.569	87	0.431	39	0.386	37	0.366	25	0.248	

*Comparision of the allele frequencies between the patient and the control groups.

4. Comparison of haplotype frequencies between CPP and control groups

In total, 21 haplotypes were constructed based on the typing results and their relationships with CPP were also investigated (Table 5). Haplotype *GGGC-ACCC* was detected less frequently in CPP group compared to control group (p=0.024). The odds ratio of haplotype *GGGC-ACCC* for CPP was 0.309 (0.106-0.903) with respective 95% confidence intervals. None of the other haplotypes exhibited significant correlation.

Table 5. Haplotype analysis

Haplotypes[1]	Patients		Controls		p-value
	Counts	Frequencies	Counts	Frequencies	
TGGGT-CCG	132	0.462	76	0.376	0.061
TGGC-ACCC	103	0.360	87	0.431	0.115
TGGC-AACC	14	0.049	8	0.040	0.665
GGGC-ACCC	5	0.018	11	0.055	0.024
TACG-ACCC	7	0.025	6	0.030	0.724
TGGG-ACCC	4	0.014	1	0.005	0.409
TGGCTACCC	2	0.007	3	0.015	0.653
TGGGT-CTG	3	0.011	2	0.010	0.999
TGGGTACCG	4	0.014	1	0.005	0.409
TGGGTACCC	3	0.011	1	0.005	0.646
TGGCTAACC	2	0.007	1	0.005	0.999
TGGCT-CCG	0	0.000	2	0.010	0.171
TGGGTAACC	2	0.007	0	0.000	0.514
TGGGT-CCC	0	0.000	1	0.005	0.414
TACG--CCC	1	0.004	0	0.000	0.999
GGGC--CCC	0	0.000	1	0.005	0.414
TGGCTAACG	1	0.004	0	0.000	0.999
TGGG-ACCG	0	0.000	1	0.005	0.414
TGGGTAATC	1	0.004	0	0.000	0.999
TACC-ACCC	1	0.004	0	0.000	0.999
GGGG-ACCC	1	0.004	0	0.000	0.999

[1]The haplotypes are designated according to the sequence of each SNP position as follows: 55648176 *T/G* - 55648180 *G/A* - 55648181 *G/C/A* - 55648184 *C/G* - 55648186 *-/T* - 55648254 *A/-* - 55648343 *C/A* - 55648386 *C/T* - 55648429 *C/G*

5. Comparison of serum kisspeptin levels

In, SNP 55648184 *C/G,* serum kisspeptin levels of subjects with *C/G* or *G/G* (n=45) were higher than those of subjects with *C/C* (n=35) (4.20 ± 1.94 *vs.* 2.91 ± 0.98 pmol/l, p<0.001) (Figure 1.).

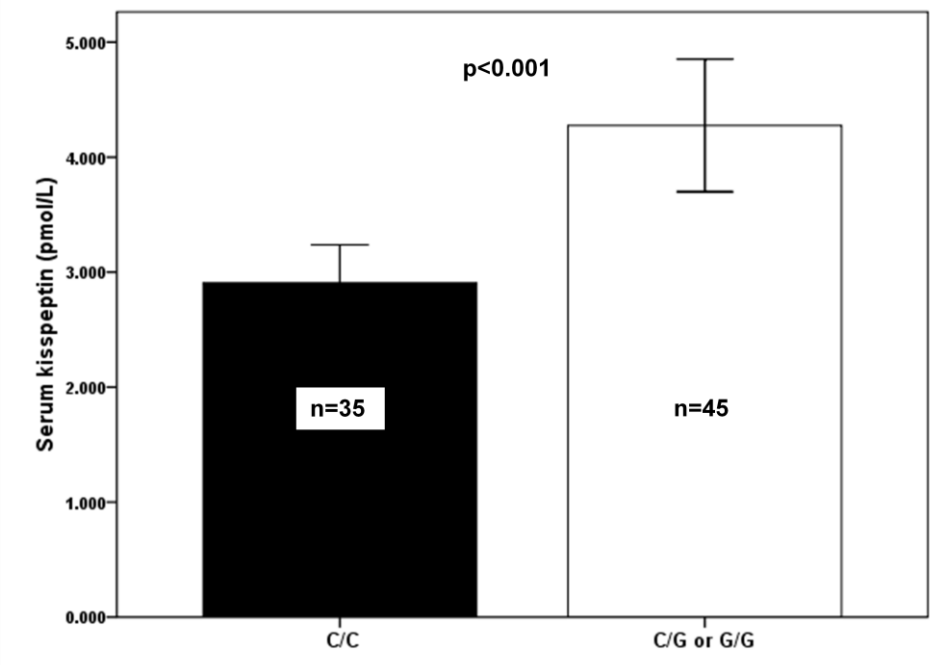

Figure 1. Comparison of serum kisspeptin levels between subjects with 55648184 *C/C* and subjects with 55648184 *C/G* or *G/G*. Serum kisspeptin levels of subjects with *C/G* or *G/G* were higher than those of subjects with *C/C* (4.28 ± 1.94 *vs.* 2.91 ± 0.98 pmol/l, p<0.001).

In SNP 55648186 *-/T*, serum kisspeptin levels of subjects with *T/T* or *-/T* (n=40) were higher than those of subjects with -/- (n=40) (4.21 ± 1.94 *vs.* 3.14 ± 1.29 pmol/l, p=0.005) (Figure 2.).

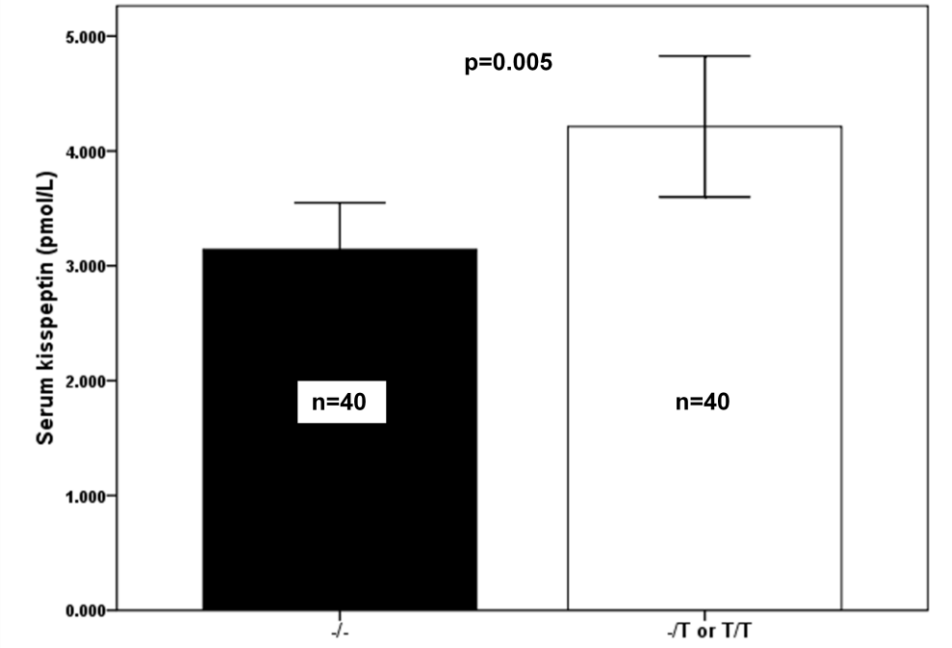

Figure 2. Comparison of serum kisspeptin levels between subjects with 55648186 -/- and subjects with 55648186 *-/T* or *T/T*. Serum kisspeptin levels of subjects with *-/T* or *T/T* were higher than those of subjects with -/- (4.21 ± 1.94 *vs.* 3.14 ± 1.29 pmol/l, p=0.005).

In SNP 55648176 *T/G*, serum kisspeptin levels of subjects with *T/T* (n=71) were higher than those of subjects with *T/G* or *G/G* (n=9) (3.86 ± 1.73 *vs.* 2.20 ± 0.65 pmol/l, p<0.001) (Figure 3.).

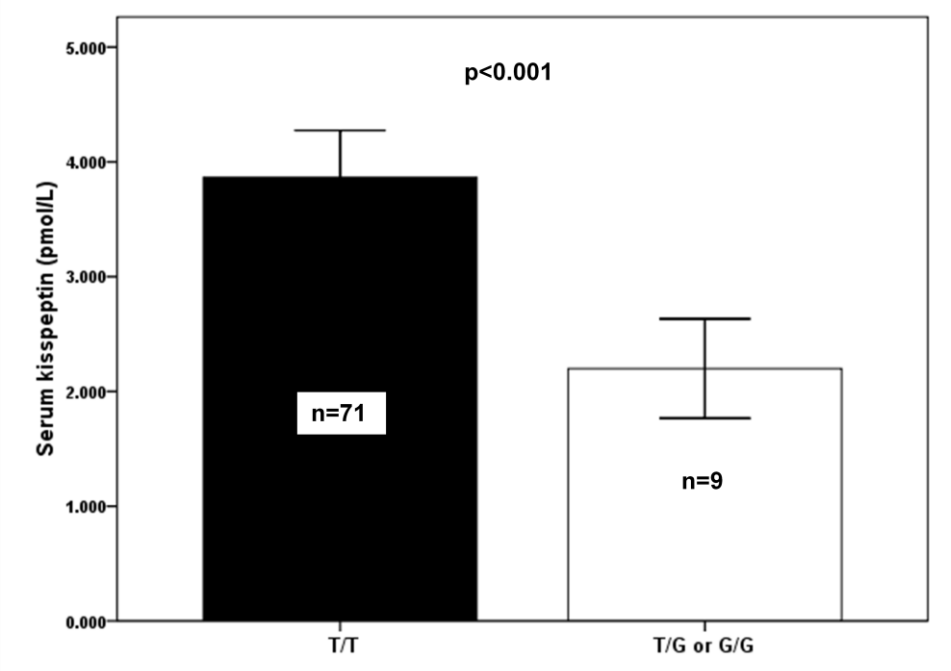

Figure 3. Comparison of serum kisspeptin levels between subjects with 55648176 *T/T* and subjects with 55648176 *T/G* or *G/G*. Serum kisspeptin levels of subjects with *T/T* were higher than those of subjects with *T/G* or *G/G* (3.86 ± 1.73 *vs.* 2.20 ± 0.65 pmol/l, p<0.001).

IV. DISCUSSION

In 2003, kisspeptin was initially demonstrated to perform a function in the reproductive axis, wherein mutations in the *GPR54* gene result in idiopathic hypogonadotrophic hypogonadism[4, 7]. These mutations suggested that kisspeptin and its receptor GPR54 are the crucial regulators of puberty and the hypothalamus-pituitary-gonadal axis. Since 2003, further mutations were found in *GPR54* gene and their functions demonstrated to cause idiopathic hypogonadotrophic hypogonadism[8, 21, 22], and an activating mutation leading to CPP[16]. However, no definite causative mutation has been detected in another promising candidate gene, *KISS1*, in humans with idiopathic hypogonadotrophic hypogonadism or CPP. In this study, the nine SNPs were detected by the sequencing of *KISS1* gene, and the frequencies of each SNP were calculated and compared between CPP and control groups; each polymorphism was also genotyped.

According to the calculated statistical results, SNP 55648184 *C/G*, 55648186 *-/T* and a novel SNP, 55648176 *T/G* were found to be statistically significant. Although they were detected in both CPP and control groups, the allele frequencies of these groups differed statistically. SNP 55648184 *C/G* and 55648186 *-/T* were detected more frequently in CPP group and SNP 55648176 *T/G* was detected less frequently in CPP group compared to control group.

In my previous study[23], it was demonstrated that serum kisspeptin level was significantly higher in girls with CPP than in their age-matched prepubertal controls (4.61 ± 1.78 vs. 2.15 ± 1.52 pmol/l, $P<0.001$) in accordance with the first report on kisspeptin serum level in prepubertal girls and in girls with CPP in 2009[24]. In this study, when significant difference of allele frequencies between CPP and control groups was noted for each polymorphism, serum kisspeptin levels were compared between subjects harboring a certain polymorphism (homozygote or heterozygote) and those lacking that polymorphism in order to evaluate the clinical significance of each

polymorphism. In two SNPs (55648184 *C/G* and 55648186 *-/T*) which were detected more frequently in CPP group, serum kisspeptin levels of subjects with the polymorphism were higher than those of subjects lacking that polymorphism. In SNP 55648176 *T/G* which was detected less frequently in CPP group, serum kisspeptin levels of subjects with the polymorphism were lower than those of subjects lacking that polymorphism.

All of these SNPs are located at the untranslated region and they are close to each other in distance. Untranslated region can contain elements for controlling gene expression. It is assumed that these SNPs are contained in the regulatory sequences that affect the transcription of the rest of the DNA. The change of DNA sequence by the polymorphisms would alter the function of the regulatory sequences. Subsequently, the production of kisspeptin had been altered.

SNP 55648343 *C/A* was first reported in a study of Chinese CPP patients in 2007[18], and they demonstrated that p.P110T was an infrequent polymorphism with an allele frequency of 0.057 in all sequenced subjects. This allele frequency was quite similar to that of Korean population assessed in 2010, in which the allele frequency was calculated as 0.046[17]. Moreover, p.P110T was detected statistically significantly more commonly in controls than in the CPP patients in both two studies[17, 18]. It has been suggested that p.P110T may exert a protective effect on pubertal precocity[17, 18]. However, these findings have yet to be confirmed by functional studies and more evidences are required. In this study, the allele frequency was 0.059 similar to those of the previous studies but no significant differences in the frequencies were noted to exist between the two groups (p=0.331). This SNP seems not to have a critical effect on the activity of kisspeptins.

Nonsynonymous SNP 55648429 *C/G* was not found to be statistically associated with CPP (p=0.079). The previous association studies in Korean and Chinese population also found no relations to CPP[17, 18]. This polymorphism introduced a substitution occurred at the 81st site from proline to arginine, which was observed in kisspeptin-54 but not in the other three forms kisspeptins (kisspeptin-14,13 and 10) (Figure 4.). Therefore, the amino acid change by SNP 55648429 *C/G* would not seriously influence the bioactivity of kisspeptins. This supposition was supported by the results of a previous study showing that kisspeptin-54 was unstable and could readily degrade into kisspeptin-14, 13 and 10[5]. Thus, kisspeptin-14, 13 and 10 might be more important than kisspeptin-54.

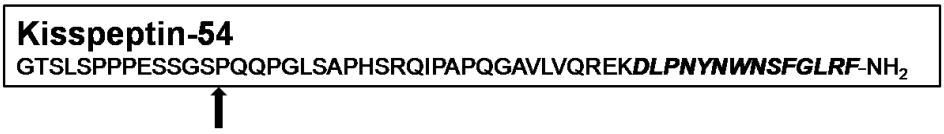

Figure 4. The amino acid sequence of kisspeptin-54. The predicted peptide of kisspeptin-14 is in italicized and bold letters. The 81st amino acid changed by SNP 55648429 *C/G* is marked with an arrow.

SNP 55648254 *A/-* leads to the frameshift mutation from the 139th site of KISS1 protein. But, there were no significant differences in the allele frequencies between CPP and control groups (p=0.140). Considering that 139th amino acid is not contained by the three natural *in vivo* forms of kisspeptin, kisspeptin-54, 14, and 13, this frameshift mutation seems not to be able to alter the function of kisspeptins (Figure 5.).

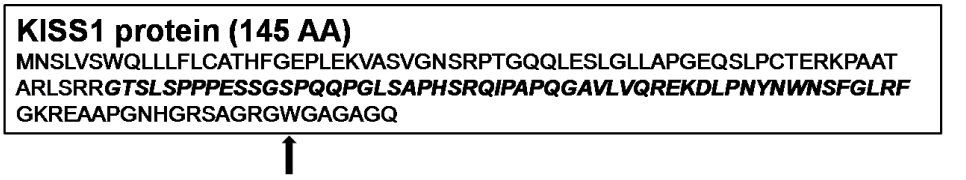

Figure 5. The amino acid sequence of KISS1 protein. The predicted peptide of kisspeptin-54 is in italicized and bold letters. The 139th amino acid is marked with an arrow.

The association study of haplotypes with CPP revealed that haplotype *GGGC-ACCC* was detected less frequently in CPP patients than controls (p=0.024). These findings suggest that haplotype *GGGC-ACCC* exerts a protective effect on CPP. Haplotype *GGGC-ACCC* is a combination of *G* allele of SNP 55648176 *T/G*, *C* allele of SNP 55648184 *C/G*, wild type allele of SNP 55648186 *-/T* and wild type alleles of the other SNPs. According to the genotyping results, *G* allele of SNP 55648176 *T/G*, *C* allele of SNP 55648184 *C/G* and wild type allele of SNP 55648186 *-/T* are detected less frequently in CPP group compared to controls. Therefore, the results of haplotype analysis suggest that the SNPs with significance do not have an effect on CPP separately but work in combination with one another. As mentioned above, SNP 55648176 *T/G*, 55648184 *C/G* and 55648186 *-/T* are located at the untranslated region and they are close to each other in distance. Untranslated region can contain the regulatory sequences for controlling gene expression. Accordingly, it is assumed that these SNPs work together to affect the transcription of the rest of the DNA.

In the previous Chinese study[18], eight SNPs were detected in *KISS1* gene, and only two SNPs (55648429 *C/G* and 55648343 *C/A*) were identified to be shared with this Korean study. But, six SNPs in exon 3 of this study were shared with the previous Korean study[17]. Especially, SNP 55648386 *C/T* was a novel SNP. The genetic background of these two ethnic groups appeared to differ profoundly, even though Koreans and Chinese are both ethnically Asians.

In this study, on the contrary to the previous reports, SNP 55648184 *C/G* and 55648186 *-/T* were identified to be a meaningful polymorphism and SNP 55648343 *C/A* showed no significant differences in allele frequency. From these contradictory results, it may be assumed that other ligands may be capable of compensating for kisspeptins and activating GPR54. Recently, *KISS1* knockout mouse models have been developed and have been shown to

evidence, to varying degrees, characteristics of idiopathic hypogonadotrophic hypogonadism[25, 26], whereas *GPR54* knockout mice consistently evidence characteristics of hypogonadotrophic hypogonadism[7, 26]. The less complete phenotype detected in the *KISS1* knockout mice supports this assumption.

A possible limitation of the present study was that the sample scale was relatively small. Therefore, the association between SNPs and CPP should be validated by further evidence obtained from large-scaled studies. Efforts to identify other ligands activating GPR54 instead of kisspeptin are also needed.

V. CONCLUSION

In brief, this study tried to identify the sequence variations and haplotypes of *KISS1* gene associated with CPP and also attempted to clarify the effect of each sequence variation by investigating serum kisspeptin levels in Korean girls. A novel SNP, 55648176 *T/G* was identified for the first time. Three SNPs 55648184 *C/G*, 55648186 *-/T* and 55648176 *T/G* were identified with significant differences in allele frequencies between CPP and control groups. The clinical significance of these polymorphisms was confirmed by showing significant difference in serum kisspeptin levels between subjects with these polymorphisms and subjects lacking them. Haplotype *GGGC-ACCC* was identified to exert a protective effect on CPP.

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<ABSTRACT (IN KOREAN)>

중추성 성조숙증을 가진 한국 여아에서 *KISS1* 유전자 다형성

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키스펩틴과 G단백질공역수용체-54는 사춘기 때 생식샘 자극축의 활성화에 중요한 역할을 한다. 중추성 성조숙증은 시상하부의 생식샘 자극 호르몬 방출 호르몬 분비의 조기 활성화에 의하여 발생한다. 최근 연구에서 중추성 성조숙증 여아에서 *G단백질공역수용체-54* 유전자의 기능획득 돌연변이가 발견되었다. 이것은 중추성 성조숙증의 발병에 키스펩틴 - G단백질공역수용체-54 체계가 관여함을 의미한다. *KISS1* 유전자도 중추성 성조숙증의 발병에 *G단백질공역수용체-54* 유전자처럼 기여할 것으로 생각된다. 본 연구의 목적은 중추성 성조숙증을 가지고 있는 한국 여아와 대조군에서 *KISS1* 유전자의 돌연변이와 단일 유전자 변이를 포함한 변이를 찾고 혈중 키스펩틴 농도를 측정함으로써 환자군과 대조군에서의 유전자 변이의 발생 빈도 차이의 효과를 밝히는 것이다. 중추성 성조숙증을 가진 143명의 한국인 여아와 101명의 대조군에서 *KISS1* 유전자의 엑손의 염기 서열을 분석하였고 각각 40명의

환자군과 대조군에서 혈중 키스펩틴 농도를 측정하였다. 모두 9개의 단일 유전자 변이가 발견되었다. 단일 유전자 변이 55648176 T/G는 본 연구에서 처음으로 발견되었다. 단일 유전자 변이 55658184 *C/G*와 55648186 *-/T*는 환자군에서의 발견 빈도가 대조군에서 보다 높았고, 유전자 변이를 가지고 있는 대상이 없는 대상보다 혈중 키스펩틴 농도가 높았다. 단일 유전자 변이 55648176 *T/G*는 환자군에서의 발견 빈도가 대조군에서 보다 낮았으며, 유전자변이를 가지고 있는 대상이 없는 대상보다 혈중 키스펩틴 농도가 낮았다. 유전자 분석 자료를 기초로 모두 21개의 단상형(haplotype)을 구성하였다. 단상형 *GGGC-ACCC*는 환자군보다 대조군에서 더 높은 빈도로 발견되었고, 중추성 성조숙증에 보호 작용을 하는 것으로 보인다. 본 연구에서 저자는 한 개의 새로운 단일 유전자 변이를 발견하였고 환자군과 대조군에서 대립 유전자의 발견 빈도가 다른 세 개의 단일 유전자 변이를 밝혀냈다. 이와 같은 단일 유전자 변이와 중추성 성조숙증과의 연관성을 입증하기 위해서는 보다 많은 대상으로 한 연구가 필요하며, 아울러 키스펩틴을 대신하는 G단백질공역수용체-54의 새로운 리간드를 찾는 연구도 함께 필요하다.

핵심되는 말: *KISS1* 유전자, 키스펩틴, G단백수용체-54, 중추성 성조숙증, 단일 유전자 변이