CLINICAL STUDY

Association study of the polymorphisms in the *KISS1* gene with central precocious puberty in Chinese girls

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Abstract

Objective: The kisspeptin/GPR54 pathway has been proven to be crucial in the process of puberty onset, yet the polymorphisms in the *KISS1* gene and their relationships with central precocious puberty (CPP) have not been investigated. This study was performed to reveal the relationship between the gene and the disease.

Design and Methods: 272 Chinese Han girls diagnosed to be CPP patients were recruited as Case Group I, 43 unrelated African women as Case Group II, and 288 unrelated normal Chinese Han girls as Control Group. Polymorphism scans of the *KISS1* gene were performed for the first time by bidirectional resequencing of the whole gene in a subset of the patients, and then by ligase detection reaction some of the polymorphisms identified were typed in the two groups and the respective haplotypes were constructed. The relationships of the typed polymorphisms and the haplotypes with CPP were evaluated by an association study between genotypes and phenotypes.

Results: By resequencing, eight polymorphisms were identified, five of which were typed forming 18 haplotypes. Although one novel nonsynonymous single nucleotide polymorphism substituting one amino acid in kisspeptin (P110T) was found to be statistically related to the disease ($P=0.025$), no further supporting evidence has yet been found. The other polymorphisms and all the haplotypes were not found to be related.

Conclusion: The polymorphism scanning and typing of *KISS1* uncovered several potentially meaningful polymorphisms, but the conclusion was not solid and further studies are necessary for function validation of these polymorphisms.

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Introduction

When kisspeptin was first identified in 1996 (1), it was mainly considered as a tumor metastasis suppressor. Since it was found to be the ligand of orphan G-protein coupled receptor GPR54 in 2001 (2), researchers came to notice the possible role of kisspeptin/GPR54 signal pathway in the regulation of endocrine function, which was proved by recent studies to demonstrate that kisspeptin would directly activate the onset of puberty in mammals via GPR54 by stimulating the secretion of gonadotropin-releasing hormone (GnRH; (3–5)).

The onset timing of puberty varies depending on numerous variables. Generally, it would be defined as precocious puberty if any signs of secondary sexual maturation appears at an age more than 2 standard deviation units below the population mean (<8 for girls and <9 for boys in China), and would be further defined as central precocious puberty (CPP) if the process is driven by activation of hypothalamic GnRH secretion.

There have been reports indicating that CPP patients would be shorter in stature when fully grown and more susceptible to breast cancer (6). The incidence of CPP in Chinese children was about 1% according to epidemiologic data in 2003 (7). Some studies have identified several associated genes or polymorphisms involved in development, body composition, gonadal steroid metabolism, and many other biological processes (8–10), yet the exact mechanism remains a mystery. The important function of kisspeptin in puberty onset makes it necessary to investigate the polymorphisms in the gene and their relationships with CPP.

The *KISS1* gene consists of three exons, of which only part of the second and third exons are finally translated into a 145-amino acid protein with a putative signal sequence, from which three forms of kisspeptins containing 54, 14, and 13 amino acids are derived (Fig. 1). The three peptides exhibit the same affinity for the receptor since they share a common C-terminal

KISS1 Protein (145 AA)

MNSLVSWQLLLFLCATHFGEPLEKVASVGNSRPTGQQLESGLLAPGEQSLPCTER**K**PAAT
 ARLS**RR**GTSLSPPP**ESS**GS**P**QQPGLSAPH**S**RQIPAPQGA**V**LVQRE**K**DL**P**NYN**W**NSFGLRF
 GK**R**EAAPGNHGR**S**AGRGWGAGAGQ

Kisspeptin-54

GTSLSPPP**ESS**GS**P**QQPGLSAPH**S**RQIPAPQGA**V**LVQRE**K**DL**P**NYN**W**NSFGLRF-NH₂

Kisspeptin-14

DL**P**NYN**W**NSFGLRF-NH₂

Kisspeptin-13

L**P**NYN**W**NSFGLRF-NH₂

Kisspeptin-10

YN**W**NSFGLRF-NH₂

Figure 1 Amino acid sequence of the KISS1 product and derived peptides. The predicted signal peptide of KISS1 is in italicized and bold letters. Dibasic motifs representing potential cleavage sites by furins and prohormone convertases are underlined and in bold letters. The two amino acids changed by 54650055 G/T and 54650141 G/C are marked within rectangles.

decapeptide designated kisspeptin-10, which is the minimum sequence necessary for receptor activation.

In this study, the three exons, exon-intron boundaries, and 1.4 kb of 5' flanking region of the *KISS1* gene were amplified and sequenced in a cohort of 36 Chinese CPP girls, by which the mechanism of how the kisspeptin/GPR54 system functions in the onset of puberty may be better understood.

Material and methods

Experimental subjects

Three groups of subjects were involved in this study. Case Group I consisted of 272 Chinese sporadic CPP girls recruited from three hospitals in Shanghai and Harbin, China; Case Group II consisted of 43 normal African adult female subjects; and Control Group consisted of 288 independent normal Chinese Han girls recruited from East China University of Science and Technology. The study protocols were reviewed and approved by the Ethics Committees for Human Research of the hospitals and the college, and informed consent was obtained from all the subjects or their guardians.

The CPP patients were all diagnosed according to the same criteria and at diagnosis they were between 5.9 and 8.5 years of age. The patients all showed dramatic growth acceleration, with Tanner breast stage scores ≥ 2 based on standardized Tanner breast stage assessment. Bone ages were measured and data of 215 subjects (79.04%) were found to be at least 1 year ahead of their true ages, and a few of the patients even had vaginal bleeding. A GnRH stimulation test was carried out in all subjects to confirm that the hypothalamic-pituitary-gonadal axis had been activated, by which significantly high luteinizing hormone (LH) peak values (23.2 ± 12.7 mIU/ml) were observed after 40 min of the injection, when

compared with the cut-off value of 15 mIU/ml (11), and the LH/follicle-stimulating hormone (FSH) ratios were all above 0.66 (12). To exclude any possible brain abnormalities, magnetic resonance imaging was performed and no brain diseases were found. Most of the parents of the patients were questioned about their pubertal ages, yet none of them recalled precocious puberty in childhood.

The DNA samples of the African subjects were obtained from the Chinese National Human Genome Center at Shanghai. The 43 subjects were all normal African women with a mean age of 36.8 ± 12.5 years. The puberty onset timing of the subjects was not obtained.

The control subjects were selected from female volunteers who were first grade students in East China University of Science and Technology based on a freewill questionnaire regarding their age of menarche. The mean age of the control subjects was 18.24 ± 1.38 and the mean age of menarche was above 12 years.

In total, 36 CPP subjects were randomly selected from Case Group I for sequencing.

Peripheral blood samples were collected from all subjects and genome DNA samples were isolated with phenol-chloroform method.

Sequencing

Six pairs of overlapping primers covering all the sequenced regions were designed and synthesized (Sangon, Shanghai, China), with which PCR was performed in a 20 μ l system by 35 cycles of amplification. The products were purified for bidirectional sequencing on an ABI 3100 sequencer (Applied Biosystems, Foster City, CA, USA).

Genotyping

Typing of the selected single nucleotide polymorphisms (SNPs) was performed with a method developed by

ligase detection reaction (LDR; (13)), the mechanism of which is shown in Fig. 2. Multiplex PCR was performed to amplify four fragments containing the five SNPs with HotStarTaq polymerase (Qiagen Inc.) in a volume of 20 μ l by 40 cycles of amplification.

The PCR products were used as templates for LDR. For each polymorphism, one universal fluorescence-labeled probe and two allele-specific probes of different lengths were designed according to the flanking sequence. The LDR was carried out in a volume of 20 μ l containing probes, PCR products and thermostable *Taq* DNA ligase (New England Biolabs, Ipswich, MA, USA) according to the instructions. Recognizing respective alleles, the probes would be joined by ligase into corresponding products distinguishable on ABI 3100 sequencer. The electrophoresis results were analyzed by GeneMapper ID software version 3.2 (Applied Biosystems). Primers and probes were also synthesized by Sangon, and oligo sequences and detailed reaction conditions can be supplied on request.

Deviations from the Hardy–Weinberg equilibrium were tested by comparison of observed and expected genotype frequencies with χ^2 test.

Haplotype construction and statistical analysis

Haplotypes were estimated from genotype data for individual participants using the PHASE program version 2.0 (14).

The comparison of the allele frequencies among the three groups was performed by Fisher's exact test. The SPSS 11.5 software package (SPSS Inc., Chicago, IL, USA) was used to perform statistical analysis with $P < 0.05$ as the minimal level of statistical significance.

Results

Sequencing of the *KISS1* gene revealed eight SNPs as listed in Table 1, among which four were identified for

Figure 2 Flow chart of genotyping method based on ligase detection reaction. A sample which was wild-type homozygous at 54 650 055 and heterozygous at 54 650 141 was selected to explain the mechanism of the genotyping method. The trace map at the bottom was generated with the software GeneMapper ID version 3.2.

Table 1 Single nucleotide polymorphisms (SNPs) identified by sequencing.

No.	Position ^a	Allele	Location	dbSNP ID	Frequency in sequenced samples	Typed	Note
1	54 656 335	A/G	5' flanking region	/	0.897/0.103	Yes	
2	54 656 397	C/T	5' flanking region	rs3924 586	0.651/0.349	Yes	
3	54 656 436	G/A	5' flanking region	rs3924 585	0.939/0.061	No	
4	54 656 861	A/T	5' flanking region	/	0.983/0.017	No	
5	54 655 907	C/T	Exon 1	/	0.944/0.056	No	Untranslated
6	54 655 965	A/—	Exon 1	rs5780 218	0.5/0.5	Yes	Untranslated
7	54 650 055	G/T	Exon 3	/	0.943/0.057	Yes	AA change: P110T
8	54 650 141	G/C	Exon 3	rs4889	0.542/0.458	Yes	AA change: P81R

^aThe positions of the SNPs were defined according to contig NT_004487.18.

the first time (sequencing traces shown in Fig. 3). Two SNPs (54 650 055 G/T and 54 650 141 G/C) were found to be nonsynonymous, leading to substitution of P110T and P81R respectively.

Five SNPs of relatively high heterozygosity were selected and genotyped in the three groups, and the results fitted the expectation given by the Hardy–Weinberg equilibrium. Allele counts and frequencies are shown in Table 2a. With Fisher’s exact test, the relationships between the polymorphisms and the phenotypes were evaluated, among which the nonsynonymous polymorphism 54 650 055 G/T showed statistical significance with CPP ($P=0.025$), while the other four SNPs showed none.

In total 18 haplotypes were constructed in Case Group I and Control Group based on the typing data, and their relationships with CPP were also investigated (Table 2b), yet none of them showed significant correlation.

Discussion

GPR54 remained an orphan receptor until three independent groups (2, 15, 16) identified its natural ligand kisspeptin. Numerous studies on various species including mice, rats, sheep, primates and humans had reported that the kisspeptin/GPR54 pathway would stimulate the secretion of GnRH and start the onset of puberty (3, 17–19). Several mutations were found in the GPR54 gene and their functions demonstrated, yet no such studies had been carried out on the KISS1 gene. This study was the first time that the polymorphisms in KISS1 and their relationships with CPP were investigated in Chinese girls.

Whole gene resequencing was carried out in part of the CPP patients to screen polymorphisms, after which the frequencies of the eight SNPs found in the sequencing were calculated in the sequenced cohort, and five were considered informative due to their

Figure 3 Sequencing results of novel polymorphisms. Typical sequencing traces of the four novel single nucleotide polymorphism are shown, wild-type homozygous subjects on the top and heterozygous genotype on the bottom. The arrows indicate the positions of polymorphisms.

Table 2 Genotyping results and haplotype analysis.

		Allele counts (frequencies)				Genotype counts (frequencies)						P-value	
SNP	Group	1		2		11		12		22		Case I – Control	Case II – Control
(a) Genotyping results													
54 656 335 A/G A=1, G=2	Case I	519	0.954	25	0.046	247	0.908	25	0.092	0	0	0.660	0.251
	Case II	80	0.930	6	0.070	37	0.860	6	0.140	0	0		
	Control	553	0.960	23	0.040	265	0.920	23	0.080	0	0		
54 656 397 C/T C=1; T=2	Case I	379	0.697	165	0.303	138	0.507	103	0.379	31	0.114	0.209	0.796
	Case II	61	0.709	25	0.291	22	0.512	17	0.395	4	0.093		
	Control	421	0.731	155	0.269	152	0.528	117	0.406	19	0.066		
54 655 965 A/– A=1, Del=2	Case I	261	0.480	283	0.520	57	0.210	147	0.540	68	0.250	0.187	0.417
	Case II	42	0.488	44	0.512	9	0.209	24	0.558	10	0.233		
	Control	253	0.439	323	0.561	57	0.198	139	0.483	92	0.319		
54 650 055 G/T G=1, T=2	Case I	523	0.961	21	0.039	251	0.923	21	0.077	0	0	0.025	0.006
	Case II	86	1	0	0	43	1	0	0	0	0		
	Control	536	0.931	40	0.069	248	0.861	40	0.139	0	0		
54 650 141 G/C G=1; C=2	Case I	316	0.581	228	0.419	91	0.335	134	0.493	47	0.173	0.856	0.640
	Case II	48	0.558	38	0.442	13	0.302	22	0.512	8	0.186		
	Control	338	0.587	238	0.413	101	0.351	136	0.472	51	0.177		
		Case I				Control							
Haplotype	Count	Frequency		Count		Frequency				Total		P-value	
(b) Haplotype analysis													
AC–GC	172	0.3162		178		0.3090				350		0.797	
AC–GG	57	0.1048		81		0.1406				138		0.070	
AC–TC	1	0.0018		1		0.0017				2		1.000	
AC–TG	2	0.0037		7		0.0122				9		0.180	
ACAGC	9	0.0165		18		0.0313				27		0.122	
ACAGG	111	0.2040		103		0.1788				214		0.288	
ACATC	0	0		4		0.0069				4		0.125	
ACATG	17	0.0313		24		0.0417				41		0.427	
AT–GC	20	0.0368		17		0.0295				37		0.509	
AT–GG	11	0.0202		20		0.0347				31		0.149	
ATAGC	24	0.0441		16		0.0278				40		0.150	
ATAGG	95	0.1746		84		0.1458				179		0.193	
GC–GC	1	0.0018		0		0				1		0.486	
GC–GG	4	0.0074		1		0.0017				5		0.205	
GCAGG	4	0.0074		0		0				4		0.055	
GCATC	1	0.0018		4		0.0069				5		0.375	
GT–GC	0	0		1		0.0017				1		1.000	
GT–GG	15	0.0276		17		0.0295				32		0.860	

relatively high degree of heterozygosity and were selected as 'tagging' SNPs for genotyping. Though the frequency of 54 656 436 G/A was a little higher than that of 54 650 055 G/T in the sequenced cohort, the latter was selected for typing since it was an amino acid substituting polymorphism.

According to the statistical results calculated with the typing data, the nonsynonymous SNP 54 650 055 G/T substituting the 110th amino acid of *KISS1* protein from proline to threonine was found to be statistically related to CPP. Though the P110T site was contained by all the three natural *in vivo* forms of kisspeptin, kisspeptin-54, 14, and 13 (Fig. 1), the association could not be validated without further support from functional data, especially considering that the sample scale was relatively small and that a *P*-value of 0.025 was not significant enough.

A different ethnic group was involved for investigation to provide more solid evidence. From the aspect of human evolution and migration, delayed puberty onset would be observed as humans moved to colder regions, as described in a recent review (20) which showed that African–American children had earlier pubertal milestones than white children, with the data of Mexican–American children in between. The facts can be explained by the assumption that individuals tend to delay the onset of puberty when facing tougher environments – less food and lower temperature. Forty-three healthy African female adults were then selected as Case Group II despite the lack of their pubertal data. The five tagging SNPs were also typed with the same method in all the subjects, and the results (also shown in Table 2) showed that four SNPs were of similar frequencies as observed in Chinese subjects, except 54 650 055 G/T,

which was not found in any of the African subjects. The *P*-value between Case Group II and Control Group was 0.006. Based on the data, it may be reasonable to hypothesize that the mutation could in some degree protect the carrier from earlier puberty onset.

The other nonsynonymous SNP did not exhibit significant relationship either between Case Group I and Control Group, or between Case Group II and Control Group. The polymorphism introduced a substitution at the 81st site from proline to arginine, which was included by kisspeptin-54 but not by the other three forms. From the result, it may be assumed that kisspeptin-14 and 13 are possibly more important than kisspeptin-54 in humans; thus, the amino acid change would not seriously affect the bioactivity, supported by a previous report that kisspeptin-54 was unstable and would easily degrade into the other two forms (2).

The association study of haplotypes constructed with the five SNPs typed showed no significant relationship between the 18 haplotypes, which could be partially attributed to the relatively small scale of population typed, causing the correlated alleles to disperse into different haplotypes. Owing to this, it was more difficult to confirm the relationship between the haplotypes and the disease.

In brief, this study tried to reveal the relationships between the polymorphisms and haplotypes in *KISS1* gene and CPP in Chinese girls. The results would deepen our understanding in both the *KISS1* gene and the mechanism of the disease. Yet, the limit of association study calls for more research in the future to confirm the actual function of the polymorphism.

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