

## Association of single nucleotide polymorphisms in estrogen receptor 1 gene with the risk of idiopathic short stature.

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### Abstract

Estrogen is a hormone in hypothalamic-pituitary-gonadal (HPG) axis that plays an important role in the regulation of bone maturation and closure of the growth plate. The aim of this study was to investigate the association between single nucleotide polymorphisms (SNPs) in estrogen receptor 1 (*ESR1*) gene and susceptibility to idiopathic short stature (ISS) in the Chinese population. Six SNPs of the *ESR1* gene (*rs6557177*, *rs1884049*, *rs3020429*, *rs3798575*, *rs3778090*, *rs3798757*) were genotyped and we found a significant positive association between *rs6557177* genotype and susceptibility to ISS compare to control group in preliminary study (ISS=200, control=220,  $\chi^2=6.262$ ,  $P=0.044$ ). Validation of *rs6557177* was conducted by further expanding the sample size to 400 ISS patients and 380 control subjects genotyped by SNaPshot Multiplex System. The frequencies of the *rs6557177* CC genotype and C allele were observed to increased in ISS group compared with the control group ( $\chi^2=9.913$ ,  $P=0.007$ ;  $\chi^2=5.57$ ,  $P=0.018$  OR: 1.502; 95% CI: 1.07-2.108 respectively). However, no significant correlation was observed between the *rs6557177* and clinical characteristics of the study participants. We demonstrate for the first time that the presence of a C allele or CC genotype at *rs6557177* of *ESR1* constitutes risk factor for developing ISS in Chinese children.

**Keywords:** Estrogen receptor 1, Idiopathic short stature, Single nucleotide polymorphisms.

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### Introduction

Short stature is one of the most popular endocrine disease presenting to pediatric endocrinologists. The prevalence of the short stature around the world is estimated to be 3-5% [1]. The characterization of idiopathic short stature (ISS) is in which the height of a patient is less than -2SDS below the equivalent mean height for the same age, sex, and population group without knowing diseases, and almost 60-80% of short stature fitting the classification of ISS [2]. A typical human linear growth has four major stages: fetal, infancy, childhood, and pubertal growth. Previous reports have shown that hypothalamic-pituitary-gonadal (HPG) axis and growth hormone-Insulin-like growth factor (IGF) axis is the most vital hormonal axis governing growth [3]. Any level of abnormality of GH-IGF axis may cause short stature [4], such as GHR [5], IGF-1R [6,7], IGFALS [8] were associated with ISS, but fewer studies have investigated the association of HPG axis with the risk of ISS.

Estrogens are important endocrine regulators both involved in GH-IGF axis, and gonadotropic axis [9]. Many studies had implied that *ESR1* plays a central role of estrogen actions in bone, such as skeletal growth in the growth spurt, epiphyseal fusion during puberty [10-12]. Genome-wide linkage analysis of large samples has shown strong evidence of the association of *ESR1* gene with stature [13]. A mutation of the *ESR1* gene was previously described as a woman who showed growth retardation with no pubertal growth spurt and breast development [14]. However, there is no study has investigated the correlation of the *ESR1* gene with the risk of ISS.

Therefore, we designed a case-control study consisting of 400 children with ISS and 380 normal children and analyzed the SNPs of *ESR1* gene to determine the correlation of *ESR1* gene alleles and frequencies, and analyzed the clinical characteristics of these patients. Our study findings would pave the way for future research on the genes responsible for increased susceptibility to ISS.

## Materials and Methods

### Subjects

The case-control study design was adopted in this experiment. A total of 780 samples were recruited from unrelated individuals of the southern Chinese Han population. This study was approved by the Ethics Committee of Jiangxi Children's Hospital and written informed consent was obtained from all participants' guardians.

### ISS Group

A total of four hundred children who were clinically diagnosed with ISS between 2008 and 2016 at Jiangxi Provincial Children's Hospital agreed to participate in this study. The mean age of case group was  $9.52 \pm 2.20$  years. None of the children had yet entered puberty, and the heights of their parents were all within the normal range. Familial short stature and physical, developmental delay of adolescence were excluded for all subjects.

The inclusion criteria [15] were as follows: i) height less than 2 SD of the mean height of children of the same age and sex [16], ii) at least one GH peak value  $>10$  ng/mL in the GH provocation test, iii) normal routine blood, thyroid function, liver, and kidney function, iv) birth weight and length within the normal range, and v) no other inherited metabolic diseases, chromosomal abnormalities, congenital skeletal abnormalities, or chronic diseases.

### Control group

Three hundred and eighty normal children who came for regular physical examinations in our hospital during the same period were selected as control subjects. The mean age of control was  $9.3 \pm 2.04$  years. The inclusion criteria were as follows: (i) height within  $\pm 2$  SDs of the mean height of the normal children of the same sex and (ii) BMI within the normal range. Children whose heights were between  $\pm 2$  SD of the height of other children of the same sex, age and those

whose body mass index (BMI) was within the range of 17-23 were included in this group.

### Genomic DNA extraction

Two milliliters fasting venous blood was drawn from the ISS and control group subjects, EDTA-2K anti-coagulated, and genomic DNA was extracted according to the routine phenol-chloroform-isoamyl alcohol method, which was stored at  $-20^{\circ}\text{C}$  until use.

### SNP selection and genotyping

The *ESR1* gene covers 295.7 kb of genomic sequence on chromosome 6. The practical SNP data was from the International HapMap Project ([www.hapmap.org](http://www.hapmap.org)) and Tagger program to select a subset of tag SNPs based on linkage disequilibrium (LD) patterns ( $r^2 > 0.8$ ) across *ESR1* gene region, and used the HapMap CHB subset for our tag SNPs selection (the minor allele frequency  $> 0.05$ ) [17]. Six tag SNPs (*rs6557177*, *rs1884049*, *rs3020429*, *rs3798575*, *rs3778090*, *rs3798757*) were dressing by screening and ordered for genetic variations in the *ESR1*. Genotyping of *ESR1* gene was performed by using the SNaPshot Multiplex System (ABI 3730 sequencer, Applied Biosystems, Forster City, CA, USA). After designing primers of different lengths, the SNaPshot reaction was performed targeting different mutational sites.

The SNaPshot polymerase chain reaction (PCR) cycle conditions were:  $96^{\circ}\text{C}$  for 10 s, followed by 25 cycles of  $96^{\circ}\text{C}$  for 10 s,  $50^{\circ}\text{C}$  ( $53^{\circ}\text{C}$ ) for 5 s, and  $60^{\circ}\text{C}$  for 30 s, and finally  $60^{\circ}\text{C}$  for 30 s and reducing to  $4^{\circ}\text{C}$ . The total volume of PCR amplification was 5  $\mu\text{L}$  containing 1.3  $\mu\text{L}$  dH<sub>2</sub>O, 1.2  $\mu\text{L}$  Pooled Primers, 2  $\mu\text{L}$  Pooled PCR Products and 0.5  $\mu\text{L}$  Reaction Mix. A pair of primers were designed (forward: 5'-actcagatattcgtgtcctt-3' and reverse: 5'-ttggatacaaccttgaatgc-3') to amplify a 251 bp fragment from *ESR1* that contained *rs6557177*. The sequencing result of *rs6557177* locus was analyzed using GeneMapper Software Version 4.0 (Applied Biosystems, Forster City, CA, USA).

**Table 1.** Genotypic and allelic association analyses of *ESR1* SNPs in Preliminary experiment.

SNPs ID	ISS (n=200)	Control (n=220)	OR (95%CI)	P value	Genotype	ISS (n=200)	Control (n=220)	P value
<i>rs6557177</i>					CC	8	2	0.044*
C	64	46	1	0.017*	CT	48	42	
T	336	394	1.631 (1.087-2.448)		TT	144	176	
<i>rs1884049</i>					CC	5	5	0.195
C	65	55	1	0.121	CT	55	45	
T	335	385	1.358 (0.922-2.002)		TT	140	170	
<i>rs3020429</i>					CC	4	1	0.388
C	40	38	1	0.496	CT	32	36	

T	360	402	1.175 (0.737-1.874)	TT	164	183	
<i>rs3798575</i>				GG	6	12	0.194
G	78	109	1	0.067	GA	66	85
A	322	331	0.736 (0.53-1.022)	AA	128	123	
<i>rs3798757</i>				GG	3	6	0.198
G	64	91	1	0.081	GA	58	79
A	336	351	0.731 (0.513-1.040)	AA	139	136	
<i>rs3778090</i>				CC	31	33	0.812
C	158	166	1	0.598	CT	96	100
T	242	274	1.078 (0.816-1.423)	TT	73	87	

OR: Odds Ratio; 95% CI: 95% Confidence Interval; \*P<0.05

**Statistical analysis**

Height, IGF-1, IGF-binding protein 3 (BP3) [18] and E2 were measured, and genetic height was used as the reference unit to calculate the SDS. The SDS of IGF-1=(actual IGF-1-the mean IGF-1 at the same age)/standard deviation of IGF-1 at the same age.

The differences in alleles, genotypes and dominant and recessive modes were assessed by the chi-square test between

ISS patients and controls with the odds ratio (OR) and the 95% confidence interval (95%). The Student t-test or the rank sum test was adopted to analyze the association between genotype and clinical parameters. Statistically, significant differences were indicated when P<0.05. All analyses were performed with SPSS17.0.

**Table 2.** Chi-square test for *rs6557177* locus of the *ESR1* gene.

SNP	ISS (n=400)		Control (n=380)		$\chi^2$	P	OR	95% CI	Boferroni
	N	%	N	%					
CC	260	65	205	54	9.913	0.007*			0.013*
CT	124	31	156	41					
TT	16	4	19	5					
Allele	648	81	562	74	5.570	0.018*	1.502	1.00 (1.070-2.108)	0.023*
C	12	19	198	26					
T									
Dominant	384	96	361	95	0.730	0.393	1.351	1.00	0.497
CC+CT	16	4	19	5	9.888	0.002*	1.585	(0.676-2.699)	0.012*
TT	260	65	205	54				1.00	
Recessive	140	35	175	46				(1.189-2.114)	
CC									
CT+TT									

OR: Odds Ratio; 95% CI: 95% Confidence Interval; \*P<0.05.

**Results**

**Hardy-Weinberg equilibrium and chi-squared test in Preliminary experiment**

Genotype distributions of these six *ESR1* polymorphisms were in Hardy-Weinberg equilibrium in both patients (n=200) and controls (n=220) (P>0.05), Analysis at the screening stage of the 200 ISS patients and 220 control subjects revealed that

*rs6557177* was significantly correlated to ISS ( $\chi^2=6.262$ , P=0.044) showed in Table 1. Targeting this locus, validation was conducted by further expanding the sample size to 400 ISS patients and 380 control subjects. The validation revealed no statistical significance in the differences in the distribution frequencies of the alleles, the distribution frequencies of

genotypes, or case-control associations of genotype in the dominant and recessive modes.

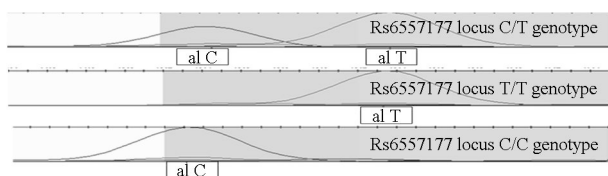
**Table 3.** Descriptive clinical and laboratory data of studied cases and controls.

Clinical parameter	ISS	Control
gender		
male	208	200
female	192	180
Age (years)	9.52 ± 2.20	9.3 ± 2.04
HtSDS	-3.15 ± 1.01	1.20 ± 1.03
weightSDS	-1.62 ± 1.33	1.33 ± 1.47
BMI (kg/m <sup>2</sup> )	15.77 ± 4.24	20.73 ± 4.24
IGF-1SDS	-1.48 ± 1.65	1.22 ± 1.70
IGFBP3SDS	-1.08 ± 1.60	1.25 ± 1.17
TH (cm)	163.78 ± 5.77	172.86 ± 4.16
THSDS	-1.37 ± 1.03	1.82 ± 2.11
E2 (pg/ml)	15.79 ± 4.26	16.79 ± 2.28

Abbreviations: SDS: Standard Deviation Score; HtSDS: Height Standard Deviation Integral Value; E2: Estradiol; IGF-1: Insulin-Like Growth Factor 1; IGFBP3: Insulin-Like Growth Factor Binding Protein; TH: Target Height; BMI: Body Mass Index.

### SNP locus genotyping

The SNP sequencing results at the *rs6557177* locus of the *ESR1* gene were analyzed using GeneMapper4.0 and are shown in Figure 1.



**Figure 1.** SNP locus genotyping; C/T, T/T and C/C genotype results for different samples at the *rs6557177* locus of the *ESR1* gene.

### Association study of *rs6557177* with ISS

The results of the *rs6557177* analysis showed a statistically significant difference between ISS and control groups in the C/T allele distribution frequencies ( $\chi^2=5.570$ ,  $P=0.018$ ,  $OR=1.502$ ,  $95\% CI=1.070-2.108$ ) and the genotype distribution frequencies also showed significantly difference between ISS and control groups ( $\chi^2=9.913$ ,  $P=0.007$ ), whereas the C recessive mode did show statistical significance ( $\chi^2=9.888$ ,  $P=0.002$ ,  $OR=1.585$ ,  $95\% CI=1.189-2.114$ ). After Bonferroni correction for multiple tests, the difference in C/T alleles, genotype and recessive mode in ISS patients remained statistically significant, which suggested that the C allele, genotype and the C recessive mode of *rs6557177* were relevant to susceptibility to ISS (Table 2).

### Comparison of clinical characteristics under *rs6557177* C Recessive mode

The clinical characteristics of the participants, including height, body weight, BMI, and levels of IGF-1, IGFBP3 were summarized (Table 3). The differences in HtSDS, BMI, IGF-1 SDS, IGF-BP3 SDS, and TH SDS of the CC genotype compared to (CT+TT) genotype at loci *rs1976667* of ISS were not statistically significant ( $P>0.05$ ) (Table 4).

**Table 4.** Correlation analysis of Clinical Characteristics Under *rs6557177* C Recessive mode (CC/CT+TT).

	CC(n=400)	CT+TT(n=380)	F/t-value	P
HtSDS	-3.22 ± 1.182	-3.11 ± 0.987	-1.012	0.312
BMI	15.300 ± 2.648	15.54 ± 2.343	-0.729	0.466
IGF-1 SDS	-1.58 ± 1.808	-1.53 ± 1.520	1.148	0.252
IGF-BP3 SDS	-1.12 ± 1.591	-0.98 ± 1.532	-1.264	0.207
TH SDS	-2.27 ± 4.414	-1.42 ± 0.938	-1.839	0.067
E2(pg/ml)	20.38 ± 16.3	23.20 ± 17.491	-1.181	0.248

CC/CT+TT: CC Genotypes Compared With CT+TT Genotype; HtSDS: Height Standard Deviation Integral Value; E2: Estradiol; IGF-1: Insulin-Like Growth Factor 1; IGFBP3: Insulin-Like Growth Factor Binding Protein

### Discussion

The heritability of height is estimated to be 80% [13,19], although 180 loci were confirmed by recent GWAS study, only explain for approximately 10% of the stature variation [20]. According to large longitudinal cohorts of height studies results, SNPs plays a critical role in childhood growth [21] which implies the complicated genetics of short stature. ISS is thought to be a polygenic disease, both genetic and environmental factors contribute to its pathogenesis, but genetic factors play a more significant part in the development of ISS [22]. Previous reports have shown that mutations in the genes along the GH-IGF axis and genes that regulate the development of the cartilage growth plate are involved in the development of ISS [4,23,24]. At present, studies are focused on the GH-IGF-1 axis genes, but from GWAS results, most genes and biological pathways associated with height were located in growth plate [21], such as BMP2, SOX8, WNT4, et al. To reduce the expensive burden of growth hormone therapy, it's profitable to reveal the etiology of ISS.

The *ESR1* gene is located at position 6q25.1, which includes eight exons, seven introns. Obviously, Estrogen take a vital part in the HPG axis in the regulation of bone maturation and closure of the growth plate [11], and Estrogen signaling participated in longitudinal bone growth and for bone remodeling via ER $\alpha$ . Genome-wide linkage analysis of large samples has shown strong evidence of the association of *ESR1* gene with stature [13]. Schuit et al. [25] studied the polymorphisms in the *ESR1* gene and reported that ER $\alpha$  affected adult height in normal individuals. Dahlgren et al. [26] reported that *rs2179922* which was in intron 4 of *ESR1* showed

an association with male stature in two Swedish population cohorts. Previous reports have also revealed that SNPs of *ESR1* could influence the onset of menstruation in girls, and body weight, fat accumulation, and BMI in children and adolescents [27,28]. Furthermore, *ESR1* polymorphism is associated with osteoporosis [29]. Kang et al. [30] showed that in boys, *ESR1* engaged in constitutional delay of growth and puberty. And Hero et al. [31] observed that a two-year long treatment that used a potent aromatase in boys with ISS has significantly improved the predicted adult height.

The absence of *ESR1* in mice caused insulin resistance and glucose intolerance in both gender. Multicystic ovaries without corpora lutea, hemorrhagic and hypoplastic uteri were presented in a female *ESR1* knockout mice; however, the male *ESR1* knockout mice showed obesity and reduced fertility [32]. Until now, there were only two individuals were reported *ESR1* mutations. The first was a woman who showed growth retardation, with no pubertal growth spurt, no breast development, and no insulin resistance [14]. In contrast, the other case, who was a man with *ESR1* mutation, showed average height, with continued linear growth until adulthood, osteoporosis, delayed epiphyseal closure, and insulin resistance [33].

The frequencies of the *rs6557177* CC genotype and C allele were observed to increased in ISS group compared with the control group ( $\chi^2=9.913$ ,  $P=0.007$ ;  $\chi^2=5.57$ ,  $P=0.018$  OR: 1.502; 95% CI: 1.07-2.108 respectively). Carrying the CC genotype significantly influenced the genetic susceptibility to ISS ( $P=0.018$ , OR=1.502, 95% CI 1.070-2.108), which was most likely a moderate risk factor ( $1<OR<2$ ), and presented a C recessive genetic mode of inheritance. These results indicated that ISS carrying the CC genotype at the *rs6557177* locus were probably more likely to develop ISS. However, no significant correlation was observed between the *rs6557177* and clinical features of the study participants, other clinical features related to *ESR1* gene maybe need to confirm.

The *rs6557177* locus is in the intron of the *ESR1* gene. There is no study has investigated the structure and function of the encoded protein at this locus to date. Our study found that the *rs6557177* locus of the *ESR1* gene might be relevant to the genetic susceptibility to ISS. Currently, there are no studies on the encoded proteins at the *rs6557177*, and thus further study on the relationship between structure and function is required to clarify the association of the *rs6557177* with genetic susceptibility to ISS.

In conclusion, our study revealed that polymorphism of *rs6557177* in *ESR1* gene might be linked with ISS risk and provided new insights into the mechanism underlying the pathogenesis of ISS. Moreover, our data would serve as a theoretical basis for developing novel methods for treatment of ISS based on gene mutations by regulating the transcription level of *ESR1* gene.

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