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Estrogen Receptors (ESRs) Mutations in Adolescent Idiopathic Scoliosis: A Cross-Sectional Study

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Background: Adolescent idiopathic scoliosis (AIS) is the most common spinal deformity, but its etiology is unclear. Multiple genetic mutations have been reported to be associated with AIS.

Material/Methods: We enrolled a cohort of 113 surgically treated AIS patients with available parental subjects from the Peking Union Medical College Hospital. We performed whole-exome sequencing in 10 trio families and whole-genome sequencing in 103 singleton patients. Luciferase assay was used to detect the functional alterations of candidate *ESR1* and *ESR2* variants.

Results: Using a *de novo* strategy, a missense variant in *ESR1* (c.868A>G) was selected as a candidate gene for AIS. The main Cobb angle of this patient was 41° (T6–T10). Another potential pathogenic variant in *ESR2* (c.236T>C) was identified. The main curve of the patient was 45° at T10–L3. The transactivation capacities of the mutated *ESR1* and *ESR2* protein were both significantly decreased ($p=0.026$ and 0.014 , respectively).

Conclusions: Potential pathogenic variants in *ESR1* and *ESR2* were identified in 113 AIS patients, suggesting that genetic mutations in *ESR1/2* were associated with the risk of AIS.

MeSH Keywords: **Estrogen Receptor alpha • Estrogen Receptor beta • Mutation, Missense • Scoliosis**

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Background

Scoliosis is a three-dimensional deformity with a curvature of the spine that is 10 degrees or greater on a coronal radiographic image using Cobb angle measurement [1]. Adolescent idiopathic scoliosis (AIS) is the most common type of scoliosis, affecting adolescents from 10 to 18 years of age [2]. The global incidence of AIS is approximately 1% to 3% [3,4]. AIS is much more common in females, and the reported female-to-male ratio is 4: 1 [1]. This ratio surges to 8: 1 for a subset of the affected IS population whose Cobb angle is more than 30° [5]. Scoliosis has a negative impact on a patient's appearance, induces low back pain, neurological symptoms, impaired cardiopulmonary function and low quality of life [6].

Although the mechanism underlying the pathogenesis of AIS is still unclear the active role played by genetic factors has been widely reported. Previous studies have identified several susceptible genes, such as *PAX1* [7,8], *GPR126* [3,9], *LBX1* [10], *BCN2* [11] and *CALM1* [12]. Because the prevalence of AIS is much higher in females, an estrogen receptor (ESR)-related etiology of AIS has been reported in the past [13,14]. Although several studies have found that *ESR1* polymorphisms are correlated with the development of scoliosis [15,16], few rare deleterious variants has been linked to AIS. Rapidly advancing whole-exome sequencing (WES) and whole-genome sequencing (WGS) have made investigations of potential disease-causing variants much more efficient.

In the present study, we conducted WES in 10 trios and WGS in 103 sporadic AIS patients. Genetic mutations of *ESR1* and *ESR2* were detected in 2 AIS patients, and a functional analysis of the potential candidates was also conducted.

Material and Methods

Human subjects

A total of 10 trio families (each family is composed of the proband patient and both of his/her healthy parents) and 103 singletons (proband only), admitted to Peking Union Medical College Hospital (PUMCH), as a part of the Deciphering Disorders Involving Scoliosis and Comorbidities (DISCO) study (<http://www.discostudy.org/>), were included in this study. These patients underwent standard physical examination and image evaluations. Written informed consent was obtained from each individual. The Ethics Committee of PUMCH approved the study.

Blood sample collection

Genomic DNA was extracted from peripheral blood leukocytes of each subject by a blood DNA extraction kit (QIAamp DNA

Blood Mini Kit; Qiagen, Germany), and procedures strictly followed the manufacturer's protocols. Purified DNA was qualified by Nanodrop2000 (Thermo Fisher Scientific, Waltham, MA, USA) and quantified by Qubit 3.0 using the dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA, USA). DNA samples were stored at 4°C while not in use.

Whole-Exome Sequencing (WES)

WES was performed for 10 trio families. Illumina paired-end libraries were prepared from DNA samples and subjected to exome capture using a SureSelect Human design (All Exon V6+UTR r2 core, 91 Mb, Agilent), followed by sequencing on an Illumina HiSeq 4000 platform (Illumina, San Diego, CA, USA) with 150-bp pair-end reads mode.

Whole-Genome Sequencing (WGS)

WGS was conducted for the peripheral blood DNA samples of 103 singletons. Sequencing libraries were prepared using the KAPA Hyper Prep kit (KAPA Biosystems, Kusatsu, Japan) according to the manufacturer's protocols. Illumina HiSeq X-Ten sequencer (Illumina, San Diego, CA, USA) was used for multiplex sequencing. The primary sequencing data were analyzed using a standard analysis workflow.

Variant filtering and annotation

The variant-calling and annotation were performed according to the in-house-developed Pipeline (PUMP) [17]. Rare variants (MAF<0.001) were selected for analysis based on the following public databases: the 1000 Genomes Project (The 1000 Genomes Project Consortium 2015), the Exome Sequencing Project (<http://evs.gs.washington.edu/EVS/>), the Exome Aggregation Consortium (ExAC) (<http://exac.broadinstitute.org/>) and in-house databases from phenotypically well-characterized subjects. Computational prediction tools GERP⁺⁺ [18], CADD [19], PolyPhen-2 [20], SIFT [21] and ClinVar [22] were used to forecast the pathogenicity of these variants. We also annotated the detected variants using a customized database based on the Human Gene Variant Database (HGMD) and Online Mendelian Inheritance in Man (OMIM) (available at: <https://omim.org/>).

Sanger validations

We confirmed the candidate variants by Sanger sequencing from genomic DNA obtained from probands and parents from trios. We used an Axygen AP-GX-50 kit to purify the amplicons. Then, we sequenced them by Sanger sequencing (ABI3730XL instrument, Thermo Fisher Scientific, Waltham, MA, USA).

Table 1. Summary of *ESR1* and *ESR2* variants identified by WGS.

Patient ID	Chr	Gene	cDNA change	AA change	Mutation type	REF	ALT	ExAC_pLI	SIFT	Polyphen2	CADD	ExAC	1000G	gnomAD
AIS1188	6	ESR1	c.868A>G	p.Asn290Asp	Missense	A	G	0.99	T	P	13.47	0	0	0
AIS80	14	ESR2	c.236T>C	p.Leu79Ser	Missense	T	C	0	D	P	10.51	0	0	0.0001

Chr – chromosomal localization; cDNA change – nucleotide change; AA change – amino acid change; REF – the reference allele; ALT – the alternative allele; ExAC_pLI – the probability of being loss-of-function intolerant (pLI) score from Exome Aggregation Consortium (ExAC); SIFT – sorting intolerant from tolerant; PolyPhen-2 – polymorphism phenotyping v2; CADD – Combined Annotation Dependent Depletion; T – tolerated; D – deleterious; damaging, P – possibly damaging; Public data base (ExAC_HomoAlt, 1000G_ALL and gnomAD_genome_ALL).

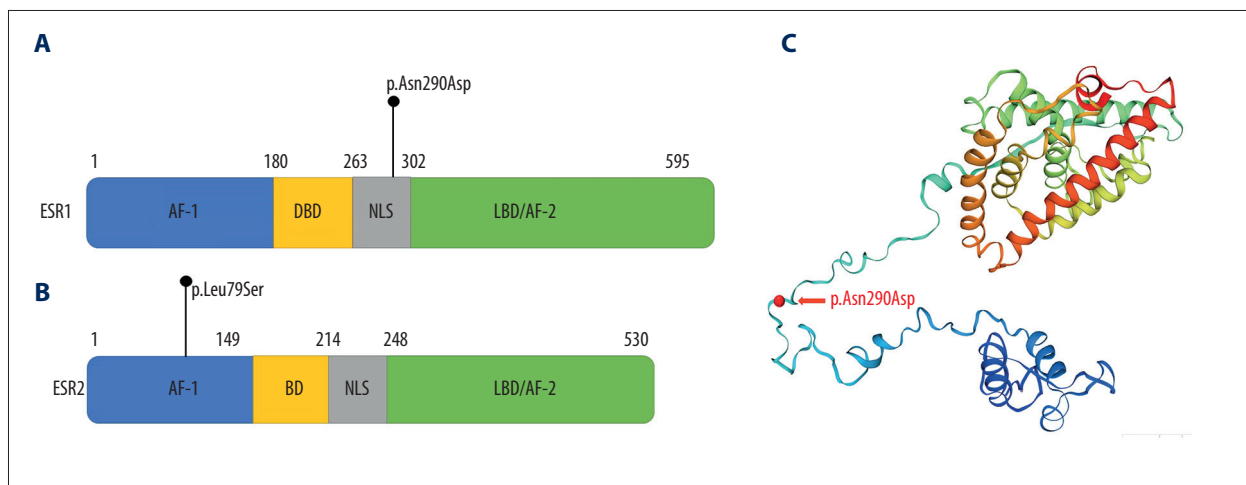


Figure 1. The schematic view of *ESR1* and *ESR2* missense variants. **(A, B)** Diagram of *ESR1/2* domains with the locations of the identified missense variants. AF-1 – activation function-1; DBD – DNA-binding domain; NLS – nuclear localization signal; LBD – ligand-binding domain; AF-2 – activation function-2. **(C)** Space distribution of missense variant in the NLS domain in *ESR1* protein. The model of *ESR1* protein (182–545 amino acids) was built by Swiss-Model (<https://www.swissmodel.expasy.org/>). The *de novo* missense variant (c.868A>G, p.Asn290Asp, red) was located close to the DNA-binding region.

Serum estradiol examination

Blood samples of patients were collected at 2 different time-points during the menstrual cycle [23]: 1) One in the early follicular phase 2–5 days after the onset of the menstrual cycle, and 2) Another in the preovulatory phase 11–16 days before the onset of the next menstrual cycle. All blood samples were obtained by venipuncture between 8 and 10 a.m. after an overnight fast. These samples were centrifuged within 30 min after venipuncture. The serum obtained was frozen at -80°C until further analysis. Serum estradiol level was measured by electrochemiluminescence immunoassay method (ECLIA) on an autoanalyzer (Elecsys 2010, Roche, Germany).

Plasmid construction

We used the pEGFP-C1-based vector for human *ESR1* and *ESR2* plasmids (wild-type and mutant). We used ERE-TK-Luc (3 copies

of Estrogen Response Element up-stream luciferase reporter and minimal TK promoter) [24] as luciferase reporter plasmids. We constructed these reporter plasmids on pGL3-Basic Vector. We verified all of these plasmids by DNA sequencing.

Plasmid transfections and Luciferase dual assays

The HEK 293T cell line (purchased from the Cell Bank of the Chinese Academy of Sciences, Shanghai, China) was maintained in DMEM medium containing 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA), streptomycin (50 $\mu\text{g}/\text{ml}$), and penicillin (50 U/ml) in 24-well plates. We co-transfected 500 ng of *ESR* plasmids and 500 ng of reporter plasmids in each well. Then, we harvested the cells and used Promega's luciferase assay system for luciferase assays 48 h post-transfection, as described before [25].

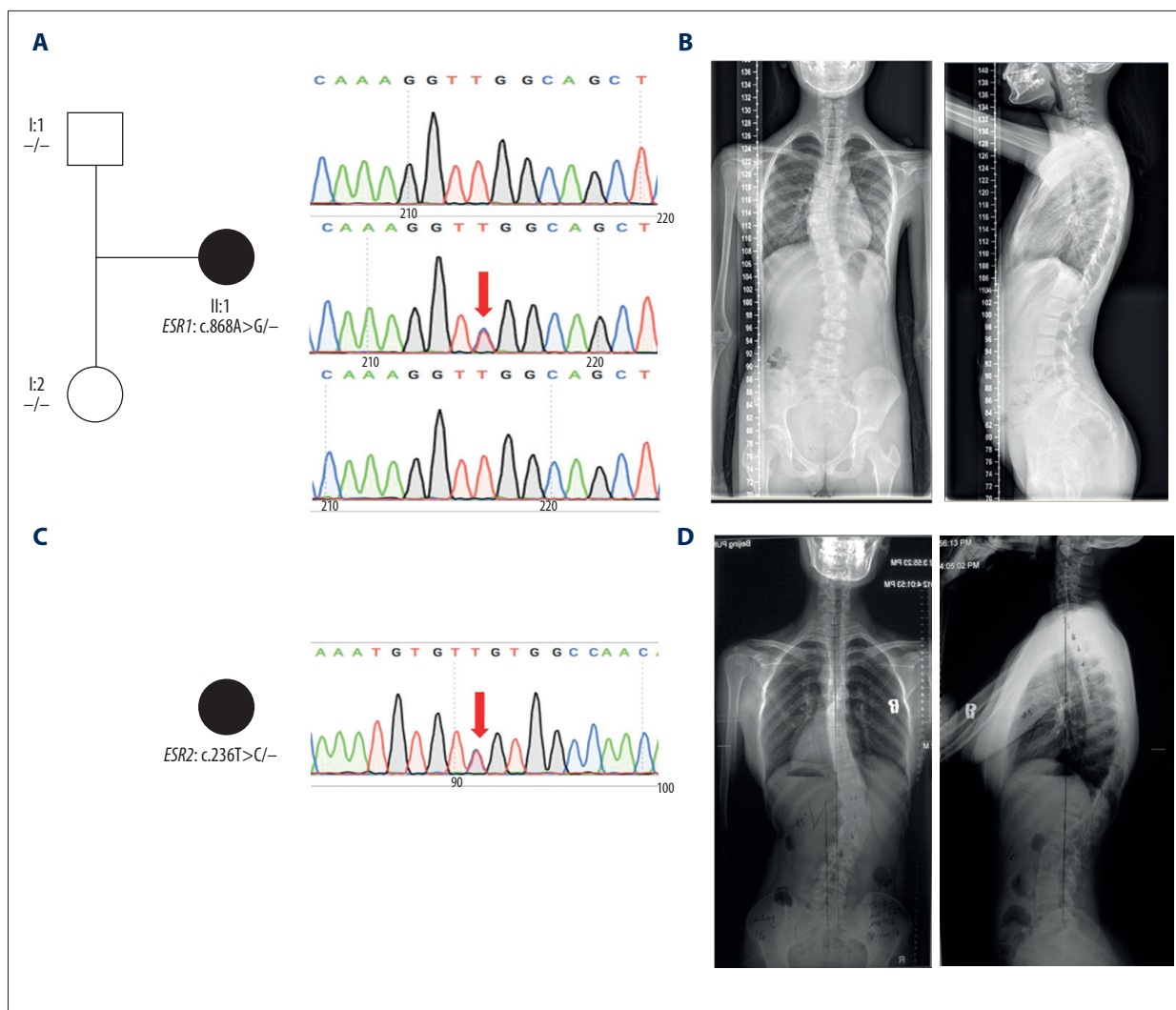


Figure 2. Variant information and spinal radiograph of AIS1188 (A, B) and AIS80 (C, D). (A) Depicting the *de novo* *ESR1* missense mutation (hg19, chr6: 152265415, c.868A>G) in a heterozygous sample. The mutation was not found in the father or mother. (B) Cobb angle=13° (T1–T5), Cobb angle=41°(T6–T10), Cobb angle=32° (T12–L4). (C) Depicting the *ESR2* missense mutation (hg19, chr14: 64749468, c.236T>C) in a heterozygous sample. (D) Cobb angle=45° (T10–L3). The red arrows indicate the mutation positions.

Statistical analysis

The data are presented as means±SD. Statistical significance was determined by one-way analysis of variance (ANOVA). All tests were performed using SPSS 22.0 software, and a P value of less than 0.05 was considered to be statistically significant.

Results

Identification of a *de novo* missense variant in *ESR1*

By analyzing WES data of 10 trio cases, a *de novo* missense variant (c.868A>G, p.Asn290Asp) in *ESR1* was identified

(Table 1, Figure 1). This variant, novel to both public and in-house databases, located at the nuclear localization signal (NLS) domain of *ESR1*, which contains the hinge region and plays an important role in nuclear localization signal of estrogen receptor [26]. Notably, molecular modeling showed that this variant (c.868A>G, p.Asn290Asp) located in the NLS domain, close to the DNA-binding domain (Figure 2). Thus, these variants might be pathogenic by disrupting nuclear localization-mediated downstream transactivation or by affecting DNA-binding affinity via other mechanisms.

This variant was identified in a 13-year-old female AIS patient (AIS1188) with 3 curves. The Cobb angle of main thoracic curve of AIS1188 was 41° from T6 to T10 (Figure 2). There

Table 2. Serum levels of estradiol in patient AIS1188.

Patient ID	Serum estradiol in early follicular phase (pg/mL)	Serum estradiol in preovulatory phase (pg/mL)
AIS1188	40.2	156.3

Serum estradiol in early follicular phase: decreased estradiol level during menses (normal range: 27–122 pg/mL). Serum estradiol in preovulatory phase: first rise of estradiol level during menses (normal range: 49–291 pg/mL).

were no positive findings from physical examination or whole-spine MRI scanning. The patient presented mild prolapse of the mitral valve and mitral insufficiency identified by echocardiography. Both of her parents had normal spine phenotype.

Serum level of estradiol in patient AIS1188

This patient, coded as AIS1188, underwent serum estradiol tests during the early follicular phase and preovulatory phase. The menstrual cycle of AIS1188 was 28 days, with a menses duration of 5 days. AIS1188's serum concentration of estradiol in the early follicular phase was 40.2 pg/mL (normal range: 27–122 pg/mL), and it was 156.3 pg/mL (normal range: 49–291 pg/mL) in the preovulatory phase. Both parameters were within the respective clinical normal ranges, suggesting that the *ESR1* variant does not affect the estradiol level (Table 2).

Identification of a rare missense variant in *ESR2*

One missense mutation (c.236T>C, p.Leu79Ser) in *ESR2* was identified in an 18-year-old female among the patients who underwent WGS (AIS80, Table 1, Figure 1). According to public

databases, this variant is rare and was predicted to be deleterious (gnomAD=0.0001, CADD=10.51). This variant is located in the activation function (AF)-1 domain of *ESR2*, which has a ligand-independent activation function [27]. In addition, the AF-1 domain is a coregulatory region that binds various ESR coactivators and corepressors that modulate ESR-mediated transcriptional activity [28]. The main Cobb angle of this patient was 45° (T10–L3) (Figure 3). The patient had no intraspinal anomalies identified by whole-spine MRI.

Mutant *ESR1* and *ESR2* proteins altered transactivation potential

We performed a co-transfection experiment in 293-T cells to determine whether the mutant can alter transactivation activation. The transactivation capacities of the mutated *ESR1* and the *ESR2* protein (p.Asn290Asp and p.Leu79Ser, respectively) were significantly lower than that of the wild-type for the ERE-TK-Luc reporters (p=0.026, p=0.014, respectively; Figure 3). These results suggest that *ESR1* (c.868A>G, p.Asn290Asp) and *ESR2* (c.236T>C, p.Leu79Ser) contribute to AIS through influencing downstream targeted genes.

Discussion

In this study, we conducted WES in 10 trio families and WGS in 103 sporadic AIS patients. Two likely pathogenic variants of *ESR1* (c.868A>G, p.Asn290Asp) and *ESR2* (c.236T>C, p.Leu79Ser) were identified. Furthermore, we demonstrated that the transactivation capacities of mutated *ESR1* and *ESR2* protein were significantly decreased, indicative of a mutant-induced impairment of physiological functions of *ESR1* and *ESR2*.

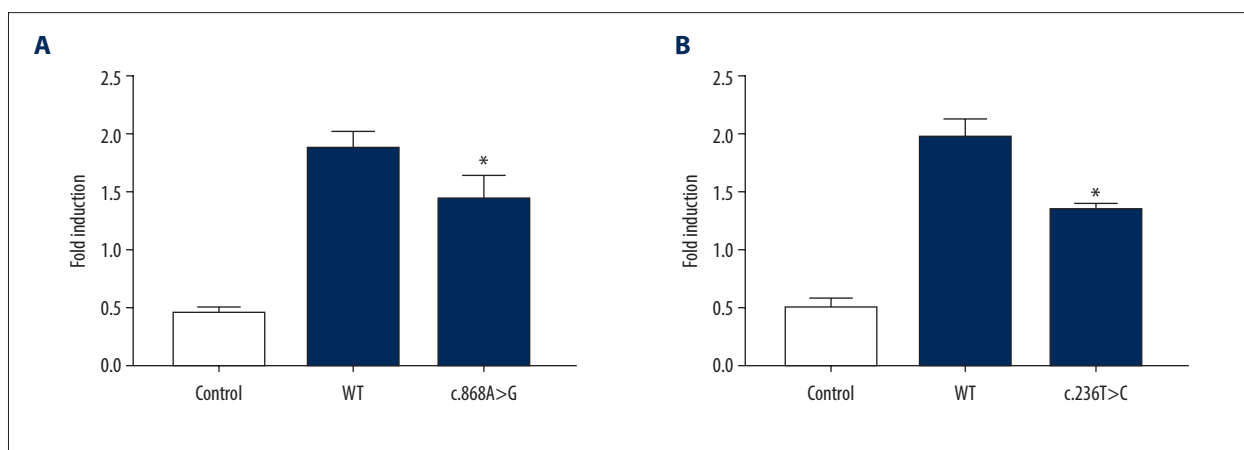


Figure 3. *In vitro* functional analysis for *ESR1* and *ESR2* missense variants. Dual luciferase assay. (A) The activity of c.868A>G construct was significantly decreased compared with that of WT (p<0.05). (B) The transcriptional activity of c.236T>C construct was also significantly decreased compared with that of WT (p<0.05). This experiment was repeated twice (* p<0.05).

Estrogen is an important molecule for information transmission *in vivo*. It acts as a chemical messenger that regulates its target cells, tissues or organs. Its physiological functions are dependent upon a high degree of receptor-ligand affinity, by which estrogen can recognize and bind to specific protein estrogen receptors (ESR) located either on the plasma membrane or in the nucleus of the cell. Classical ESRs (e.g. ESR1 and ESR2) are all nuclear receptors. The ESR structural domain is composed of AF-1, a DNA-binding domain (DBD), NLS and a ligand-binding domain (LBD)/AF-2. AF-1 and LBD/AF-2 are both transactivation domains. DBD is responsible for binding specific estrogen response elements (EREs) within the promoters of target estrogen-responsive genes. The NLS domain plays an important role in nuclear localization signaling of the estrogen receptor. Many studies reported that estrogen was involved in the onset and progression of AIS [15,16]. Cutler et al. found that the serum concentration of estrogen was about 8-fold higher in females than in males by measuring the estrogen content in healthy pre-pubertal males and females [29]. This finding demonstrated that pubertal growth spurts of both sexes were primarily stimulated by estrogen, and the higher estradiol levels of girls might result in early epiphyseal maturation. This study led researchers to explore the relationship between estrogen level and AIS, and it is now commonly accepted that the onset and progression of scoliosis can be affected by serum estrogen content [14,30–32]. We found that the 2 missense variants of *ESR1* (c.868A>G, p.Asn290Asp) and *ESR2* (c.236T>C, p.Leu79Ser) induced loss-of-function mutations that resulted in ESR losing a part of its transcriptional activity, which might contribute to AIS phenotype.

Previous studies of the relationship between ESR and AIS were mostly genome-wide association studies (GWAS) [33,34]. Two *ESR1* SNPs, located at recognition sites of endonuclease Xba I (A/G, rs9340799), Pvu II (C/T, rs2234693) and *ESR2* AluI site polymorphism, have been extensively studied around the world [34–36]. Inoue et al. detected the Pp (PvuII) and Xx (XbaI) sites in 304 Japanese female AIS patients, and the mean maximum Cobb angle and risk of curve progression for patients with genotypes XX and Xx were found to be greater than in those with genotype xx [16]. Wu et al. analyzed both sites in 202 AIS patients and 174 healthy controls, and found the frequency of XX genotype was significantly higher in patients than in controls. The frequency of XX genotype in female patients with Cobb angle $\geq 40^\circ$ was higher than in those whose Cobb angle was less than 40° , suggesting that the XbaI site polymorphism of *ESR1* gene is associated with risk of AIS [15]. However, a recent meta-analysis showed that rs9340799 did not appear to be a likely susceptibility variant for AIS predisposition, but it might still be associated with curve severity, progression and treatment outcomes of AIS [36]. Zhao et al. found that distribution of the rs2234693 site of the *ESR1* gene was significantly higher in AIS patients with Cobb angle $\geq 40^\circ$

than in healthy controls [35]. Whether the *ESR* gene polymorphism is associated with the occurrence/onset and development of AIS is inconsistent across studies and is yet to be confirmed by large multi-center studies.

Previous studies with *ESR1* and *ESR2* knockout (KO) mice have shown phenotype abnormality in vertebrae morphology, indicating that *ESR1* and *ESR2*-mediated transcriptional activity plays an important role in bone formation [37,38]. The present study has certain limitations that should be considered. The primary limitation is that we were only able to demonstrate that there was a decrease in the transactivation capacity of the mutated *ESR1* and *ESR2* proteins, and we did not demonstrate the exact means by which this change occurs. Further studies are needed to analyze the relationship between ESR defect and AIS. In addition, more demographically diverse populations are needed in replication studies to validate our results.

We identified 2 heterozygous variants of *ESR1* (c.868A>G, p.Asn290Asp) and *ESR2* (c.236T>C, p.Leu79Ser) in 113 AIS patients. We also demonstrated that transactivation capacities of the mutated proteins were both significantly reduced, which might lead to functional abnormalities of *ESR1* and *ESR2*, thus ultimately disrupting their nuclear localization-mediated transcription activation or lowering their DNA-binding affinities. In addition, our serum estradiol assessment showed that the serum estradiol concentration of AIS1188, which carried a missense mutation of *ESR1* (c.868A>G, p.Asn290Asp), was in the normal range, suggesting that this missense variant of *ESR* did not affect serum estradiol levels. In summary, the 2 missense variants of *ESR1* (c.868A>G, p.Asn290Asp) and *ESR2* (c.236T>C, p.Leu79Ser) induced a partial loss of the transcriptional activity of ESR, which might contribute to an AIS phenotype.

Conclusions

We identified 2 missense variants in *ESR1* and *ESR2* from 113 AIS patients and found decreased transcriptional activity, suggesting that perturbations of *ESR1/ESR2* might contribute to the etiology of AIS.

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Conflicts of interests

None.

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