Investigating Role of IRX Family in Development of Female Adolescent Idiopathic Scoliosis: Which One Is Real Cause?

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BACKGROUND: **Previous studies showed that several variants located around the IRX family may have functional roles in the development of adolescent idiopathic scoliosis (AIS). However, there was lack of knowledge concerning the target gene of the region on 5p13.3 and the role of IRX genes in the etiology of AIS. This study aimed to validate the relationship between the IRX family and AIS in a large-scale general population and to further investigate the target gene of the region, which was associated with AIS.**

METHODS: **SNP rs12517904 and rs117273909 were genotyped in 1323 patients and 1670 age-matched healthy controls. Paraspinal muscle was collected from 70 AIS patients and 20 congenital scoliosis patients. Student's *t*-test was used to compare the *IRX1* expression between AIS patients and controls. The 1-way analysis of variance test was used to compare the expression of the IRX genes among different genotypes.**

RESULTS: **For rs12517904, patients were found to have a significantly higher frequency of allele T than the controls (37.6% vs. 34.7%, $P = 0.02$). Allele T can significantly add to the risk of AIS with an odds ratio of 1.14. AIS patients were found to have significantly lower *IRX1* expression than the controls. Patients with genotype TT were found to have significantly lower *IRX1* expression than those with genotype GG.**

CONCLUSIONS: **Our large-scale case control study validated that the *IRX1* gene could be the disease-associated gene of AIS. The variant rs12517904 of the *IRX1* gene is functionally associated with the development of AIS in the Chinese population. The role of *IRX1* in the onset of AIS is worthy of further investigation.**

INTRODUCTION

Adolescent idiopathic scoliosis (AIS) is a 3-dimensional structural spinal deformity that occurs around the time of puberty.[1] Genetic factors were believed to contribute to AIS as indicated by the familial aggregation of the patients. Through genetic association study, several susceptible genes have been identified to be associated with the occurrence and development of AIS, such as MATN1, TGFB1, IL6, and MMP3.[2-4] However, few of these candidate genes could be successfully replicated in different populations.[5-8] In recent years, genome-wide association studies (GWASs) have been used to identify common genetic variants contributing to AIS risk, which successfully unveiled a number of disease-related genomic regions in Caucasian, Japanese, and Chinese populations, encompassing LBX1, GPR126, BNC2, PAX1, CHL1, BCL2, and PAX3.[9-14] To be noted, only a small amount of AIS risk could be explained by these common variants. More disease-related variants need to be discovered to decipher the genetic etiology of AIS.

In recent years, contribution of rare variants to complex disease has been of great interest, since rare variants are thought to exert a greater contribution to inherited disease risk.[15] Buchan et al[16] firstly performed the whole-exome sequencing in familial idiopathic scoliosis, which demonstrated that the rare variants in FBN1 and FBN2 may contribute to AIS risk and severity. These results were successfully replicated in both European ancestry and Chinese population.[17] Patten et al[18] identified 3 rare functional variants in the POC5 that were strongly associated with AIS in a large family with multiple members affected with IS. A replication study based on

Key words
- Adolescent idiopathic scoliosis
- IRX1
- SNP

Abbreviations and Acronyms
AIS: Adolescent idiopathic scoliosis
DNA: Deoxyribonucleic acid
GWAS: Genome-wide association study
PCR: Polymerase chain reaction
RNA: Ribonucleic acid

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Table 1. Distribution of Genotype and Allele Frequency of rs12517904 and rs117273909 in Patients and Controls

	Genotype			*P*	Allele		*P*	Odds Ratio (95% CI)
rs12517904	TT	GT	GG	0.04	T	G	0.02	1.14 (1.02—1.26)
Patients ($n = 1323$)	179	639	505		997	1649		
Controls ($n = 1670$)	201	756	713		1158	2182		
rs117273909	AA	AC	CC	0.98	A	C	0.98	1.01 (0.40—2.56)
Patients ($n = 1323$)	1	6	1316		8	2638		
Controls ($n = 1670$)	1	8	1661		10	3330		

95% CI indicates 95% confidence interval.

Chinese population showed that common variant of POC5 is also associated with the susceptibility of adolescent idiopathic scoliosis.[19] Similarly, rare variants of AKAP2 and VANGL1 were reported to be implicated with AIS.[20,21] However, these results were not successfully replicated in the Chinese population,[22,23] indicating that large cohort studies are necessary to determine the contribution of rare variants to AIS.

In a recent study, Justice et al[24] identified through genome-wide linkage analysis of 7 families that a 3.5-Mb region on 5p13.3 containing IRX1, IRX2, and IRX4 was associated with IS. Several rare variants were identified to be associated with idiopathic scoliosis. The variant with the most significant association, rs12517904, was located 6.5 kb downstream from IRX1. Moreover, the results of the zebrafish transgenesis showed that a functional variant rs117273909, located in the 841 kb gene desert between *IRX1* and *IRX2*, has an effect on enhancer activity. To be noted, the sample size of the affected family is relatively small and the real disease-associated gene in 5p13.3 remains unknown. The primary aims of this study were to validate the relationship between IRX family and AIS in a large-scale general population and further investigate the target gene of the region that was associated with AIS.

MATERIALS AND METHODS

Subjects

The current case control study was approved by the ethics committees of The Affiliated Drum Tower Hospital of Nanjing University Medical School. A total of 1323 female AIS patients and 1670 age-matched healthy controls who visited our scoliosis center between June 2001 and July 2015 were enrolled in this study. The inclusion criteria were 1) aged between 10 and 18 years and 2) With a main curve Cobb angle of >20 degrees. Patients with congenital scoliosis (CS), neuromuscular scoliosis, and scoliosis secondary to other known etiology were excluded. The healthy participants were recruited during their routine examinations before high school admission. All control subjects were verified through the Adam forward bend test by 2 experienced orthopedic surgeons (Q.Y. and Z.Z.) to rule out any spine deformity. Demographic data including menarche age, curve magnitude, and body mass index of the patients were recorded at their first visit to our center.

Sample Collection

Ethylenediamine tetraacetic blood samples were collected for deoxyribonucleic acid (DNA) analysis, with informed consent obtained from the participants or their parents. Genomic DNA was extracted from peripheral blood leukocytes using the commercial kit (QIAGEN) according to the manufacturer's instructions. Paravertebral muscles were collected in 70 AIS patients during the surgical interventions at the apical level. Besides, 20 age-matched CS patients undergoing posterior spinal correction surgery were recruited as the controls. All the samples were collected at the concave and convex sides of the apex for patients with AIS and CS. The total ribonucleic acid (RNA) was extracted subsequently using Trizol reagent (Invitrogen) according to the manufacturer's protocol. To avoid the contamination of genomic DNA, all the samples were treated with DNaseI (Invitrogen, Carlsbad, California, USA). Total RNA was then reverse-transcribed from 2 ug of RNA using the PrimeScript RT Master Mix kit (TaKaRa, Tokyo, Japan). A flowchart diagram of the "Method" section is included in **Supplementary Figure 1**.

Genotyping of Target Variations in IRX Family

SNP rs12517904 and rs117273909 of IRX genes reported by Justice et al[24] was genotyped using allelic-specific multiple ligase detection reactions according to the standard protocol. Fifteen percent of the samples were randomly validated to ensure the reliability of the genotyping results.

Genotyping of Common Variations in IRX Family

SNPs of *IRX1*, *IRX2*, and *IRX4* genes were selected by the Haploview v4.2 software based on Chinese Han Beijing population data from the HapMap database. The GWAS database reported in our previous study was used to extract the genotyping information of AIS patients and normal controls.[14] The genotyping results of each SNP were then calculated accordingly.

Tissue Expression of IRX Family

The tissue expression of *IRX1*, *IRX2*, and *IRX4* genes were measured with real-time polymerase chain reaction (PCR) using gene-specific primers listed as follows: forward 5′-CCGGTACGCGACAACTCTC-3′, reverse 5′-GACCCTTAATCAGGCGGACG-3′ for the *IRX1* gene, forward 5′- TCCAGACCACATCTTGTGCC-3′, reverse 5′-TGTTAGAGTCTGTGGGCTGAC-3′ for the *IRX2* gene, forward 5′-TCTACTGCCCGGTCTACGAG-3′, reverse 5′-GCAGATCCCGAACCATCCTTG-3′ for the *IRX4* gene, and forward 5′- GAGTCAACGGATTTGGTCGT-3′, reverse 5′-TTGATTTTGGAGGGATCTCG-3′ for the endogenous control gene glyceraldehyde-3-phosphate

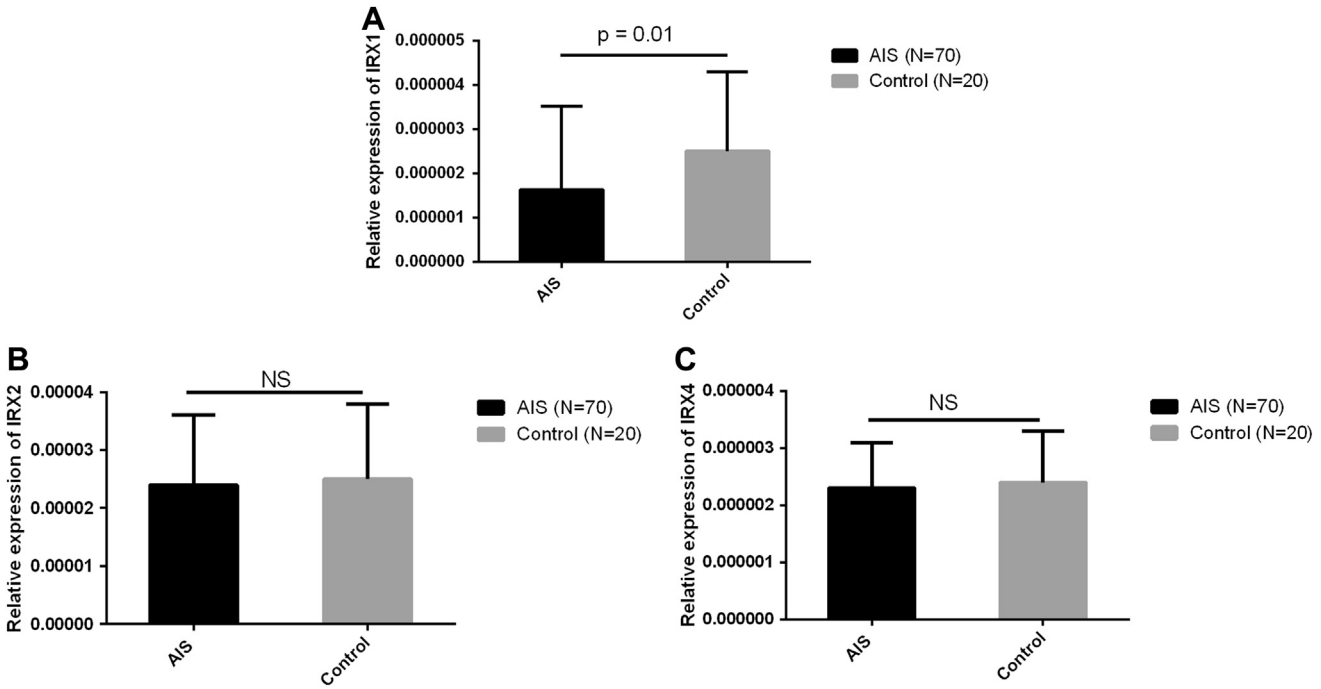

Figure 1. The mRNA expression of IRX family for patients and controls. (**A**) Adolescent idiopathic scoliosis (AIS) patients had significantly lower expression of *IRX1* as compared with the controls (0.0000016 ± 0.0000014 vs. 0.0000025 ± 0.0000018, $P = 0.01$). (**B** and **C**) There was no significant difference of IRX2/IRX4 expression between the AIS patients and controls (0.000024 ± 0.00012 vs. 0.00026 ± 0.000013, $P = 0.49$ for IRX2; 0.0000024 ± 0.00012 vs. 0.00023 ± 0.000011, $P = 0.34$ for IRX4).

dehydrogenase. Quantitative PCR analysis was performed using SYBR Premix Ex Taq II (Takara) in a 20-μL PCR mixture according to the manufacturer's protocol. Samples were analyzed on the Roche LightCycler 480 II instrument (Roche Diagnostics). Relative mRNA expression was analyzed based on the $2^{-\Delta Ct}$ method using glyceraldehyde-3-phosphate dehydrogenase expression as an internal control.

Statistical Analysis

The genotype frequencies of SNPs were tested for Hardy–Weinberg equilibrium separately in both patients and healthy controls. The odds ratio values and 95% confidence intervals were calculated on the basis of allele frequency table. The Student's t-test was used to compare the expression of IRX genes between patients and controls. AIS patients were classified into 3 groups according to the genotypes of each SNP, and the 1-way ANOVA test was used to compare the expression of the IRX genes among different genotypes. Statistical analysis was performed with SPSS20.0 software (SPSS Inc, Chicago, Illinois, USA). A 2-tailed $P < 0.05$ was considered statistically significant.

RESULTS

Clinical Characteristics of Subjects

The mean age was 14.3 ± 3.2 years for the patients and 14.5 ± 3.9 years for the healthy subjects in the ligase detection reaction analysis, respectively. The mean Cobb angle of the patients was 38.5 ± 12.3 degrees. For expression analysis, the mean age was 14.6 ± 1.3 years for the AIS patients and 14.9 ± 0.9 years for the controls ($P = 0.48$). The mean curve magnitude was 58.5 ± 17.3 degrees for AIS patients and 59.1 ± 18.2 degrees for the controls ($P = 0.32$).

Association of Target Variants with Adolescent Idiopathic Scoliosis

As shown in Table 1, the genotype and allele frequency of rs12517904 were significantly different between the patients and controls. Patients were found to have a remarkably higher frequency of

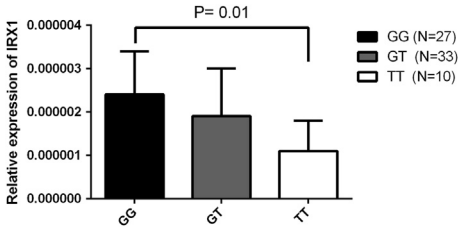

Figure 2. Relationship between the genotype of rs12517904 and *IRX1* expression. Patients with genotype TT of rs1251790 have a significantly decreased expression of *IRX1* than those with genotype GG ($P = 0.01$).

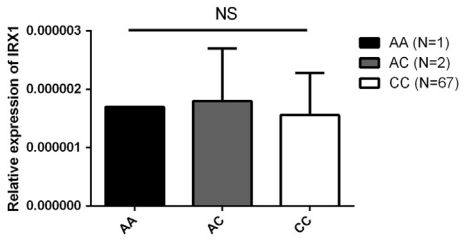

Figure 3. Relationship between the genotype of rs117273909 and *IRX1* expression. No significant differences were found among 3 genotypes.

allele T than the controls (37.6% vs. 34.7%, $P = 0.02$) with an odds ratio of 1.14. For rs117273909, neither the genotype nor the allele frequency was significantly different between the patients and controls.

Expression of *IRX1, IRX2,* and *IRX4* in Adolescent Idiopathic Scoliosis and Controls

A total of 70 AIS patients and 20 controls were recruited in this study for tissue expression analysis. AIS patients were found to have significantly lower expression of the IRX1 as compared with the controls (0.0000016 ± 0.0000014 vs. 0.0000025 ± 0.0000018, $P = 0.01$) (Figure 1A). No significant difference of IRX2/IRX4 expression between the AIS patients and controls existed (0.000024 ± 0.00012 vs. 0.00026 ± 0.000013, $P = 0.49$, for IRX2; 0.0000024 ± 0.00012 vs. 0.00023 ± 0.000011, $P = 0.34$, for IRX4) (see Figure 1B and C).

As for rs12517904, the mean value of *IRX1* expression was 0.0000024 ± 0.0000010 for genotype GG, 0.0000019 ± 0.0000011 for genotype GT, and 0.0000011 ± 0.0000007 for genotype TT. Patients with genotype TT have a significantly decreased expression of *IRX1* than those with GG ($P = 0.01$) (Figure 2). As for rs117273909, no significant differences were found among 3 genotypes (Figure 3).

Association of Common Variants with Adolescent Idiopathic Scoliosis

A total of 362 SNPs were analyzed for *IRX1*, *IRX2*, and *IRX4*. HWE test showed no significant difference regarding the genotype frequency in either the patients or controls ($P > 0.05$). No SNP among the common variants of IRX family was found to be significantly associated with AIS (Supplementary Table 1).

DISCUSSION

Recently the GWASs and whole exome sequencing became available to investigate the role of both common variants and rare variants in complex diseases. In this study, for the first time we evaluated the relationship between the variants of IRX genes and AIS susceptibility in the Chinese Han population. We demonstrated that the expression of *IRX1* was decreased in patients with AIS. Moreover, our results showed that rs12517904, which is located 6.5 kb downstream of IRX1, also contributes to AIS susceptibility in the Chinese Han population.

IRX1 encodes a member of the Iroquois protein family, which has been associated with many developmental processes in vertebrates.[25] Through genome-wide linkage analysis of 7 IS families, Justice et al[24] identified a 3.5-Mb region on 5p13.3 as a regulatory element, which contains 3 known genes, *IRX1*, *IRX2*, and *IRX4*. Thus this regulatory region is likely to be associated with the etiology of IS. To further identify the target gene of this region, we investigated the tissue expression of the *IRX1*, *IRX2*, and *IRX4* genes in the paraspinal muscles of both AIS patients and controls. Compared with controls, patients had a significantly decreased mRNA expression of *IRX1*, while the tissue expression of IRX2 and IRX4 was comparable between the 2 cohorts. These results suggested that *IRX1* was more likely to be the disease-associated gene of the regulatory region on 5p13.3.

To further characterize the role of *IRX1* in AIS, we investigated the frequency of genetic variants of IRXs in both patients and controls. Variant rs12517904 of IRX1 was reported to be significantly associated with familial IS.[24] On the basis of our large-scale population, we successfully replicated the association between this SNP and the susceptibility of AIS in the Chinese population. Patients were found to have a remarkably higher frequency of allele T than the controls. Moreover, patients with genotype TT have a significantly decreased expression of *IRX1* than those with GG. These results demonstrated that rs12517904 may play an important role in regulating the expression of *IRX1*. In addition to rs12517904, we also evaluated the association between other common variants of IRX family and AIS. None of these common variants was found to have significantly different genotype frequency between the patients and normal controls.

To summarize, we have successfully confirmed that the *IRX1* gene could be the disease-associated gene of AIS. Our findings provided new clues concerning the etiology of AIS. In future studies, the function of *IRX1* in the development of AIS is worthy of investigation via a cellular or an animal model. One limitation of this study is that the sample size of the controls in the expression analysis was relatively small. It should be noted that we have strictly matched the patients and controls in terms of age and Cobb angle. Further studies are required to include more tissues of control group for expression analysis.

CONCLUSIONS

The *IRX1* gene could be the disease-associated gene of AIS. The variant rs12517904 of the *IRX1* gene is functionally associated with the development of AIS in the Chinese population. The role of *IRX1* in the onset of AIS is worthy of further investigation.

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