

Original Article

Assessment of varied long noncoding RNA and messenger RNA expression levels in adolescent idiopathic scoliosis

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Abstract: Adolescent idiopathic scoliosis (AIS) is the most common form of spinal deformity, which involves lateral deviation of the vertebrae and axial rotation. Approximately 1 million children exhibit some degree of spinal deformity in the United States. Although numerous potential etiologies for AIS have been formulated, the primary cause of AIS remains unknown. In this study, we recruit female AIS patients and normal volunteers, measure lncRNAs and mRNA expression level changes in order to discover genomes involved in the pathophysiology of the AIS disease. A total of 2116 lncRNAs were examined in microarray. The amount of up-regulated and down-regulated lncRNAs was 1015 and 1101 respectively in the AIS group compared to the NC group. NONHSAT137367 was the most significant down-regulated lncRNA (fold change=11.27617). NONHSAT103134 was the most up-regulated lncRNA (fold change=5.174644). Compared to the NC group, a total of 540 mRNAs expression levels were significantly changed in all tested AIS samples. Interestingly, we found out that ARC, SET and TPM3 genes had significant expression differences in the AIS patients comparing to the NC subjects, which had not been reported before. This result is confirmed by qPCR analysis. Altered lncRNA expressions play a role in the pathogenesis and development of the AIS disease. ARC, SET and TPM3 genes functions in the progressive AIS disease status needs to be further studied in a bigger scale study groups, and possibly by using other sample types besides peripheral blood.

Keywords: lncRNA, mRNA, AIS, microarray, SRC, SET, TPM3

Introduction

Adolescent idiopathic scoliosis (AIS) is the most common form of spinal deformity, which involves lateral deviation of the vertebrae and axial rotation [1-4]. AIS is usually diagnosed during the pubertal growth spurt at ages 10-16 years without an identifiable cause impacting 2%-4% of adolescents [5, 6]. AIS affects girls predominantly [7]. Approximately 1 million children exhibit some degree of spinal deformity in the United States [8]. Mild scoliosis is often asymptomatic, while progressive scoliosis may result in back pain, reduced mobility, cardiac dysfunction and decreased pulmonary function [9, 10].

The Scoliosis Research Society (SRS) and the International Scientific Society on Scoliosis Orthopaedic and Rehabilitation Treatment (SOSORT) recommend brace treatment for curves that are likely to progress [11, 12].

Progressive AIS is considered when the curve to beyond 20° Cobb angle in an adolescent who has considerable growth remaining. 51% non-progressive AIS developed into progressive AIS [13, 14]. The genetic determination for AIS progressiveness is unclear.

Etiological concepts in AIS regarding genetics, molecular biology, bio-mechanics and neurology were proposed. Connective tissue structural abnormalities [15, 16], calcium and bone metabolism dysfunctions [17, 18], disorders in hormonal and growth factors signaling [19-21] were caused by single nucleotide polymorphism (SNP) at different chromosomal loci, and were associated with AIS. Observation of familial aggregation and genetic twin studies revealed that AIS is likely a autosomal dominant inheritance disease [22-24]. A genetic survey study revealed that overall risk of IS to first-, second- and third-degree relatives were 11, 2.4 and 1.4% respectively [25]. This indicated that AIS

Table 1. Detailed information of five paired participants

Groups	Gender	Age	Menarche	Disease History (Year)	Classification	Cobb Angle (°)
AIS	F	14	Y	1	PUMCc2	45
		16	N	1	PUMCc1	40
		15	N	2	PUMCc2	44
		14	N	3	PUMCb	40
		16	Y	2	PUMCc2	45
NC	F	15	Y	N/A	N/A	N/A
		14	N			
		16	N			
		15	Y			
		14	Y			

is a complex genetic disorder. Although numerous potential etiologies for AIS have been formulated, the primary cause of AIS remains unknown.

Major portion of the genome is transcribed, and protein-coding sequences only accounts for a minor portion of the cellular output. Long noncoding RNAs (lncRNAs) are transcripts longer than 100 nucleotides, which do not contain functional open reading frames [26]. Altered lncRNA expression levels can imbalance genome transcript output, and further result in aberrant expression of proteins, which may contribute to human diseases [27, 28]. Circulating lncRNAs can reflect local pathophysiological conditions [29]. Circulating RNAs in patient peripheral blood samples was used to evaluate local circumstances of spine [30]. LncRNA provides a unique research angle to study disease pathophysiology.

In this study, we recruit AIS patients and normal volunteers, measure lncRNAs and mRNA expression levels between the study groups in order to discover genomes involved in the pathophysiology of AIS disease.

Materials and methods

Patient recruitment

Written informed consent was obtained from all participants. The study was approved by the Institutional Review Board of Changhai Hospital of the Second Military Medical University. Five AIS children and five normal kids were used for Agilent human lncRNA+mRNA microarray analysis. Both study group subjects are girls. Detailed information of the two study groups is summarized in **Table 1**. The diagnosis of AIS

was made only when other causes of scoliosis, including vertebral malformation, neuromuscular disorder, and syndromic disorders were ruled out. Radiographs were taken in scoliosis patients. Patients were included in this study with Cobb angle over 40° [14].

RNA extraction

Total RNA was extracted using RNasey Mini Kit (Qiagen p/n 74104) according to the instructions recommended by

the manufacturer. The RNA purity and concentration were evaluated with NanoDrop ND-1000 spectrophotometer. The quality of the total RNA was used by RNA integrity number (RIN) and 28S/18S. $RIN \geq 7$, $28S/18S \geq 0.7$ and total RNA concentration over 0.08 $\mu\text{g}/\mu\text{l}$ were selection criteria for RNA samples.

RNA labeling and hybridization

Double-stranded cDNAs (containing T7 RNA polymerase promoter sequence) were synthesized from 1 mg total RNA according to the manufacturer's instructions and labeled with a fluorescent dye (Cy3-dCTP) (Quick Amp Labeling Kit, One-Color, Agilent p/n 5190-2305). Labeled cDNA was denatured at 95°C for 3 min in hybridization solution. Agilent human lncRNA Array V3.0 was hybridized in an Agilent Hybridization Oven overnight at 42°C and washed with two consecutive solutions (0.2% SDS, 2x SSC for 5 min at 42°C, and 0.2x SSC for 5 min at room temperature).

LncRNA microarray analysis

Labeled and fragmented cRNA was washed and scanned according to manufactures protocol. The arrays were scanned with the Agilent Microarray Scanner (Agilent p/n G2505C).

Real-time PCR

The differentiated expressed genes were confirmed in all participants. Total RNA (2 μg) was reversely transcribed into cDNA using a PrimeScript RT reagent kit containing a gDNA Eraser (TaKaRa) according to the manufacturer instructions. qPCR was performed using SYBR Premix Ex Taq on Thermal Cycler Dice TP800 instrument. All experiments were performed in

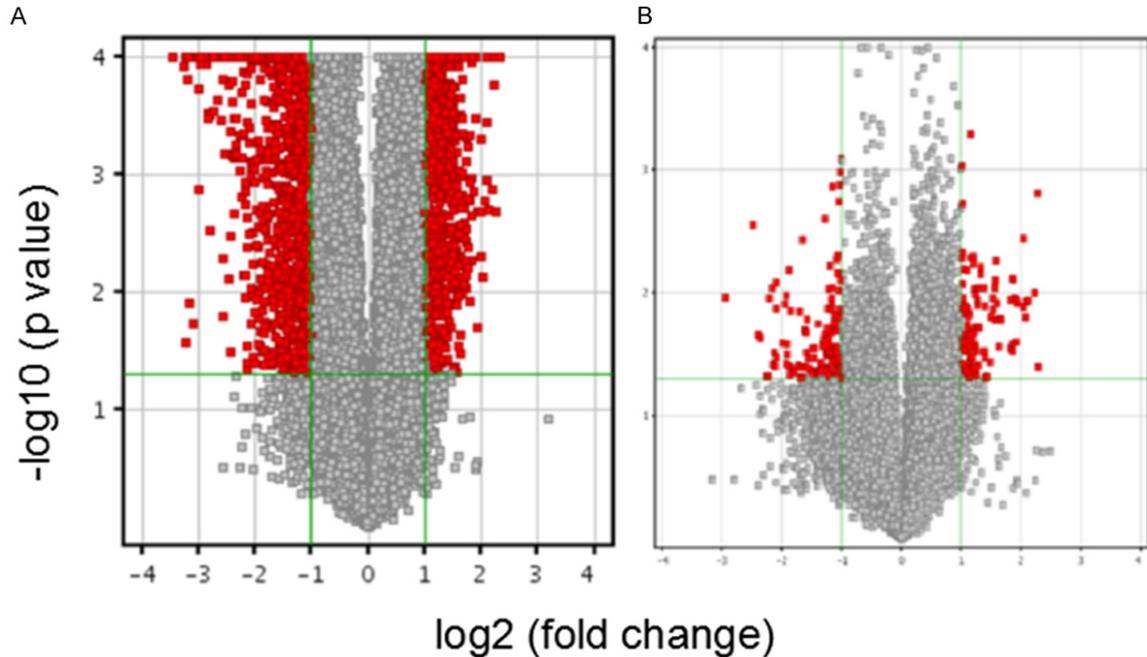


Figure 1. The volcano plots of differentially expressed genes between the AIS and NC groups. A. The volcano plot of lncRNA expression difference profile; B. The volcano plot of mRNA expression difference profile.

triplicates. All samples were normalized to GAPDH. The median in triplicate was used to calculate relative lncRNAs concentrations ($\Delta Ct = Ct \text{ median lncRNAs} - Ct \text{ median GAPDH}$). Folds change was calculated using $2^{-\Delta\Delta Ct}$ methods. The differences of lncRNAs expression between patients and control were analyzed using Student's-t-test within SPSS (version 16.0 SPSS Inc.). A value of $P \leq 0.05$ was considered as statistically significant.

NONHSAT137367 forward primer TTCCGAGAAATTTTCAG, reverse primer TCGTTATCAGAGTGGAGAC; NONHSAT103134 forward primer ATCAGTTCAAATGGGTT, reverse primer GAGGCTGGATAAAGGTT; SRC forward primer GTGACCTGACCTGCCGTCT, reverse primer GGAGGAGTGGGTGTCGCTGT; SET forward primer TCTCTCCGAGCAGCGCA, reverse primer TGGCAGCAGGAGACCC; TPM3 forward primer TGTGAGACCATTTGGGTGAA, reverse primer TGTGTTTCGTTAGGCAACAGC; GAPDH forward primer AAGGGAAGGTTGCTGGATAGG, reverse primer CACATCCACCCTCCACATC.

Data analysis

Feature Extraction software (version 10.7.1.1, Agilent Technologies) was used to analyze array images to get raw data. Genespring were

employed to finish the basic analysis with the raw data. To begin with, the raw data was normalized with the quantile algorithm. The probes that at least 1 conditions out of 2 conditions have 75% flags in "P" were chosen for further data analysis. Differentially expressed genes or lncRNAs were then identified through fold change as well as P value calculated with t-test. The threshold set for up- and down-regulated genes was a fold change ≥ 2.0 and a P value ≤ 0.05 . Afterwards, GO analysis and KEGG analysis were applied to determine the roles of these differentially expressed mRNAs. Finally, Hierarchical Clustering was performed to display the distinguishable genes' expression pattern among samples.

Results

LncRNA profiles

A total of 2116 lncRNAs were examined in microarray (Table S1). The amount of up-regulated and down-regulated lncRNAs was 1015 and 1101 respectively in the AIS group compared to the NC group. The volcano plot was presented in Figure 1A. Compared to NC group, a total of 346 lncRNAs were consistently up-regulated or down-regulated in all tested AIS samples (≥ 2 -fold). NONHSAT137367 was the

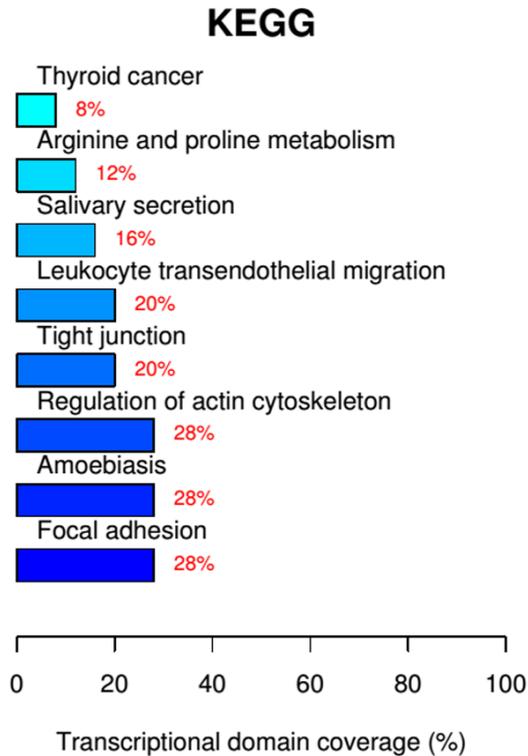


Figure 2. KEGG analysis indicates the varied mRNA genes are involved in several biological pathways.

most significant down-regulated lncRNA (fold change=11.27617). NONHSAT103134 was the most up-regulated lncRNA (fold change=5.174644).

mRNA profiles

The expression of coding transcripts was examined with microarray containing 33,982 coding transcripts probes. Compared to NC group, a total of 540 mRNAs were consistently up-regulated or down-regulated in all tested AIS samples (Table S2). The volcano plot was presented in Figure 1B. GO analysis and KEGG analysis were applied to determine the roles of these differentially expressed mRNAs (Table S3). Pathway analysis indicates that the dysregulated mRNAs are involved in thyroid cancer, arginine and proline metabolism, salivary secretion, leukocyte transendothelial migration, tight junction, action cytoskeleton regulation, amoebiasis, and focal adhesion. Coding RNAs related to major biological processes are listed in Figure 2. Hierarchical Clustering was performed to display the distinguishable genes' expression pattern among samples (Figure 3). According to previous publications, ACTN3,

CYP19A1, DCX, EGR2, FOXD3, GJA8, MEIS3, ZC4H2 had significant expression differences in AIS patients comparing to the NC subjects [30]. However, in this study we did not find significant expression differences of these genes. We found that ARC, SET and TPM3 genes had significant expression differences in AIS patients comparing to the NC subjects. The functions of these three genes in the AIS pathological process need further investigation.

qPCR validation

Data from microarray were validated by qPCR in thirty pairs of samples. qPCR results revealed that NONHSAT103134, ARC, SET and TPM3 were up-regulated, and NONHSAT137367 was down-regulated in AIS samples ($p < 0.01$), as compared to the control, which was consistent with those from microarray analysis (Figure 4).

Discussion

Microarray has been recognized as a feasible and useful approach to study genetics of diseases and seek biomarkers [32, 33]. Since hundreds of lncRNAs have been discovered, altered lncRNAs expression has been studied in various diseases, such as cardiovascular disease and hepatocellular carcinoma [34, 35]. Although lncRNA microarray had been used to study AIS, the result was not satisfactory [31].

In this study, a total of 2116 lncRNAs was examined including 346 lncRNAs with significant expression differences (≥ 2 folds) in AIS. These lncRNAs might be involved in the development and progression of AIS. NONHSAT137367 was the most significant down-regulated lncRNA (fold change=11.27617). NONHSAT103134 was the most up-regulated lncRNA (fold change=5.174644). Although the lncRNAs expression differences may result from patient selection bias, the target genes of these genes need to be determined.

In this study, compared to the NC group, a total of 540 mRNAs was consistently up-regulated or down-regulated in all tested AIS samples. Hierarchical clustering indicates that the dysregulated mRNAs were related to thyroid cancer, arginine and proline metabolism, salivary secretion, leukocyte transendothelial migration, tight junction, action cytoskeleton regulation, amoebiasis, and focal adhesion. Interestingly, we found out that ARC, SET and TPM3

LncRNA and mRNA expression in AIS

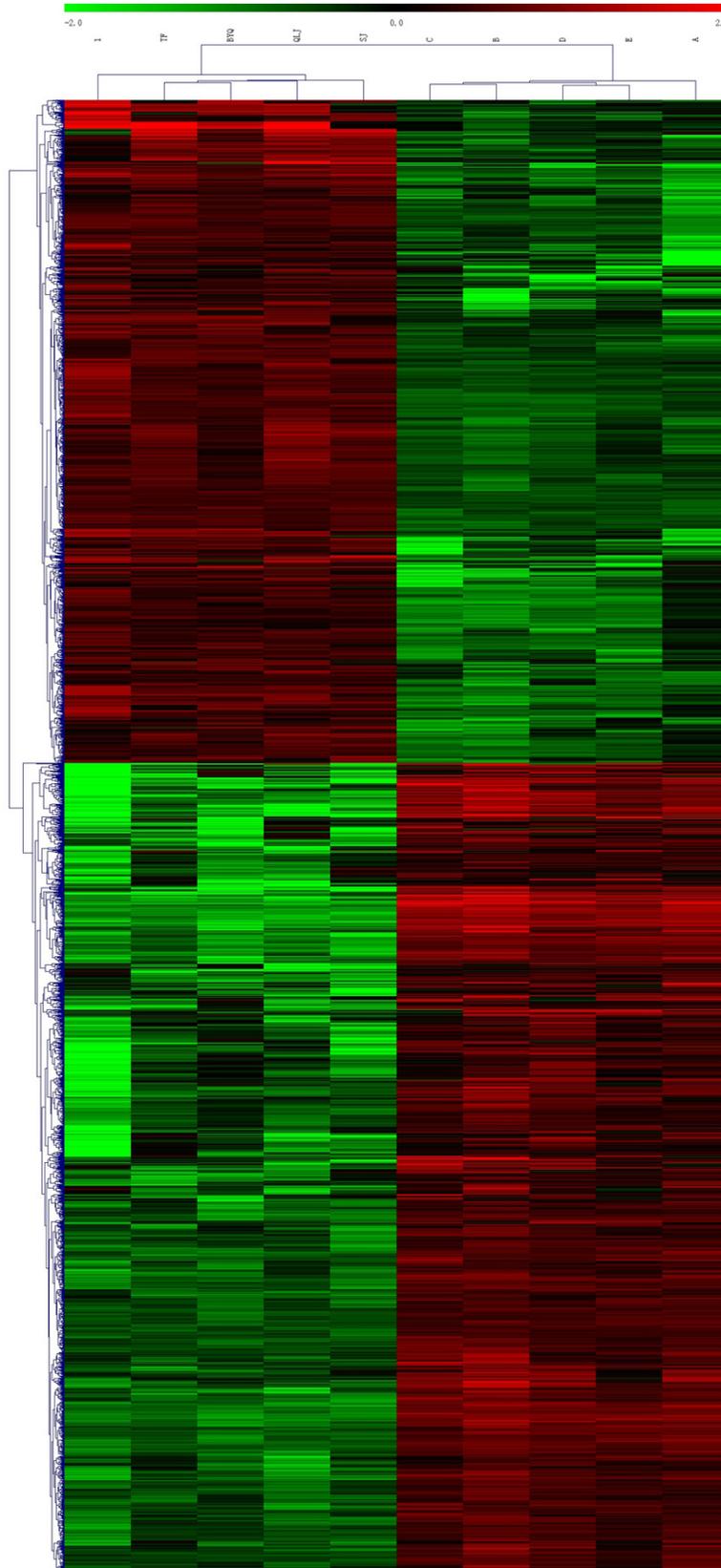


Figure 3. Hierarchical Clustering was performed to display the distinguishable genes' expression pattern among samples.

genes had significant expression differences in AIS patients comparing to the NC subjects, which had not been reported before. qPCR analysis was used to confirm the expression differences of these three genes in thirty AIS patient' peripheral blood samples. Furthermore, ARC, SET and TPM3 are more likely highly expressed in the progressive AIS patients with Cobb angle over 40°C (data not shown). The indicative value of ARC, SET and TPM3 needs to be studies in bigger size of samples.

Arc (Activity-Regulated Cytoskeleton-associated protein) is a plasticity protein, which is widely considered to be an important protein in neurobiology. Arc mRNA is localized to activated synaptic sites in an NMDA receptor-dependent manner [36-38]. SET gene is believed as a oncogene, which interacts with phosphatase 2A and plays a role in Cdk5/P35 activity [39, 40]. TPM3 encodes a member of the tropomyosin family of actin-binding proteins involved in the contractile system of striated and smooth muscles and the cytoskeleton of non-muscle cells [41, 42]. Tropomyosins are dimers of coiled-coil proteins that polymerize end-to-end along the major groove in most actin filaments in order to provide cellular stability. TM-P3 deletion and mutation causes hypercontractile congenital muscle stiffness and muscle weakness [41, 42]. ARC, SET and TPM3 genes function in the development of AIS needs to be studied thoroughly.

LncRNA and mRNA expression in AIS

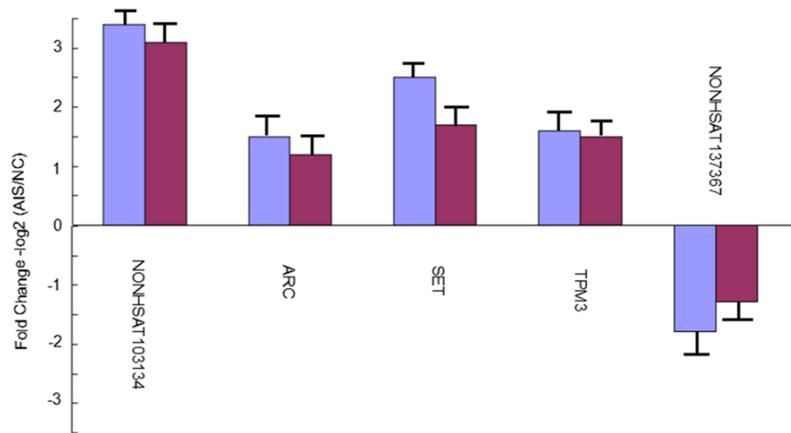


Figure 4. Data from microarray were validated by qPCR in thirty pairs of samples. qPCR results revealed that NONHSAT103134, ARC, SET and TPM3 were up-regulated, and NONHSAT137367 was down-regulated in AIS samples ($p < 0.01$).

In summary, altered lncRNA expressions play a role in the pathogenesis and development of the AIS disease. ARC, SET and TPM3 genes functions in the progressive AIS disease status needs to be further studied in a bigger scale study groups, and possibly by using other sample types besides peripheral blood.

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Disclosure of conflict of interest

None.

Authors' contribution

Dr. F Wang, Dr. X-M Xu and Dr. X-Y Zhou did most the lab works; Dr. X-D Zhu, Dr. Y-S Bai did data analysis and patients recruitment; Dr. X-Z Wei and Dr. M Li wrote the manuscript.

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