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LBX1 mRNA EXPRESSION IN PARAVERTEBRAL MUSCLES OF PATIENTS WITH ADOLESCENT IDIOPATHIC SCOLIOSIS : A PRELIMINARY STUDY

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Abstract

Objectives : To investigate *LBX1* mRNA expression in bilateral paravertebral muscles in adolescent idiopathic scoliosis (AIS) and control subjects to clarify its association with development and progression of scoliosis.

Summary of background data : Paravertebral muscle abnormalities in AIS patients have been investigated through various methods. Despite the roles of *LBX1* in skeletal muscles, the association with idiopathic scoliosis is still unclear.

Methods : Fourteen AIS patients (average age, 15.9±2.2 years ; average Cobb angle, 48.2±8.9°) and 7 controls (average age, 26.4±9.7 years) were included. Muscle samples were harvested from bilateral paravertebral muscles at the apical vertebral level. *LBX1* mRNA expression was evaluated by the real-time PCR. *LBX1* expressions in bilateral paravertebral muscles were compared in each group. The expression ratio, the expression at the convex side relative to the concave side, was compared between groups. Correlation between expression ratio and Cobb angle was analyzed.

Results : *LBX1* expression on the convex side was higher than that on the concave side in AIS group ($p=0.020$), and the expression ratio of *LBX1* in the AIS group was higher than that of controls ($p=0.012$). However, there was no significant correlation between the expression ratio of *LBX1* and Cobb angle ($r=-0.3826, p=0.177$).

Conclusions : In the AIS group, *LBX1* mRNA expression was asymmetric. The AIS group had higher expression ratios than the controls. These findings suggest the possible functional role of paravertebral muscles in the development or progression of the spinal curve.

Key words : *LBX1*, Paravertebral muscle, Adolescent idiopathic scoliosis

Introduction

Adolescent idiopathic scoliosis (AIS) is a three-dimensional spinal deformity found in 2 to 3% of school-age children[1]. The true etiology of idiopathic scoliosis remains unknown and is considered to be multifactorial. Genetic factors, melatonin, connective tissue abnor-

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malities, abnormalities in skeletal muscles including the paravertebral muscles, thrombocyte abnormalities, neurological mechanisms, growth imbalance, and biomechanical factors have been implicated in the etiology[2]. Paravertebral muscle abnormalities or imbalance has been investigated since 1976 when Spencer and Eccles reported decreased type 2 fibers in the paravertebral muscles of AIS patients[3]. In the same year, Fidler and Jowett reported an increased proportion of type 1 fibers in the multifidus muscle on the convex side as compared to the concave side[4], which has since been verified by other researchers[5,6]. In 2006, Kouwenhoven *et al*. found that muscular weakness triggers spinal decompensation in neuromuscular scoliosis, and they reported similar curve patterns and apical levels between neuromuscular scoliosis and AIS[7]. In electromyography analysis, higher myoelectric activity of the paravertebral muscles was found on the convex side[8,9]. In addition, Shimada identified neurogenic changes in 25.9% of patients with idiopathic scoliosis[10]. These results suggest a possible functional role of paravertebral muscles in the development or progression of scoliosis and the possibility that neurogenic factors are involved in idiopathic scoliosis.

In 2011, Takahashi *et al*. identified a susceptibility locus for AIS at chromosome 10q.24.31 in the region containing ladybird homeobox 1 (*LBX1*) through genome-wide association study in Japanese subjects[11]. They also found specific *LBX1* expression in human spinal cord and skeletal muscle in various human tissues. However, there have been no studies focused on relationship between *LBX1* expression in paravertebral muscles of AIS and scoliotic deformity. In this study, we aimed to compare *LBX1* mRNA expression in bilateral paravertebral muscles in AIS and clarify the relation with curve severity.

Table 1. Characteristics and comparison of the studied groups

	AIS (n=14)	Control (n=7)	p-value
Gender, female/male (n)	12/2	2/5	0.017
Age at Scoliosis Onset, years (mean±SD [range])	12.4±2.0 (10-17)		
Age at the time of surgery, years (mean±SD [range])	15.9±2.2 (12-20)	26.4 ± 9.7 (16-41)	0.030
Cobb angle, degrees (mean±SD [range])	48.2±8.9 (37-70)		
Risser grade 2/3/4/5 (n)	1/1/4/8		

SD, Standard deviation

Materials and Methods

This study was approved by the ethics committee of our institute. Informed written consent was obtained from each patient and their parents. The AIS group consisted of 2 male patients and 12 female patients. The average ages at scoliosis onset (diagnosis) and at surgery were 12.4±2.0 years (range, 10-17 years) and 15.9±2.2 years (range, 12-20 years), respectively. The average Cobb angle at the time of surgery was 48.2±8.9° (range, 37°-70°). The apical vertebrae were located between Th8 and L2. There were 7 Lenke type 1, 1 Lenke type 2 and 6 Lenke type 5 patients[12]. Risser grades in the AIS group are shown in Table 1[13]. Magnetic resonance imaging around the brainstem and whole-spine myelography with computed tomography were performed to exclude diseases or malformations of the nervous system. All patients were treated with spinal orthosis until surgery. Patients with prior spine surgery and other surgeries that affected the onset of scoliosis were excluded. All patients underwent posterior spinal correction and instrumented fusion. Bilateral paravertebral muscles were harvested from the main curvature of the apex site. Seven patients (5 male patients and 2 female patients) who underwent posterior spinal surgery without scoliosis were served as the control group. In the control group, the average age was 26.4±9.7 years (range, 16-41 years), and there were 3 spinal tumors, 1 lumbar disc herniation, 1 traumatic fracture of the spine, 1 cervical flexion myelopathy and 1 ossification of the spinal ligaments. The mRNA expression of *LBX1* was evaluated by real-time polymerase chain reaction (PCR).

Tissue specimens and experimental material

During surgery, bilateral multifidus muscles were obtained from the apical level in the AIS group and from the center of the surgical site (C6, C7, T5, T7, T11, L1 and L4 levels) in the control group. Muscle samples were placed in separate sterile tubes and immediately stored in liquid nitrogen. Frozen samples were stored at −80°C until analysis.

RNA isolation and cDNA synthesis

Muscle samples were homogenized with ISOGEN (Nippon Gene, Toyama, Japan) and then centrifuged at 12,000 g at 4°C for 10 minutes. Chloroform (200 μL) was added to the collected supernatant, and the mixture was centrifuged at 12,000 g at 4°C for 15 minutes. Isopropanol (500 μL) was added to an equivalent amount of supernatant. Then, 1 mL of ethanol was added after the supernatant was discarded and centrifuged at 7,500 g at 4°C for 5 minutes. The sediment was dried at room temperature and then diluted with 100 μL of DEPC-treated water and quantified by spectrophotometry (A260/280). The RNA concentration was determined by absorbance at 260 nm by using a U-2000 spectrophotometer (Hitachi Ltd., Tokyo, Japan). Isolated total RNA was treated with DNase I (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer's instructions. Then, cDNA was synthesized with the First Strand cDNA Synthesis Kit (GE Healthcare, Buckinghamshire, UK), according to the manufacturer's instructions.

Real-time PCR

Quantitative PCR primer assay for human *LBX1* (QIAGEN, Japan) was used for reaction according to the manufacturer's instructions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenous control gene for normalization. Primers of GAPDH were synthesized by Greiner Japan in Tokyo. The sequences of the sense (-F) and antisense (-R) primers were as follows : GAPDH-F (5´-TCCACCTTTGACGCTGGGGC-3´), GAPDH-R (5´-GGCCATGAGGTCCACCACCCT-3´). The expected size of PCR products for GAPDH was 111 bp. Before real-time quantitative PCR, products were analyzed by electrophoresis on a 2.0% agarose gel containing ethidium bromide. The LightCycler 480 (Roche Diagnostics, Laval, QC, Canada) and LightCycler 480 SYBR Green 1 Master (Roche Diagnostics, Laval, Canada) were used for quantitative analysis of *LBX1* mRNA expression. Reaction conditions were as follows : 5 min at 95°C (denaturation step) ; 45 cycles of 10 s at 95°C, 10 s at 58°C, 10 s at 72°C ; 5 s at 95°C ; 65°C to 98°C at a rate of 2.2°C/s (melting curve analysis).

Statistical analysis

All results were expressed as mean±SD. The *LBX1* mRNA expression on the concave side (left side for the control group) was compared with the convex side (right side for the control group). The paired *t* test was used to compare *LBX1* mRNA expression in bilateral paravertebral muscles. The expression ratio, calculated as the level of expression at the convex side relative to the concave side, was compared between the groups by the Student's (or Welch) *t* test. Finally, relationships between Cobb angle and expression ratio were analyzed by Pearson's correlation coefficient. All analyses, with two-sided *p* values, were performed by using the Statistical Package for the Biosciences (SPBS v9.54) [31]. *P* values < 0.05 were considered statistically significant.

Results

Comparison of LBX1 mRNA expression in bilateral paravertebral muscles

In the AIS group, the *LBX1* mRNA expression on the convex side and concave side paravertebral muscles at the apical level was 1.54±0.76 (range, 0.47-2.52) and 1.01±0.83 (range, 0.16-3.53), respectively. The *LBX1* mRNA expression on the convex side was significantly higher than that on the concave side ($p=0.020$) (Fig. 1). In the control group, the *LBX1* mRNA expression was 0.94±0.50 (range, 0.27-1.60) on the right side paravertebral muscles and 1.07±0.45 (range, 0.62-1.7) on the left side. There was no significant difference of *LBX1* mRNA expression in the control group ($p=0.509$) (Fig. 1).

Comparison of LBX1 mRNA expression ratio between the two groups

The expression ratio of *LBX1* was significantly higher in the AIS group (1.99±1.24, range 0.71-4.83) than in the control group (0.95±0.43, range 0.18-1.49) ($p=0.012$). (Fig. 2).

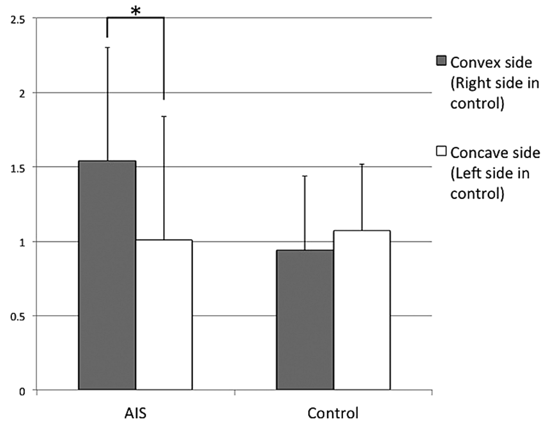

Fig. 1. The *LBX1* mRNA expression on the convex side of paravertebral muscles in the AIS group was significantly higher than that on the concave side ($p=0.020$). (Right side is taken as the convex side for the control group.) $^{*}p < 0.05$

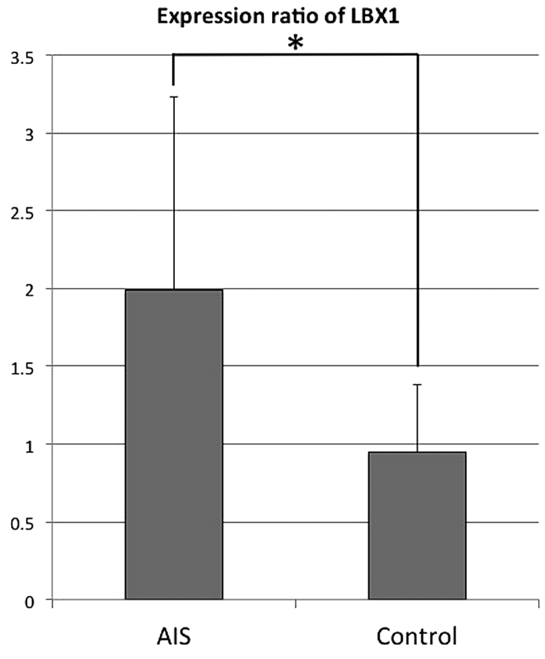

Fig. 2. The expression ratio of *LBX1* mRNA in the AIS group was significantly higher than that of the control group ($p=0.012$). $^{*}p<0.05$

Correlation between LBX1 mRNA expression and Cobb angle

In the AIS group, the expression ratio of *LBX1* was not significantly correlated with Cobb angle ($r=-0.3826$, $p=0.1770$).

Discussion

Although mouse *Lbx1* and human *LBX1* were first identified as a homeobox gene family related to the Drosophila lady bird genes[14], little is known about its physiological functions. In 2011, Takahashi *et al*. identified candidate genes for AIS. They performed genome-wide association study and indicated that the most significant single nucleotide polymorphism (SNP) was located near *LBX1*. In addition, they found high expression level of *LBX1* in both adult and fetal human skeletal muscle and spinal cord[11]. This SNP may lead to skeletal muscle and/or somatosensory dysfunctions due to *LBX1* dysfunction. The role of the paravertebral muscles in the pathogenesis of scoliosis has been the subject of much investigation. Although, the etiology of idiopathic scoliosis is considered multifactorial at present[2, 15, 16], no previous studies have investigated the expression level of *LBX1* in the paravertebral muscles of AIS patients. Here, we focused on the expression level of *LBX1* mRNA in bilateral paravertebral muscles in the AIS patients. The expression of *LBX1* mRNA on the concave side was higher than that on the convex side in the AIS group, expression ratio of *LBX1* in the AIS group was also significantly higher than in the control group. But, the expression ratio of *LBX1* was not significantly correlated with Cobb angle. Similar to previous studies investigated asymmetry of paravertebral muscles in AIS, it is always difficult to conclude whether morphologic and/or physiological changes of the paravertebral muscles in AIS are primary or secondary.

Recently, some studies identified *Lbx1* functions in the animal experiment. In the studies of mice lacking *Lbx1*, these mice showed loss of limb muscles, especially extensor muscles due to failure of muscle precursor migration. Conversely, *Lbx1* was expressed in migrated hypaxial muscle precursors. These facts suggest *Lbx1* plays an important role during lateral migration of hypaxial muscle precursors into the limb[17-19]. In humans, the erector spinae and the trasversospinal muscles including multifidus muscles are the epaxial muscles. Differently from previous studies, contribution of *lbx1*-positive myoblasts to the formation of both epaxial and hypaxial musculature was indicated in a study using Xenopus. It was suggested that regulation of these two muscles type related to *lbx1* was more similar than previously described[20]. In a study investigated postnatal *Lbx1* function, *Lbx1* was expressed in activated satellite cells after muscles were damaged by cardiotoxin. In addition, *Lbx1* expression was downregulated when satellite cells differentiate into mature myofibers. These results suggest that *Lbx1* plays important roles in not only migration of muscle precursors but also differentiation of satellite cells[21]. If *LBX1* influences paravertebral muscle functions because of physiological changes in muscle cells, development and progression of scoliosis may be modified based on so-called "Hueter-Volkmann law"[22] following alteration of mechanical load to the spine.

There are several limitations to the present study. First, this study included a relatively small sample size. Second, the mean age and the ratio of males to females of the control group was significantly higher than that of the AIS group. There have been no studies described impact of age and gender on *LBX1* mRNA expression in paravertebral muscles ; however, we needed to demonstrate symmetric expression in patients without scoliosis to demonstrate that our experiment was valid. Third, all muscle samples were obtained from AIS patients with severe spinal curvature that required surgery ; thus, the changes in mRNA expression could be the result rather than the cause of disease. The present study is a preliminary examination on *LBX1* expression in paravertebral muscles of AIS to investigate possible factors related to curve progression or severity. Further studies are required to establish whether asymmetric expression exists in AIS with mild scoliosis and these findings predict curve progression.

In conclusion, higher mRNA expression level of *LBX1* was observed in the convex side of paravertebral muscles in AIS. The expression ratio of *LBX1* was significantly higher as compared with the control group. These findings suggest the possible functional role of paravertebral muscles in the development or progression of the spinal curve. However, we are unable to conclude whether these results in the current study were primary or secondary change. Further experiments are needed to clarify these questions.

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