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Clinical Study

Paraspinal muscle ladybird homeobox 1 (LBX1) in adolescent idiopathic scoliosis: a cross-sectional study

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Abstract

BACKGROUND CONTEXT: Adolescent idiopathic scoliosis (AIS) is the leading cause of spinal deformity in adolescents globally. Recent evidence from genome-wide association studies has implicated variants in or near the ladybird homeobox 1 (*LBX1*) gene, encoding the ladybird homeobox 1 transcription factor, in AIS development. This gene plays a critical role in guiding embryonic neurogenesis and myogenesis and is vital in muscle mass determination. Despite the confirmation of the role for *LBX1* gene variants in the development of AIS, the biological basis of *LBX1* contribution to AIS remains mostly unknown.

PURPOSE: To investigate the potential role of *LBX1* in driving spinal curving, curve laterality, and progression through muscle-based mechanisms in AIS patients by analyzing its gene and protein expression.

STUDY DESIGN: This is a cross-sectional study using clinical data and biological samples from the Immunometabolic CONnections to Scoliosis study (ICONS study).

PATIENT SAMPLE: Twenty-five patients with AIS provided informed consent. Paraspinal muscle biopsies from the maximal points of concavity and convexity for gene expression and protein analysis were obtained at the start of corrective spinal surgery.

OUTCOME MEASURES: The outcome measures included the detection of paraspinal muscle *LBX1* mRNA abundance and *LBX1* protein expression and the correlation of the latter with age, sex, and curve severity.

METHODS: The measurement of mRNA abundance was done using quantitative real-time polymerase chain reaction (qRT-PCR). Additionally, protein lysates from the biopsied muscle samples were probed with a monoclonal LBX1 antibody to compare the muscle protein levels on either side of the scoliotic curve by western blot. This study received funding from the Division of Orthopedics, Department of Surgery, McMaster University, Hamilton, Ontario, Canada ($39,900 CAN for 2 years). The authors have no conflicts of interest to disclose.

RESULTS: *LBX1* mRNA abundance (concave 2.98±0.87, convex 3.40±1.10, p value 0.73) and protein expression (concave 1.20±0.13, convex 1.21±0.10, p value 0.43) were detected on both

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sides of the scoliotic curve at equivalent levels. The expression of LBX1 protein did not correlate with age (concave: correlation coefficient 0.32, p value 0.12; convex: correlation coefficient 0.08, p value 0.69), sex (concave: correlation coefficient −0.03, p value 0.08; convex: correlation coefficient 0.07, p value 0.72), or the severity of spinal curving measured using the Cobb angle (concave: correlation coefficient −0.16, p value 0.45; convex: correlation coefficient −0.08, p value 0.69).
CONCLUSIONS: *LBX1* is expressed in erector spinae muscles, and its levels are equal in muscles on both sides of the scoliotic curve in AIS. The expression of *LBX1* on the convex and concave sides of the scoliotic curve did not correlate with age, sex, or the severity of spinal curving. The molecular mechanisms by which *LBX1* contributes to the development and propagation of AIS need to be explored further in muscle and other tissues.

Keywords: Adolescent idiopathic scoliosis; ICONS study; ladybird homeobox 1; paraspinal muscle

Introduction

Adolescent idiopathic scoliosis (AIS) is the most common spinal deformity in the pediatric population, with a prevalence of around 3% globally [1–5]. The treatment of AIS in advanced cases is complex and includes surgery which is costly [6] and is associated with mortality and postoperative morbidities including neurological deficits, wound infections, and pulmonary complications [7,8]. Importantly, the complication rates are significantly higher in cases requiring a revision surgery [7,8]. There is a pressing need to understand the mechanisms driving AIS so that targeted management strategies are offered to improve outcomes.

Although the exact etiology of AIS is unknown, several lines of evidence have pointed to genetic factors as a critical pathway in its genesis. AIS is more common in twins and in those with a positive family history than the general population [9,10]. Although several genes have been implicated in AIS, these findings have not been reproduced [11–19].

Human genome-wide association studies (GWAS) have consistently linked the ladybird homeobox 1 gene (*LBX1*) to spinal curve creation and progression in AIS [20–22]. Polymorphisms in the *LBX1* gene alter the susceptibility to scoliosis in a sex- and ethnicity specific manners. For example, the T allele of rs111090870 has been linked to increased susceptibility and progression of AIS in Asians, Caucasians, and females [14,22–28]. On the other hand, the G allele of two polymorphisms near the gene, including rs678741 reduces AIS risk in females, and rs625039 increases the risk in Asians [14,22–28].

Although the exact function of *LBX1* is unknown in humans, a recent case report has suggested that *LBX1* microduplication may play a role in the development of congenital scoliosis, paraspinal muscle development, and muscle mass determination [29]. The mechanisms by which paraspinal muscle *LBX1* contribute to spinal curving in AIS remain unclear.

Murine studies have demonstrated that *LBX1* encodes a homeodomain-containing transcription factor that is important during ontogeny in cardiogenesis, myogenesis, and neurogenesis. Also, *LBX1* plays an essential role in muscle cell precursor migration and patterning of limb musculature in utero [30–36]. It is not fully clear to what degree does the human muscle *LBX1* gene mimic the functions of its murine counterpart.

Based on the limited human muscle data and the GWAS reports suggesting a role for *LBX1* in AIS genesis, paraspinal muscle *LBX1* role in curve occurrence, laterality, and severity was assessed. We tested the hypothesis that in patients with AIS, *LBX1* is expressed differentially in paraspinal muscles on convex versus concave sides of the scoliotic curve, and this differential expression is associated with curve severity.

Methods

This cross-sectional study is using clinical data and biological samples from the Immunometabolic Connections to Scoliosis study (ICONS study). The study is focused on the mechanistic analysis of the role of the immune system in scoliosis. The protocol for the study and study feasibility has been published [37,38].

Population

Participants were recruited into the study from the Orthopedic clinics at McMaster Children's Hospital, a tertiary pediatric academic center in Hamilton, Ontario, Canada.

The study included female and male participants with a confirmed diagnosis of AIS who were scheduled to have spinal surgery for scoliotic curve correction.

The exclusion criteria comprised those with an active infection or those who are on immunosuppressive therapies within 2 weeks of surgery, pregnancy, or smoking.

Study procedures

Recruitment took place between November 2013 and June 2017. During a clinic visit before surgery, the study was presented to potential participants. If there was an agreement to participate, patients or their guardians provided written informed consent for participation in the study. The participant signed a consent form if they were

16 years or older. For those participants 10–16 years of age, the participant signed an assent form, and the parent or guardian signed the consent forms. The institutional Research Ethics Board approved the study.

Following the signature of the consent forms, several measurements were taken including height to the closest 0.1 cm using a stadiometer, and the fat mass percentage and weight using a Tanita electronic scale (Tanita Corporation, USA). Blood pressure and pulse rate were documented twice in the sitting position using a digital blood pressure monitor, and the average values were reported [37,38].

The families and participants filled questionnaires related to participants' general health, past medical history, medications, family history, birth history, nutrition, physical activity, sleep, and psychological health. Data related to the verification of the diagnosis of AIS as well as imaging and other clinical details were extracted from the clinical notes [37,38].

Biological sample collection

Muscle biopsies

Muscle samples were collected from the erector spinae muscles at the maximal points of concavity and convexity of the scoliotic curves at the start of the surgery. To ensure the fidelity of the muscle biopsy samples for later analyses, they were obtained before tissue cautery, which is known to cause muscle contraction. Biopsies were also performed before epinephrine injection, which is periodically used to control bleeding from the surgical site to avoid alteration of the muscle phenotype. Following dissection from connective tissue, samples were dried from blood gently with a piece of gauze. The samples were divided into several pieces that were snap frozen immediately in liquid nitrogen. Samples were stored at −80°C until further processing for isolation of RNA and protein.

Gene expression analysis

To assess *LBX1* mRNA abundance, muscle samples were powdered and homogenized in 1 mL of Trizol reagent using the Precellys 24 Homogenizer (Bertin Technologies, Paris, France) with ceramic beads. RNA was isolated using the Qiagen RNAeasy Mini Kits (Qiagen, Valencia, CA, USA) in accordance with manufacturer's guidelines. One microgram of RNA was used to generate cDNA using the Superscript VILO cDNA Synthesis kit (Thermo Fisher Scientific, Canada). cDNA was diluted 1:10 before Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) analysis using the TaqMan™ Gene Expression Assays (Thermo Fisher Scientific, Canada). All qRT-PCR reactions were performed using a Rotor-Gene 6000 machine (Corbett Research, Mortlake, Australia).

Protein expression

Muscle samples were powdered and homogenized in homogenization buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 50 mM NaF, 5 mM Na pyrophosphate, 10% glycerol, 1% Triton X-100 and protease inhibitor cocktail [Roche]) using the Precellys 24 Homogenizer (Bertin Technologies, Paris, France) with ceramic beads. Protein lysate concentrations were measured using the BCA assay (Thermo Fisher Scientific, Canada).

SDS-PAGE was used to resolve 40 micrograms of total protein by molecular mass in a 7.5% polyacrylamide gel before electrophoretic transfer to a polyvinylidene difluoride membrane (Bio-Rad) using the Mini Trans-Blot Cell (Bio-Rad). Protein-immobilized polyvinylidene difluoride membranes were blocked for one hour at room temperature with a 5% bovine serum albumin solution in 1X TBST (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) before overnight incubation at 4°C with 1–5 ug/mL of mouse monoclonal Anti-LBX1 antibody (Abcam, ab56480) diluted in 1X TBST. Anti-mouse IgG, HRP-conjugated secondary antibody (Cell Signaling Technology) was applied to membranes for one hour at room temperature. Proteins were detected by chemiluminescence with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Canada) using the Montreal Biotech Gel Doc System (Montreal Biotech, Montreal, Canada).

Protein bands were quantified using Image J software (1.51k, NIH) and were normalized to total protein as measured by Ponceau staining (Sigma Aldrich).

Statistical analysis

Statistical analysis was performed using SPSS version 25.0. Data were tested for normality using the Kolmogorov-Smirnov and Shapiro-Wilk tests, and non-normally distributed data on either test were log transformed.

Continuous variables were analyzed and reported as mean±SD, except gene expression and western blot data that are reported as mean±SE. Dichotomous variables were analyzed and reported as numbers (%).

Correlations were performed using Spearman's correlation test. The comparison of *LBX1* concave and convex gene and protein expression data were performed using the Wilcoxon signed rank test, with the p value set at 0.05 to delineate statistical significance.

Results

The study included 25 patients with AIS. The characteristics of the study population are reported in Table 1. The majority of participants were female (n=21, 84%), and the age of menarche was 12.80±1.80. The majority of participants were Caucasian (n=20, 80%), and most curves were right-sided (n=18, 72%). All curves were significant and qualified for surgical correction (Cobb angle 68.80±15.40°).

The qRT-PCR was performed to assess for differences in the abundance of *LBX1* mRNA in muscle from the convex versus the concave sides of the spine. The analysis detected muscle *LBX1* mRNA on both sides of the scoliotic curve, with a similar gene expression pattern noted on both sides

Table 1
Summary of participant characteristics. The patient characteristics for 25 AIS patients who participated in this study are shown

Variable	Mean (Range)	Standard deviation
Age at enrollment (y)	13.80 (11.00–17.00)	1.50
Cobb angle	68.80 (40.00–104.00)	15.40
Height (cm)	159.60 (130.40–186.10)	11.60
Weight (kg)	52.32 (41.00–96.30)	11.90
BMI percentile (%)	50.50 (4.95–98.90)	29.80
Systolic blood pressure (mm Hg)	117.30 (98.00–145.00)	11.20
Diastolic blood pressure (mm Hg)	73.40 (57.00–89.00)	8.60
Pulse rate (bpm)	88.65 (53.00–126.00)	18.70

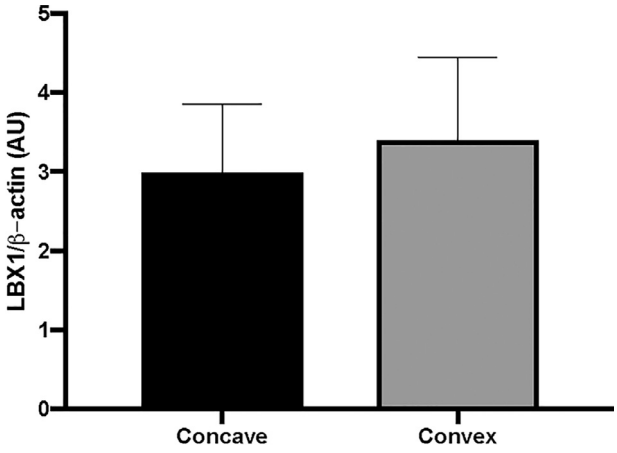

Fig. 1. LBX1 mRNA abundance on the concave and convex paraspinal muscle of AIS patients.

of the scoliotic curve (Fig. 1; concave 2.98±0.87, convex 3.40±1.10, p value 0.73).

To determine if the transcription of *LBX1* mRNA translated to a protein product, western blot analyses were performed. The protein expression levels were correlated with age, sex, and the Cobb angle. The protein levels in muscle on the concave and convex sides correlated strongly with each other (correlation coefficient 0.58, p value 0.002). The protein analysis revealed similar LBX1 protein expression in muscle from the concave and convex sides of the scoliotic curve (Figs. 2 and 3; concave 1.20±0.13, convex 1.21±0.10, p value 0.43).

Next, we assessed whether LBX1 protein expression correlated with participants' characteristics. Table 2 demonstrate that LBX1 protein did not correlate with age (concave: correlation coefficient 0.3 0.12; convex: correlation coefficient 0.08, p value 0.69), sex (concave: correlation coefficient −0.03, p value 0.08; convex: correlation coefficient 0.07, p value 0.72), or the severity of spinal curving measured using the Cobb angle (concave: correlation coefficient −0.16, p value 0.45; convex: correlation coefficient −0.08, p value 0.69).

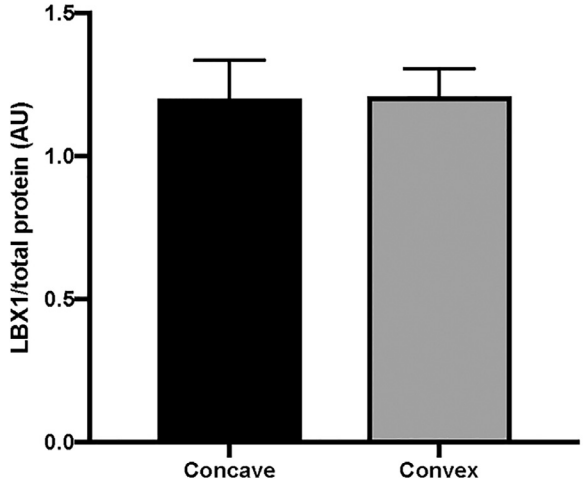

Fig. 2. LBX1 protein expression comparison between paraspinal muscles on the concave and convex sides of the scoliotic curve.

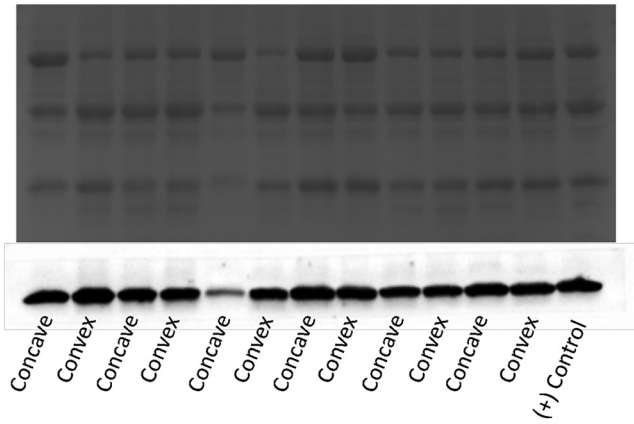

Fig. 3. LBX1 expression is equal on both sides of the scoliotic curve. Representative immunoblot (bottom) of paraspinal muscle lysates probed with mouse monoclonal Anti-LBX1 antibody (Abcam, ab56480). LBX1 levels were normalized to total protein as measured by Ponceau staining (top). (+) control lane refers to a collection of pooled lysates which was loaded on each gel and served as a standard.

Taken together, these data demonstrate that *LBX1* is expressed in paraspinal muscles in AIS. Also, the expression levels were similar between the concave and convex sides of the scoliotic curve and were not correlated with the severity of the spinal curvature.

Discussion

Adolescent idiopathic scoliosis affects millions of children around the world, yet there is no unifying mechanistic model for its development. This study investigated the role of muscle *LBX1* in AIS, a gene that has been linked with AIS in GWAS and recent meta-analyses [14,21,23,27,39].

It was demonstrated that *LBX1* was expressed in the erector spinae muscle in AIS patients. The expression levels of *LBX1* were similar in paraspinal muscles on both sides of

Table 2
LBX1 expression does not correlate with age, sex, or cobb angle in AIS. Spearman's rho analysis correlation between LBX1 expression and participant characteristics (n=25)

Variable	Concave		Convex	
	Correlation coefficient	p Value	Correlation coefficient	p Value
Age	0.32	0.12	0.08	0.69
Sex	−0.03	0.08	0.07	0.72
Cobb angle	−0.16	0.45	−0.08	0.69

the scoliotic curve and were not linked to the age of participants or their sex. Importantly, LBX1 expression did not associate with the severity of the scoliotic curve.

LBX1 gene has two exons and is located on chromosome 10 in humans [40]. It was identified by homology to Drosophila Ladybird early and late homeobox genes [33], and encodes a 281-amino acid transcription factor that is expressed in the central nervous system as well as migrating muscle precursor cells and activated satellite cells [31,32]. The latter are muscle stem cells that are present between the muscle fiber basement membrane and the sarcolemma [41].

This gene plays a critical role in muscle embryonic development, as it determines the migratory routes of hypaxial muscle precursor cells leading to the foundation of distinct muscle patterns in the limbs [33,36,42,43].

In mice, *LBX1* knockout leads to impaired development of the hindlimbs and the extensor muscles of the forelimbs due to myocellular migration failure [44,45]. Importantly, data from adult mice demonstrate that *LBX1* is quiescent in inactive satellite cells and is upregulated when there is a need for myofiber generation during development and with injury and is then downregulated when myofibers mature [41].

Although the function of *LBX1* is unknown in humans, two human phenotypes provide essential insights into its potential role and association with scoliosis. A recent case report demonstrated that a 130 kb microduplication at 10q24.31 that exclusively affects the LBX1 gene in a 12-year-old girl was associated with congenital scoliosis and paravertebral muscle hypotrophy/hypoplasia [29]. This microduplication may interfere with satellite cell migration and activity, neuronal and muscle development, and other mesoderm-derived cells or neural tube development, thus leading to the muscle and spinal phenotypes noted [29].

In addition, patients with split-hand/foot malformation 3 (SHFM3) have partial or complete duplications of several genes in the 10q24.31 region that may include *LBX1*, but do not have scoliosis or a muscle phenotype. The SHFM3 patient phenotype is likely caused by other gene duplications in the region [46–48].

The detection of bilateral and equivalent expression of *LBX1* in AIS patients is significant, as this may indicate the upregulation of satellite cell function related to paraspinal myofiber remodeling or regeneration in AIS. This possibility requires further exploration.

On the other hand, equivalent bilateral *LBX1* expression is not in disagreement with the observation that specific *LBX1* gene variants are associated with AIS development. The expression of *LBX1* is during ontogeny, and AIS-associated *LBX1* gene variants presumably alter gene expression unequally on both sides of the scoliotic curve during embryogenesis of the concave and convex paraspinal muscle cells but this expression equalizes postnatally. Whether *LBX1* gene is differentially expressed in the paraspinal muscle on both sides of the scoliotic curve during human or murine embryogenesis or at the early stages of scoliosis development is unclear and requires additional analysis.

One strength of this study include the detailed analyses of paraspinal muscles using qRT-PCR and western blotting to assess the expression of *LBX1*. A limitation of this study is the inability to perform the same analysis on muscle biopsies from non-scoliosis controls. This comparison with controls could help clarify how paraspinal muscle LBX1 protein levels are affected, if at all, by the presence of *LBX1* gene variants. The most widely validated *LBX1* risk variant, rs11190870, maps to the 3' region of *LBX1* where it is believed to influence gene expression, and surprisingly no functional *LBX1* variants have been identified so far. Another limitation of this study is the cross-sectional design, which limits the potential of ascribing a causal relationship between muscle *LBX1* and AIS. However, as humans are the only species that develop AIS, there are no alternative models that faithfully replicate the mechanisms of its development.

In conclusion, *LBX1* expression is detected in paraspinal muscle in AIS patients. *LBX1* is expressed at equivalent levels on both sides of the scoliotic curve, and these levels are not correlated with the severity of the curve. Considering its association with AIS that is noted in genetic studies and current human data, further exploration of the exact tissue-specific molecular mechanisms of how *LBX1* contributes to AIS is warranted.

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