Dipeptidyl peptidase-4 is associated with myogenesis in patients with adolescent idiopathic scoliosis possibly via mediation of insulin sensitivity

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Abstract

Background: Abnormal metabolic features have been previously described in adolescent idiopathic scoliosis (AIS) patients. As an important regulator involved in energy metabolism, DPP-4 activity was reported to be remarkably decreased in osteoblasts of AIS patients. To date, there was still a lack of knowledge concerning the role of DPP-4 in the myogenesis of AIS.

Methods: Circulation DPP-4 level was assessed in the serum of 80 AIS girls and 50 healthy controls by ELISA. Myoblasts were purified from muscle specimens of AIS patients and LDH controls, and then treated with metabolic effectors including glucose and insulin. CCK-8 assay was used to assess the cell viability and myotube fusion index was calculated to evaluate myogenesis ability. Gene expressions of downstream signals of DPP-4 were evaluated by RT-qPCR and Western blot respectively.

Results: AIS girls had remarkably down-expressed DPP-4 in both serum level (0.76 fold) and tissue (0.68 fold) level. Treatment with metabolic effectors led to significantly increased DPP-4 expression in the control cells, while there was no increase of DPP-4 in AIS cells. CCK-8 assay showed that the proliferation rate of control cells was significantly increased after being treated. Remarkably higher fusion index was also observed in the treated control cells. By contrast, the fusion index and cell proliferation rate were comparable between the treated and the untreated AIS cells.

Conclusions: Our study suggested a potential role of DPP-4 in abnormal metabolic condition of AIS patients. Compared with control cells, AIS myoblasts presented obviously impaired sensitivity to the treatment of glucose and insulin. Aberrant DPP-4 expression could lead to impaired insulin sensitivity in myoblasts and further influence the cell viability during myogenesis. The molecular mechanism connecting DPP-4 and insulin-related signaling in AIS is worthy of further investigation.

Keywords: Adolescent idiopathic scoliosis, Dipeptidyl peptidase-4, Insulin sensitivity, Metabolism, Signaling

Introduction

Adolescent idiopathic scoliosis (AIS) is a 3-dimensional deformity which occurs during the pubertal growth [1]. To date, there is no consensus on the etiology of AIS. Many factors were proposed to be involved in the development of AIS, including genetic variation, metabolism dysfunction, and abnormal neuromuscular function.
It has been well documented that AIS patients tended to have different anthropometric parameters compared with age-matched controls, such as taller stature with lower body mass index (BMI) and lower bone mineral density (BMD), while the cause of these differences remains obscure [5, 6]. Since there was no significant difference of nutrition intake between AIS girls and controls, it was therefore speculated that the abnormal energy homeostasis including appetite regulation, energy expenditure and insulin sensitivity might be impaired in AIS girls [7]. Several hormone and metabolic molecule, such as leptin [8], melatonin [9, 10], and lipid metabolite [11] have been revealed to be associated with AIS.

Dipeptidyl peptidase-4 (DPP-4) was a type of transmembrane protein that can modulate insulin-related metabolism [12]. DPP-4 inhibitor, launched and wildly used in clinical practice, was proven to improve BMD and decrease the risk of fracture in type 2 diabetes, establishing a relationship between DPP-4 and bone quality [13]. Normand et al. [14] observed lower serum expression of DPP-4 in AIS patients. Moreover, remarkable differences in DPP-4 activity and regulation between osteoblasts of AIS patients and healthy controls were found in their study.

Except for decreased bone density, AIS patients also presented lower body mass and abnormal growth pattern of paraspinal muscles. Previous studies have shown that DPP-4 could modulate muscular energy homeostasis, insulin secretion and sensitivity directly, which may further affect muscle proliferation and differentiation in adolescents [15–17]. However, to our knowledge, there was a lack of study investigating the role of DPP-4 in the modulation of myogenesis in AIS patients. In the current study, we aimed to investigate the influence of DPP-4 on the biological viability of myoblasts and to further characterize its role in the metabolic condition of AIS.

Sample collection
Blood samples were collected from 50 healthy girls and 80 AIS patients. All the blood samples were treated with sodium citrate, centrifuged to isolate serum, and stored at −80 °C until thawed and analyzed. Paravertebral muscles of the proximal vertebrae were collected from 45 AIS and 30 LDH patients during surgery with the informed consent obtained from their parents. Deep paraspinal muscle biopsies of 1.5 × 1.5 × 1.5 cm³ were stored at −80 °C directly.

Enzyme-linked immunosorbent assay (ELISA)
ELISA analysis of serum DPP-4 was conducted according to the protocol previously described by guidebook of CD26 Human ELISA kit (Abcam, Cambridge, UK). Assays were performed according to manufacturer’s protocol and samples were analyzed without dilution.

Myoblast isolation and purification
Paraspinal muscle of 3 AIS patients and 3 LDH controls were randomly selected for digestion to single cell suspension, treated with mixed enzyme containing Collagenase D (0.15 U/mg, Sigma, Darmstadt, Germany) 10 mg/mL, Dispase II (0.5 U/mg, Sigma, Darmstadt, Germany) 4.8 mg/mL, and 250 mM CaCl₂ 50 mM/mL. After centrifugation, cells were resuspended in growth media containing F12 (Gibco) complemented with 20% FBS (Gibco), 1% P/S (Gibco), 1% α-glutamine (Sigma), and 2.5 ng/mL FGF (Sigma). The culture medium was changed every 2 days. After 7–10 days, when cells reached 70–80% confluence, the purification of myoblast was conducted with immunomagnetic beads technology following the manufacture instructions of CD56 MicroBeads (Miltenyi, Gladbach, Germany). After 3–5 days of cell proliferation, the purity of myoblasts was then identified by immunofluorescence (Additional file 1, 2: Figs. S1, S2).

Immunofluorescence microscopy
The myoblasts were blocked by being incubated in PBS supplemented with 2% goat serum (block solution) for 30 min. After removal of the block solution, the samples were treated with primary antibody rabbit anti-desmin (1:200; Abcam), mouse Anti-MHC antibody (1:50; Developmental Studies Hybridoma Bank) and incubated overnight at 4°C in a humidified chamber. The sections were washed with PBS and then incubated for 1 h in a dark chamber with Alexa Fluor 594-conjugated Affinipure Goat Anti-Mouse IgG (H+L) (1:500; Jackson ImmunoResearch) and Alexa Fluor 488-conjugated Affinipure Goat Anti-Rabbit IgG (H+L) (1:500, Jackson ImmunoResearch). DAPI (Thermo fisher scientific,
Massachusetts, USA) was used to seal slides for 4 min in the dark. Images of stained cells were taken using a fluorescence microscope (Axio Observer, Zeiss, Oberkochen, Germany). 3 slides were observed in each case, and positive-staining cell was counted via ImageJ software.

**Cell culture and metabolic effector treatment**

Myoblasts were cultured in growth medium consisting of F-12 (Gibco) with 20% FBS and 1% penicillin-streptomycin at 37°C. To evaluate glucose metabolism in myoblasts, 0.5 mM insulin (Beyotime, Shanghai, China) and 10 mM glucose (Sigma-Aldrich, Oakville, ON, Canada) were added to the wells after a 24 h pre-incubation with serum-free F-12 media. Cell viability was assessed using the CCK-8 assay, for each sample, the cells incubated only with growth medium were used as the negative control.

To induce myogenic differentiation, the culture medium was switched to F12 (Gibco) supplemented with 2% horse serum (Gibco), 1% P/S (Gibco), 1% α-glutamine (Sigma). Myoblasts from AIS and control group were induced to differentiation added with metabolic effectors for 5 days, respectively. Cells incubated without glucose and insulin were used as the negative control. Fusion index was used to assess the formation of myotube as previously reported [18].

**RNA extraction and real time-polymerase chain reaction (RT-qPCR) analysis**

Total RNA was extracted from tissue and myoblasts with TRIzol reagent (Invitrogen). Gene expression levels were quantitated using SYBR Master Mix (TAKARA, Tokyo, Japan) on a Light Cycler 480 (Roche Applied Science, Mannheim, Germany) with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the endogenous control. The amplification protocol included an initial denaturation step at 95°C for 10 min, followed by 44 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 20 s and elongation at 72°C for 10 s. The average BMI was 13.9±2.5 years and 14.3±3.5 years (p = 0.44), respectively. The average BMI was 17.9±2.3 kg/m² for AIS patients and 18.5±3.0 kg/m² for healthy girls (p = 0.20).

For tissue expression analysis, as shown in Table 1, the mean age was 15.4±2.7 years for AIS patients and...
15.8±4.2 years for controls (p = 0.32). The mean BMI was 18.6±1.6 kg/cm² for AIS patients and 19.8±4.5 kg/cm² for controls (p = 0.10). The mean curve magnitude was 49.3±3.4 degrees. The mean Bone mineral density (BMD) of the AIS patients was 0.84±0.10 g/cm².

Expression of DPP-4 and STAT1 in serum and paraspinal muscles

The serum DPP-4 activity was remarkably lower in AIS girls than in the healthy controls (p < 0.05) (Fig. 1). Tissue expression analysis showed that the mRNA expression of DPP-4 and STAT1 was significantly decreased in patients as compared with that of the LDH controls (0.000020±0.000008 vs. 0.000024±0.000009, p = 0.032 for DPP-4; 0.001151±0.000436 vs. 0.001504±0.000538, p = 0.003 for STAT1) (Fig. 2A, B). Significant correlation between the expression of DPP-4 and STAT1 in paraspinal muscles was observed (r = 0.41, p = 0.005) (Fig. 2C). DPP-4 protein expression in muscle was significantly higher in controls as compared with AIS patients (Fig. 2D, E).

The influence of metabolic factors on DPP-4 expression

With the treatment for 24 h. The mRNA expression of DPP-4 was increased by 2.38 folds in the treated control cells as compared with the untreated control cells (p < 0.01) (Fig. 3A). The DPP-4 protein level increased 1.8 times more in the treated control cells. By contrast, for the AIS group, there is no significance between the treated and the untreated cells (p > 0.05) (Fig. 3B).

The effect of metabolic factors on myoblast differentiation and viability

Remarkably higher fusion index was observed in the treated control cells as compared with the untreated cells in the control group (Fig. 4A, B). By contrast, the fusion index was comparable between the treated cells and the untreated cell in the AIS group (Fig. 4A, B). CCK-8 assay showed that the proliferation rate of control cells was significantly increased after being treated. As for AIS cells, no significant increase of the proliferation rate was observed after being treated (Fig. 4C).

Regulation of Ras/ERK and Akt/mTOR pathway by metabolic effectors

To determine the influence of metabolic effectors on glucose metabolism signaling pathway, protein expression of
Akt/mTOR and Ras/ERK was evaluated by WB. As illustrated in Fig. 5A, B, for Ras/ERK pathway, the Ras protein and ERK phosphorylation expression were significantly elevated in the treated control cells as compared with the untreated control cells (n = 3/group). No significant difference of DPP4 mRNA or protein expression level was observed between the treated and untreated cells in AIS. *p < 0.05, two-tailed Student’s t test, two-tailed Student’s t test. Data are presented as mean ± standard deviation.

Relationship between DPP-4 and clinical features of AIS
As shown in Fig. 6, there was a significant correlation between the expression level of DPP-4 and BMI in AIS patients (r = 0.36, p = 0.01). However, such linear relationship was not observed between DPP-4 expression and curve severity (r = −0.17, p = 0.27) or between DPP-4 expression and BMD (r = 0.02, p = 0.89).
Discussion

Energy metabolism played an important role in muscle development and function, and insulin acts as a significant effector in this procedure [19]. Several studies have reported that the circulating DPP-4 level was related to insulin resistance [20–22]. Daniela et al. [23] reported that the pathological release of DPP-4 in Type 2 diabetes might impair insulin pathway, especially insulin sensitivity. As documented in earlier literatures, abnormal metabolism has been implicated in the etiology of AIS. To characterize the role of DPP-4 in the metabolism of AIS patients, Normand et al. [14] firstly isolated osteoblast from AIS patients and controls and reported abnormally down-regulated expression of DPP-4 in AIS patients. In the current study, we successfully validated that AIS subjects had remarkably lower DPP-4 expression...
expression in both serum and paraspinal muscles when compared with the controls. It was known that STAT1 could regulate the expression of DPP-4 by binding to its promoter region as a transcriptional factor. In line with this research, we observed that the DPP-4 and STAT1 were simultaneously down-expressed in paraspinal muscles of AIS. Moreover, the expression of STAT1 was significantly correlated with that of DPP-4. It was therefore plausible that STAT1 may be involved in the abnormal expression of DPP-4 in AIS patients. It was worthwhile to investigate the molecular mechanism underlying the regulation of STAT1 on DPP-4 in future study.

As reported in earlier literatures, DPP-4 strongly correlates with obesity in normal cohort. Kirino et al. [24] reported that DPP-4 activity displayed strong positive correlations with BMI in healthy young people. Interestingly, DPP-4 expression in AIS subjects was found significantly correlated with BMI. Patients with lower BMI were found to have decreased circulating expression of DPP-4. As for BMD and curve severity, a lack of correlation with DPP-4 expression was noted. Based on these findings, it was likely that insufficient production of DPP-4 could damage physiological energy metabolism in body mass, thereby involved in the development of AIS.

To further determine the role of DPP-4 in the etiology of AIS, we investigated the relationship between DPP-4 expression and insulin sensitivity in the patients. Insulin sensitivity was reported to play an important role in myogenesis. For the first time, we isolated myoblast from AIS patients and evaluated the influence of metabolism on cell viability. A lack of response to the treatment was observed in AIS myoblasts as evidenced by the unchanged DPP-4 expression and cell viability. By contrast, remarkably higher expression of DPP-4 and increased cell viability in normal myoblasts was triggered by insulin and high glucose. Moreover, insulin and high glucose could promote myoblast differentiation in normal myoblast. On the contrary, differentiation potential was not changed after the treatment to AIS myoblast. These outcomes might reflect impaired insulin sensitivity in AIS patients resulted from aberrant regulation of DPP-4, which may in turn affected muscle growth and differentiation.

Ras/ERK pathway and Akt/mTOR pathway were fundamental for insulin-related signal transduction in cell proliferation and differentiation [25, 26]. In the current study, we observed significant divergence regarding expression of downstream signaling between AIS and normal cells after being treated by metabolic effectors. Remarkably increased expression of Ras/ERK was observed in normal myoblasts. By contrast, for AIS myoblasts there was no difference in the expression of Ras/ERK pathway. Moreover, the Akt/mTOR pathway were comparable between the treated cells and the untreated cells in both AIS group and the control group. Our findings partially uncovered the mechanism of impairing insulin-conducted signaling in AIS. Namely, deficiency of DPP-4 may be associated with altered insulin sensitivity, thus leading to down-expressed Ras/ERK signaling in AIS tissues. The mechanism connecting DPP-4 and Ras/ERK pathway is worthy of further exploration.

Based on above results, lower DPP-4 was associated with abnormal metabolism of musculoskeletal system in AIS, thus disturbing the homeostasis of spine and making adolescent more susceptible to scoliosis. We speculated that DPP-4 might not only affect metabolic pathway, but also contribute to the development of AIS.

Two limitations of the present study need to be addressed. The sample size of the control group enrolled in the tissue expression analysis was relatively small. Since it was difficult to obtain the muscles samples from healthy girls, we recruited age-matched female LDH patients and collected the paraspinal muscles during surgery, which may lead to potential bias. Tissues from patients with spine trauma can be collected in future study to further validate our findings. Second, we preliminarily investigated the role of DPP-4 in metabolism of AIS patients through expression analysis. More in-vivo experiments are warranted to further uncover the molecular mechanism underlying the regulation of DPP-4 on insulin sensitivity of AIS patients.

**Conclusions**

Our study suggested a potential role of DPP-4 in abnormal metabolic condition of AIS patients. DPP-4 expression level was down-regulated in patients with AIS. Aberrant DPP-4 expression could affect insulin sensitivity in myoblasts and further influence the cell viability during myogenesis. The molecular mechanism connecting DPP-4 and insulin-related signaling such as Ras/ERK pathway is worthy of further investigation.

**Abbreviations**

AIS: Adolescent idiopathic scoliosis; DPP-4: Dipeptidyl peptidase-4; LDH: Lumber disc herniation.

**Supplementary Information**

The online version contains supplementary material available at https://doi.org/10.1186/s13018-022-02978-w.

*Additional file 1: Fig. S1.* The purity of myoblasts identified by immunofluorescence. The percentage of Desmin positive cells was used to evaluate the purity of primary myoblasts isolated from AIS and LDH patients (n = 3/group). The purity was more than 90%.

*Additional file 2: Fig. S2.* Impact of treatment with glucose and insulin on AKT/mTOR pathway. The p-AKT and p-mTOR protein expression was
comparable between the untreated and treated group (n = 3/group). ns, not significant. two-tailed paired Student’s t test. Data are presented as mean ± standard deviation.

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Authors’ contributions
ZCD, YQ and ZZZ were responsible for the design and writing of the manuscript. BCX, ZCW and ZHF were responsible for the collection of the data and images. LLX were responsible for the statistics and analysis of the data. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Declarations
Ethics approval and consent to participate
This study was approved by the Ethics Committee of the Nanjing University Medical School Affiliated Nanjing Drum Tower Hospital, China (No. 2019-066-01) and informed consent was taken from all the patients.

Consent for publication
We have obtained written consent to publish from the participant to report individual patient data.

Competing interests
The authors declare that they have no competing interests.

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