Overexpression of YAP and miR-130a is closely related to pulmonary hypertension in congenital diaphragmatic hernia

Junzuo Liao¹, Wenying Liu^{1,2,*}, Libin Zhang¹, Qin Li¹, Fang Hou¹

¹Department of Pediatric Surgery, Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital, School of Medicine, University of Electronic Science and Technology of China, Chengdu 610054, China

²Institute of Laboratory Animals of Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital, Chengdu 610212, China

Abstract: Purpose: The aim of this study was to investigate the expression of YAP and miR-130a in the normal lung tissues and CDH lung tissues through the rat model of CDH, and preliminarily explored the relationship between YAP, miR-130a and CDH. **Methods:** Pregnant rats were divided into two groups: control (n = 5) and CDH (n = 5). A single oral dose (125 mg/kg) of nitrofen was administered to pregnant rats on embryonic day (E) 9.5 to induce CDH. All fetuses were acquired by cesarean delivery on E21.5. Fetuses with diaphragmatic hernias in the CDH groups were chosen for analysis. Lung weight (LW) and body weight (BW) were recorded and histologic evaluations, image analysis, western blot analysis and PCR were performed after lung processing. **Results:** Five female rats in the control group produced 76 fetuses without CDH. CDH was observed in 49 of 72 rat fetuses in the CDH group. Pulmonary hypoplasia and vascular remodeling were observed in the CDH group. YAP expression in the lungs was markedly increased in the CDH group compared to the control group (P = 0.001). However, there was no significant difference in the phosphorylation level of YAP (P = 0.113) between the two groups. YAP mRNA and miR-130a expression in the lungs were markedly increased in the CDH group compared to the control group (P = 0.01, P = 0.002). **Conclusion:** A relative increase YAP activity and miR-130a expression in the CDH rats may be associated with increased pulmonary vascular resistance. The role of the feedback mechanism between YAP and miR-130a playing in the CDH-associated pulmonary hypertension deserves further study.

1 Introduction

Congenital diaphragmatic hernia (CDH) is a significant clinical problem, occurring once in every 2500 human births. In recent years, despite many advances in neonatal intensive care and postpartum treatment strategies, severe respiratory failure induced by Pulmonary hypoplasia (PH) and persistent Pulmonary hypertension of the newborn (PPHN) remained to be the leading cause of life threatening in newborn with CDH.

Vascular remodeling is an important pathologic process in the development of many cardiovascular diseases, such as pulmonary hypertension, involving cell growth, death, migration, and synthesis and degradation of extracellular matrix^[1]. Hippo signaling pathway plays an important role in determining organ size and maintaining tissue homeostasis. YAP is one of the main effector molecules of this signaling pathway and is closely related to cell growth, proliferation and apoptosis. With the development of research, more and more studies showed that this pathway was closely related to the vascular remodeling. MicroRNAs are small, non-coding RNAs which regulate post-transcriptional protein expression by inhibiting the stability or translation of mRNA. Lately, it

had been reported that there was a positive feedback mechanism between miR-130a and YAP, which could make YAP activated continuously, and its function may be related to abnormal organ enlargement, tumorigenesis and the formation of persistent pulmonary hypertension^[2].

This study intended to investigate the expression of YAP and miR-130a in the normal lung tissues and CDH lung tissues through the rat model of CDH, and preliminarily explored the relationship between YAP, miR-130a and CDH.

2 Materials and Methods

2.1. Experimental design and animal model

All animals were provided by the Institute of Laboratory Animals of Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital and approved by the Medical Ethics Committee of Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital (Chengdu, China). Ten adult female Sprague-Dawley rats weighing 241.4 ~ 286.3 g (average, 273 g) and five male Sprague-Dawley rats weighing 266.2 ~ 300.2 (average, 283.7 g) were used. All rats were bred

*Corresponding author: wenying@126.com

after a night of controlled mating. A sperm-positive vaginal smear confirmed mating and represented embryonic day (E) 0.5. The pregnant rats were randomly assigned to the control group (n=5) and experimental group (CDH group, n=5). CDH was induced in pregnant rats at E9.5 via intragastric administration of a single oral dose of nitrofen (125 mg; 99% purity; Zhejiang Chemicals, Ningbo, Zhejiang, China) dissolved in 2 ml of olive oil. Control rats received an equal amount of olive oil only. Rat fetuses were delivered via Cesarean on E21.5 (prior to full term, E22). Under an anatomic stereoscopic microscope, fetal lungs were removed and the bilateral diaphragms were carefully examined for CDH. Lung weight (LW) and body weight (BW) of each fetus were recorded. The lungs of the fetuses with CDH were removed and processed for further analysis.

2.2. Lung preparation

Lungs for histological analysis were placed into 4% paraformaldehyde, fixed at 4°C for 48 hours, and then embedded in paraffin. Paraffin-embedded fetal lungs were transversely cut into 5 µm sections with a microtome. Lung samples to be used for western blot and PCR were stored at -80°C.

2.3. Morphological analyses

Lung slices were deparaffinized and hydrated using conventional methods. The sections were stained with hematoxylin and eosin (H&E) and Verhoeff-Van Gieson (VVG) respectively.

External diameter (ED) and medial wall thickness (MT) of small pulmonary arteries with a diameter of 20 ~ 60 µm that were associated with terminal bronchioles and distal airspaces were quantified using Image-Pro Plus 6.0 (Media Cybernetics, Inc., Chengdu, China). ED was defined as the distance between the external elastic laminae, and MT was defined as the distance between the internal and external elastic laminae. Percentage of medial wall thickness (%MT) was calculated using the following formula: $2 \times MT/ED \times 100$.

2.4. Immunohistochemistry

In each group, 10 samples were randomly selected to detect the expression of YAP in fetal lung tissues by SP method. PBS was used as a blank control instead of primary antibody, and light yellow or brownish yellow staining was identified as positive expression.

2.5. Western blotting

Lung tissues were homogenized in RIPA buffer supplemented with Complete Protease Inhibitor Cocktail tablets (Roche) and phosSTOP Phosphatase Inhibitor Cocktail tablets (Roche). Protein concentrations were determined using the Pierce BCA assay (Rockford, IL). Total protein (50 µg) was linearized in Laemmli sample buffer (Bio-Rad, USA) and then separated by gel electrophoresis using prefabricated 10% SDS

polyacrylamide gels (Invitrogen). Proteins were then transferred to PVDF membranes (Hybond, USA). Immediately after transfer, the membranes were blocked with 5% bovine serum albumin (BSA) for 2 hours before antibody detection. Primary antibodies against YAP (1:500, CST), phosphorylated p-YAP (1:1000, CST), and β-actin (1:5000, Abcam) were incubated overnight at 4°C. The membranes were further incubated with a goat anti-rabbit secondary antibody (1:5,000, Abcam) at room temperature for 2~3 hours followed by extensive washing. An enhanced chemiluminescence (ECL) kit (Thermo, USA) was used for antibody detection. All antibodies used in this study were diluted in phosphate buffered saline (PBS). The gel image analysis system (Tanon, China) was used for scanning analysis and the results are presented as relative expression of the target protein calculated as: target protein expression = integrated optical density value of target protein / internal reference integrated optical density value.

2.6. Real-time PCR

Total RNA was extracted with Animal Total RNA Isolation Kit (Foregene Co. Ltd.) and purified by using Direct-zol RNA Mini Prep (Zymo Research) according to the manufacturer's protocols. Aliquots of cDNAs were amplified with primers for YAP and miR-130a (Primer sequences in Tables 1).

Quantification of miRNAs were performed using the TaqMan method with the U6 snRNA as an internal control (TaqMan MicroRNA Reverse Transcription Kit and TaqMan Universal PCR Master Mix; Applied Biosystems/Ambion, Austin, TX, United States), according to the manufacturer's protocols.

Expression levels of mRNA were quantified in total RNA using the SYBR Green method with endogenous gene β-actin as control for normalization. Real-time PCR was performed in PIKORed 96 Real-Time PCR Detection System (ThermoFisher, United States). Gene expressions were amplified by PCR for 40 cycles with each cycle at 95°C for 3 s, 60°C for 30 s, and 72°C for 20 s.

Tables 1. Primer sequences

Primer	Forward	Reverse
YAP	CGACTCCTTC	AGCCTGCTGGCAAT
	TTCAAGCCGC	GGTACATCATCA
miR-130a	CTGAG	
	CGGGGTACC	
	CAGTTGGGT	CCGCTCGAGAGCAC
	GTTGAAGGA	TACACGGCCAATG
	GA	

2.7. Statistical analysis

Data are expressed as mean ± SD. SPSS 17.0 statistical software was used for statistical analysis. One-way ANOVA was used for the comparison of multiple means, while LSD test was used for homogeneity of variance. Tamhane's T2 test was used for heterogeneity of variance. Values of p < 0.05 were considered statistically significant.

3 Results

3.1. Incidence of CDH and pulmonary vascular remodeling

We determined the incidence of CDH in the two groups: none of the 76 fetuses in the control group presented with CDH and 49 out of 72 fetuses (68.6%) presented with CDH in the CDH group.

3.2. Lung morphometric analysis

Comparison of pulmonary vascular morphometry showed that ED was not statistically different ($P > 0.05$) between the CDH group and the control groups. Compared with the control group, fetuses in the CDH group had a significantly increased %MT ($P < 0.01$) and Alveolar septal thickness ($P < 0.01$) (Table 2, Fig. 1).

Table 2. lung morphometric analysis

Group	N	ED (μm)	%MT	Alveolar septal thickness(μm)
Contr	7	25.22±	17.86±	
ol	6	8.82	4.18	13.53±3.22
CDH	4	30.19±	34.84±	
	9	11.35#	5.91&	22.02±5.06*

Values are expressed as $\bar{x} \pm s$. # $P > 0.05$, & $P < 0.01$, * $P < 0.01$

Fig. 1. Lungs from CDH rats (B) with characteristic features of fetal canalicular stage, showing poorly formed saccules and thickened septal walls, compared to the lungs of control rats (A), which show well-differentiated saccules and thin septal walls. Compared with the control group (1D), the MT of the pulmonary artery was significantly increased in the CDH group (D). (H & E: AB; VVG: CD; original magnification: $\times 400$, bar = 10 μm).

3.3. Expression and distribution of YAP in fetal lung

Immunohistochemical staining showed that YAP was expressed in bronchial, alveolar epithelial cells and pulmonary artery walls, and its distribution was seen in cytoplasm and nucleus. (Fig. 2)

Fig. 2. Immunohistochemical staining of YAP in pulmonary sections of control (A) and CDH group (B). The predominant site of YAP staining was the bronchiolar and alveolar epithelial tissues in fetal lungs. Positive staining of YAP was also detected in parts of mesenchymal and lung vascular smooth muscle cells. Immunoreactivity of YAP was stronger in the CDH group than the control group. (bar = 100 μm)

3.4. Western blot analysis of YAP

YAP expression was significantly increased in the fetal lungs of CDH group compared to the control group ($P = 0.001$), while there was no significant difference in p-YAP between the two groups ($P = 0.113$). Equal loading of electro-phoresis gels was confirmed by β -actin staining of the stripped membranes. (Fig. 3, Table 3)

Fig. 3. Western blot analysis of lysates derived from control and CDH lung tissues: A(YAP), B(p-YAP), C(β -actin)

Table 3. Western blot analysis results

Group	n	YAP	P-YAP
Control	15	1.25±0.39	0.97±0.15
CDH	15	2.33±0.36	1.12±0.21
t value		4.95	1.74
P value		0.001	0.113

3.5. Relative mRNA expression levels of YAP and miR-130a

The relative expression levels of YAP mRNA and miR-130a in lungs were significantly increased in CDH group compared to Control group ($P = 0.01$, $P = 0.002$). (Table 4)

Table 4. Relative mRNA expression levels

Group	n	YAP mRNA	miR-130a
Control	15	1.31±0.14	0.56±0.08
CDH	15	1.94±0.17	0.97±0.03

t value	5.56	7.65
P value	0.01	0.002

4 Discussion

Pulmonary hypoplasia with pulmonary hypertension is an important pathological features of congenital diaphragmatic hernia and one of the main causes of death in children with CDH². The main pathological manifestations of pulmonary hypoplasia include irregular alveolar collapse, decreased effective ventilation volume, thickened pulmonary vascular wall, narrowed pulmonary vascular lumen and decreased elasticity of pulmonary vascular. Due to ethical and technical constraints, animal models have become an indispensable tool for the study of congenital diaphragmatic hernia. Rat model is the most widely used animal model of CDH, and its pathological manifestations are very similar to that of human CDH^[4]. In this study, we also observed that most of the alveoli in the fetal rats with diaphragmatic hernia were in a collapsed state, the alveolar septum was significantly thickened, the effective ventilation area was reduced, the walls of the small pulmonary vessels were thickened, and the inner diameter of these vessels were decreased.

As we know, The Hippo signaling pathway, first discovered in the mid-1990s during genetic screening for more tumor-suppressor genes in drosophila models, is an evolutionally highly conserved signaling pathway in multicellular organisms. In mammals, YAP is the key functional effector of the hippo pathway, which mainly comprises mammalian STE20-like protein kinase 1/2 (MST1/2), Salvador family WW domain containing 1 (SAV1), large tumor suppressor 1/2 (LATS1/2), Mps one binder (MOB1), YAP/transcriptional coactivator with PDZ-binding motif (TAZ), and transcriptional enhancer associate domain family members 1-4 (TEAD1-4)^[5]. When the Hippo pathway is activated, the YAP/TAZ is phosphorylated by LATS1/2, which results in its nuclear exclusion, ubiquitination, and subsequent proteolytic degradation. With the development of research, many studies have found Hippo/YAP signaling plays an important role in cardiovascular development and vascular homeostasis. Moreover, the signaling has been found to contribute to vascular remodeling and related cardiovascular diseases, including pulmonary hypertension, atherosclerosis, aortic aneurysms, restenosis, and angiogenesis. Recently, new evidence suggests that YAP regulates proliferation and survival of pulmonary arterial vascular smooth muscle cells (VSMCs) and pulmonary vascular remodeling. In our study, we found that the expression level of YAP in the lung tissues of CDH rats was significantly higher than that of the control rats, but there was no significant difference in the phosphorylation level of YAP (p-YAP) between the two groups, indicating that the increased YAP in the lung tissues of CDH rats mainly existed in an unphosphorylated state. This suggested that a relative increase YAP activity in the CDH rats may be associated with increased pulmonary vascular resistance.

In studies of pulmonary hypertension related to congenital heart disease, it has been found that the

expression level of miR-130a in plasma of patients increased, and in animal experiments, increase of miR-130a could promote the proliferation of vascular smooth muscle cells and the remodeling of vascular extracellular matrix⁵. In addition, some studies had found that miR-130a was also involved in the regulation of vasomotor state during pulmonary hypertension. However, there have been no reports on the role of miR-130a in the lung tissues of congenital diaphragmatic hernia. In this experiment, PCR quantitative analysis showed that compared with the control group, the expression level of miR-130a in the lung tissues of CDH models was significantly increased. This suggested that pulmonary vascular remodeling may also be associated with the increase of miR-130a in CDH rat models. What more important is some studies have found that a positive feedback mechanism could be formed between miR-130a and continuous activation of YAP, which could provide an intrinsic mechanism for continuous proliferation of cells after the initial cause has disappeared⁶. In our study, we found that both YAP and miR-130a increased in the lung tissues of CDH fetal rats, so it is deserved further study whether this feedback mechanism plays a role in CDH-associated pulmonary hypertension.

In summary, through the analysis of the expression differences of YAP and miR-130a in the lung tissues of rats with congenital diaphragmatic hernia and normal rats, this experiment preliminarily revealed the possible roles of YAP and miR-130a played in the CDH-related pulmonary hypertension and provided a new basis for further study on the pathogenesis and intervention measures of CDH. Due to the limitation of experimental design, more data are needed to support our results, which are the works of our next phase of research.

Acknowledgements

This study was supported by the Applied Basic Research Project of the Science and Technology Department of Sichuan Province (2017JY0305) and the Chengdu Science and Technology Bureau Technology Benefit People Technology Research and Development Project (2015-HM01-00441-SF).

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