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Elevated Krüppel-like factor 5 expression in spatiotemporal mouse lungs is similar to human congenital cystic adenomatoid malformation of the lungs

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Abstract

Objective: The study aimed to investigate the role of high Krüppel-like factor 5 (KLF5) expression on the pathogenesis of congenital cystic adenomatoid malformation of the lungs (CCAML) in mice.

Methods: A mouse model of high KLF5 expression in the lungs was established. KLF5 expression and the pulmonary lumen diameter were examined by immunohistochemistry to determine a successful model. Basement membrane damage and activity of matrix metalloproteinase-9 (MMP-9) were examined. After an adenovirus carrying KLF5 gene transfection in lung adenocarcinoma (H441) was created, changes in expression and activity of MMP-9 were determined.

Results: In a mouse model with high KLF5 expression, the pulmonary lumen was markedly enlarged, indicating establishment of CCAML. The basement membrane was degraded, and MMP-9 activity was significantly higher in the model group compared with the control group. Moreover, mice in a cellular model after transfection also showed higher MMP-9 activity than did controls.

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Conclusion: High KLF5 expression may play a pivotal role in the pathogenesis of CCAML, partly through regulating the activity of MMP-9.

Keywords
Krüppel-like factor 5, congenital cystic adenomatoid malformation of the lungs, matrix metalloproteinase, mouse, basement membrane, pulmonary lumen

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Introduction

Congenital cystic adenomatoid malformation of the lungs (CCAML), also known as congenital pulmonary airway malformation, is a rare polycystic lesion disease, with a reported incidence of approximately one in 25,000 births.[1] Congenital malformation of the lungs is characterized by abnormal branching of terminal bronchioles and a lack of normal alveoli.[2] Dilatation of the pulmonary airways can lead to formation of cystic structures, and a larger lesion may cause a respiratory disorder, resulting in mediastinal shift, infections, and oedema.[3,4] However, the pathogenesis of CCAML remains unclear.

Krüppel-like factor 5 (KLF5) is a zinc finger transcription factor belonging to the SP/KLF transcription factor family.[5] KLF5 is associated with cell proliferation, embryonic development, and lung morphogenesis. During lung development, a lack of KLF5 generally inhibits maturation of normal alveolar vesicles, and even blocks the interaction between lung epithelium and mesenchyme. This leads to morphological and biological characteristics of lung immaturity.[6] Additionally, several studies have shown that high KLF5 expression can contribute to various lung diseases, such as lung injury and chronic obstructive pulmonary disease.[7,8] Therefore, this has led to the question of whether high KLF5 expression leads to cystic lesions of CCAML. There have been limited studies on the relationship of KLF5 expression and the pathogenesis of CCAML.

In our study, we investigated mice that had the already characterized human 3.7-kb surfactant protein C (SPC) to reverse tetracycline responsive transactivator (rtTA) expression for expressing transgenes in respiratory epithelium.[9] SPC-rtTA transgenic mice were crossed with mice with tet operator (tetO) luciferase to overexpress KLF5 under control of the tetO (TRE).[10] This study aimed to determine whether overexpressed KLF5 is associated with the pathogenesis of CCAML.

Materials and method

Ethics approval

The experimental procedures in this study conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Procedures were approved by the Institutional Animal Care and Use Committee of the Maternal and Child Care Service Center of Sichuan Province.

Animals

SPC-rtTA and TRE-KLF5 transgenic mice were obtained from the Experimental

Animal Center of West China Hospital of Sichuan University. Heterozygous offspring with SPC-rtTA/TRE-KLF5 were selected as the model group. The offspring with wild type were selected as the control group. For oral treatment in the model group, 0.05 g doxycycline (Sigma, St Louis, MO, USA) was diluted in mixed solution with 625 mL deionized water and 625 mL alcohol. From the 6.5th day after gestation, the mice received doxycycline dilution in drinking water for binding the rtTA to the tetO. At the 14.5th day after gestation, pregnant mice were anaesthetized with pentobarbital sodium of 10 μL/g (concentration of 10 mg/mL), followed by delivery of the fetuses via caesarean section. The fetuses were sacrificed by decapitation, and the tails and lung tissues were prepared.

Genotyping

The tails of fetuses were used for genotyping. Polymerase chain reaction primers specific for each promoter were as follows: SPC promoter, 5′-GACACATATAAGACCCT GGTCA and 3′-AAAATCTTGCCAGC TT TCCCC; and TRE promoter, 5′-ACC CGGGTCGAGTAG GCGTGTA and 3′-CCCGGTGTCT TCTATGGAGGTCAA.

Immunohistochemical staining of fetal lung tissue

After fixing in 4% formaldehyde overnight, blocks of lung tissue were sectioned, deparaffinized, and rehydrated in graded xylene. This was followed by staining with the primary anti-KLF5 monoclonal antibody (1:2000, Abcam, Cambridge, UK). After washing with phosphate-buffered saline (PBS), the sections were incubated with secondary antibody (1:200) for 1 hour at room temperature. Pictures were taken under a microscope (Olympus U-SPT; Olympus Corp., Tokyo, Japan) with high resolution. After staining of tissue slices, pathological images were analysed by Image-Pro 6.0 (Media Cybernetics, Inc., Rockville, MD, USA) to achieve the purpose of semi-quantitative analysis of intensity. Images were captured by the Olympus U-SPT system. Positive tissues were randomly collected from each slice, and the intensity was measured in grey values.

Detection of the pulmonary lumen diameter in a mouse model

Lung tissue was collected under a microscope (ASOM-3; Olympus Corp.) and then washed by PBS. The diameters of 20 pulmonary lumen sections were observed and measured by eyepiece graticules using a microscope.

Immunofluorescence for laminin-5 in the lungs

Laminin-5, as a marker of the basement membrane in the lungs, was detected by a laminin-5 immunofluorescence kit (Abcam) by following the manufacturer's directions.

Detection of matrix metalloproteinase-9 activity

Lung tissue slices were detected by a matrix metalloproteinase (MMP)-9 immunofluorescence kit (Abcam) and observed under a microscope (BX41; Olympus Corp.).

Cell culture in vitro transfection

Adenovirus with KLF5 (ad-KLF5; Gibco, Gaithersburg, MD, USA) or green fluorescent protein (ad-GFP; Gibco) was transfected into the human lung adenocarcinoma cell line H441 (American Type Culture Collection, Manassas, VA, USA). The cells were incubated for 48 hours before being harvested. Successful transfection was determined by high KLF5 protein expression.

Western blotting

The cells were washed with ice-cold PBS and homogenized in cell lysis buffer. Equal amounts of protein were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA). After blocking the nonspecific sites with a blocking solution, the membranes were incubated with anti-MMP-2 (1:2000) and MMP-9 (1:2000) antibodies (Abcam). Subsequently, the membrane was washed with PBS Tween followed by incubation with a secondary antibody (1:200) at room temperature for 1 hour. The blots were then analysed using Quantity One V 4.62 (Bio Rad, Philadelphia, PA , USA).

Detection of MMP-9 activity

MMP-9 samples were isolated from lung tissue, and then gelatin zymography was conducted for detecting MMP-9 activity. The proteins were separated in a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis gelatin gel (1 mg/mL) and ran in running buffer for 20 minutes at 60 V and then at 90 minutes at 120–150 V. After running the gel, it was incubated in 2.5% triton-X on a high-speed shaking platform for 1 hour at room temperature. The gel was then incubated in a developing buffer (5 mM $CaCl_2$, 0.2 M NaCl, 50 mM Tris-HCl) at 37°C overnight. Coomassie Blue R-250 was used as a staining dye and the gel was washed for 30 minutes after staining. The resulting images were analysed using Image-Pro 6.0.

Statistical analysis

Statistical analysis was performed by using IBM SPSS Statistics, Version 22.0 (IBM Corp., Armonk, NY, USA). Continuous data are expressed as mean ± standard deviation (SD). Comparisons were performed using the Student's t-test, while categorical variables were analysed by the chi-square test or Fisher's exact test. A P value of < 0.05 was accepted as statistically significant.

Results

Sixty-six fetuses were obtained from 10 pregnant mice in this study. A total of 15 fetuses were in the control group and 13 fetuses were in the model group after genotyping.

Immunohistochemistry of KLF5 expression in lung tissue

The distribution of KLF5 (black staining) was intensive and abundant in airway epithelial cells of the fetuses in the model group. However, KLF5-positive particles in the control group were sparse (Figure 1). KLF5 expression was significantly higher in fetuses in the model group compared with those in the control group (118.55 ± 10.30 vs. 83.95 ± 7.01, $P < 0.05$).

Pulmonary lumen diameter

As shown in Figure 2, cystic malformation and an unclear lumen border of lung tissue were observed in fetuses in the model group. In contrast, a regular lumen with a uniform size and clear boundary were observed in the control group. The mean pulmonary lumen diameter was significantly larger in fetuses in the model group than in those in the control group (32 ± 3.07 vs. 18.06 ± 2.06 μm, $P < 0.05$).

Laminin-5 expression in lung tissue

The immunofluorescence results of laminin-5 showed changes in the integrity of the basement membrane, with a loose arrangement in the model group (Figure 3). In contrast, the basement membrane of lung tissue

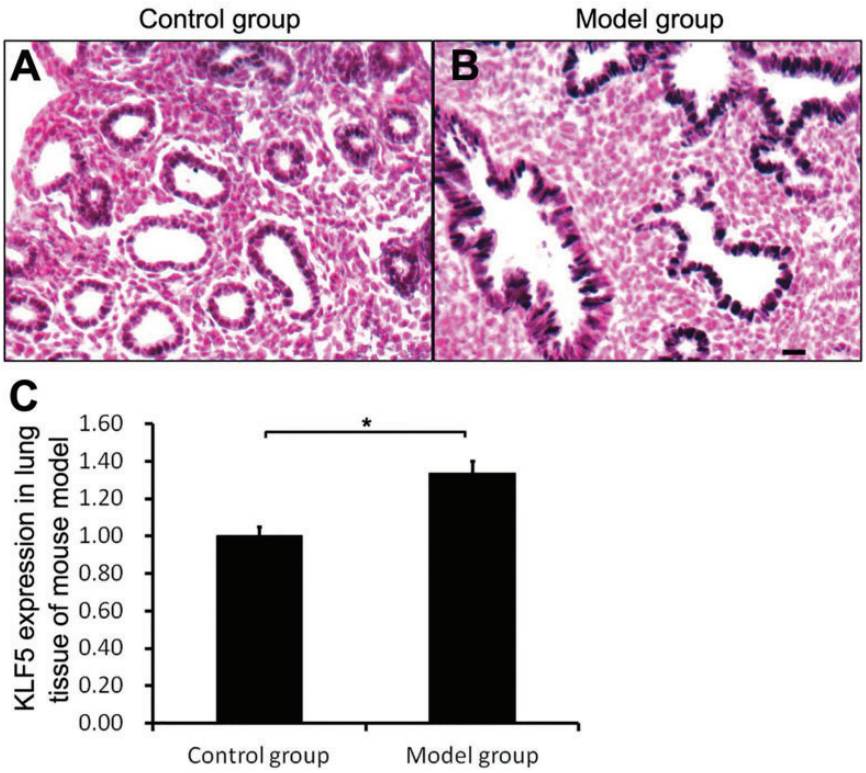

Figure 1. Measurement of KLF5 production in the pulmonary airways by immunohistochemical staining in the model and control groups. (A) Strong staining of KLF5-positive particles in luminal epithelial cells in the model group was observed. (B) Fewer KLF5-positive particles were observed in the control group than in the model group. (C) Graph showing KLF5 expression in lung tissue. *$P < 0.05$ vs. the control group. KLF5, Krüppel-like factor 5.

in the control group was intact and had a clear border.

MMP-9 activity in transfected cells in vitro

The fluorescence intensity of MMP-9 was markedly higher in fetuses in the model group than in those in the control group ($P < 0.05$, Figure 4). Cellular activity of MMP-9 was determined by gelatin zymography. MMP-9 activity was significantly higher in the model group than in the control group (3.32 ± 0.27 vs. 1 ± 0.09, $P < 0.05$, Figure 5).

Protein expression of MMP-2 and MMP-9 in transfected cells

After successful transfection with ad-KLF5, H441 cells were harvested for detecting the level of MMP-2 and MMP-9 expression. MMP-2 and MMP-9 protein expression levels were similar in both groups ($P > 0.05$, Figure 6).

Discussion

KLF5 is critical not only in the physiology of lung development, but also in the pathological processes of various diseases. Wan et al.[6] showed that KLF5 regulates genes

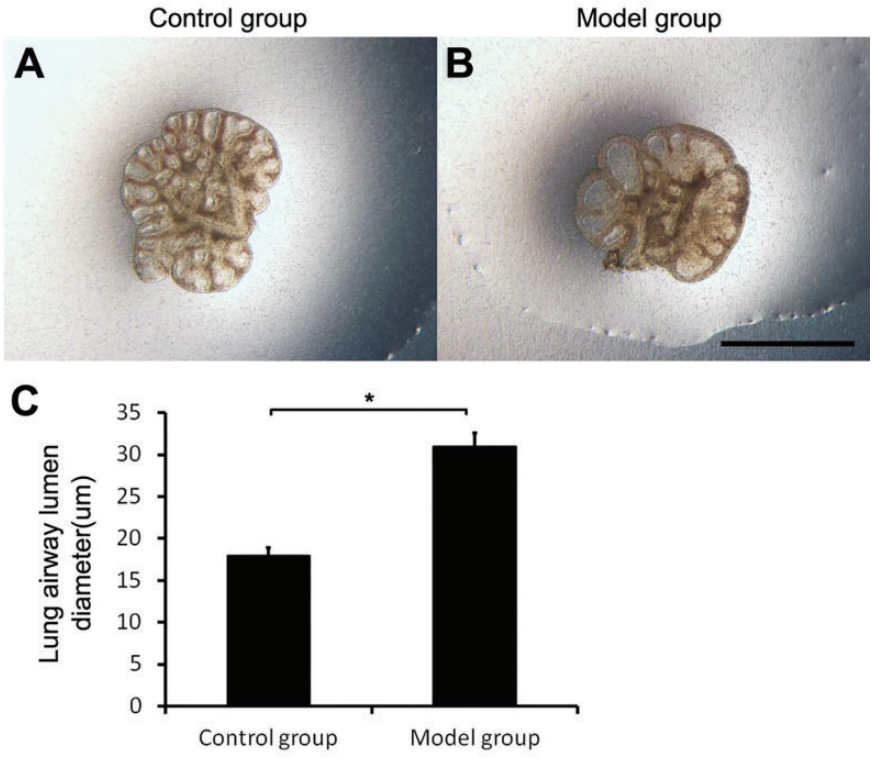

Figure 2. Measurement of the pulmonary lumen diameter in the model and control groups. (A) Measurement of the pulmonary lumen diameter in the control group. (B) Measurement of the pulmonary lumen diameter in the model group. (C) Graph showing comparison of the pulmonary lumen diameter between the groups. $^{*}P < 0.05$ vs. the control group.

controlling paracrine interactions during lung morphogenesis. A lack of KLF5 expression can lead to immaturity of the respiratory epithelium. Abe et al.[8] demonstrated that KLF5 expression was upregulated in small airways and pulmonary vessels of patients with chronic obstructive pulmonary disease. Moreover, high KLF5expression in cardiac fibroblasts under pressure overload contribute to development of cardiac hypertrophy and fibrosis.[8,11] However, the relationship between CCAML and KLF5 has not been investigated. In our study, inducible, lung epithelial, cell-specific KLF5 transgenic mice (SPC-rtTA/TRE-KLF5) were generated.[12] The pseudoglandular stage of the fetus generally begins from 9.5 to 16.5 days after gestation,[13] and this plays a crucial role in the development of CCAML.[14] In our study, doxycycline was administered 2 days ahead of the pseudoglandular stage to achieve effective drug concentrations and to sufficiently bind the rtTA to the TRE (tetO) during the pseudoglandular stage. This mouse model was established to observe changes in the pulmonary airways with high KLF5 expression. A successful model was shown by immunohistochemical staining. Excessive cystic malformation also indicated that the CCAML model can be constructed under high KLF5 expression.

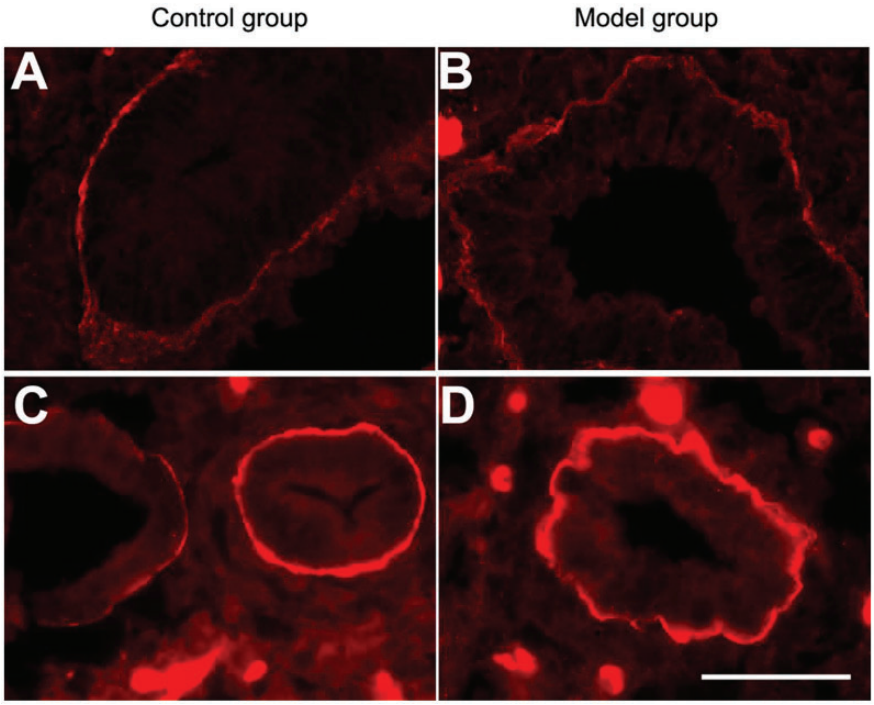

Figure 3. Measurement of laminin-5 distribution in the pulmonary basement membrane by immunofluorescence in the model and control groups. (A) The basement membrane of the main airway is smooth and the boundary is intact in the control group. (B) The basement membrane of the main airway is incomplete, unsmooth, and wrinkled in the model group. (C) The basement membrane of the peripheral airway is intact and smooth in the control group. (D) The basement membrane of the peripheral airway is incomplete and wrinkled in the model group.

MMPs, as a family of zinc-dependent proteolytic enzymes, are involved in various pulmonary pathologies. MMPs can degrade all components of the extracellular matrix, including the basement membrane.[15] MMP-9 is a potent proteinase, which degrades denatured collagens, and activates other MMPs and cytokines.[16] MMP-9 levels are dramatically upregulated under various inflammatory conditions.[17] Shinoda et al.[18] reported that KLF5 caused cartilage matrix degradation by regulating MMP-9. Furthermore, repression of MMP-9 in endothelial cells maintained the integrity of the developing vasculature in a previous study.[19] In our study, the pulmonary basement membrane was markedly degraded and MMP-9 activity was significantly enhanced in the model group. This finding suggested that KLF5 was capable of degrading the basement membrane partly by regulating MMP-9.

In lung adenocarcinoma cells in our study, the high KLF5 expression model was generated by transfecting adenovirus with KLF5. Our finding of higher activity of MMP-9 in the model group than in the control group supported the relationship between KLF5 and MMP-9. However, there was no significant difference in expression levels of MMP-2 and MMP-9 levels between the groups. Further studies need to be performed to investigate this discrepancy between results.

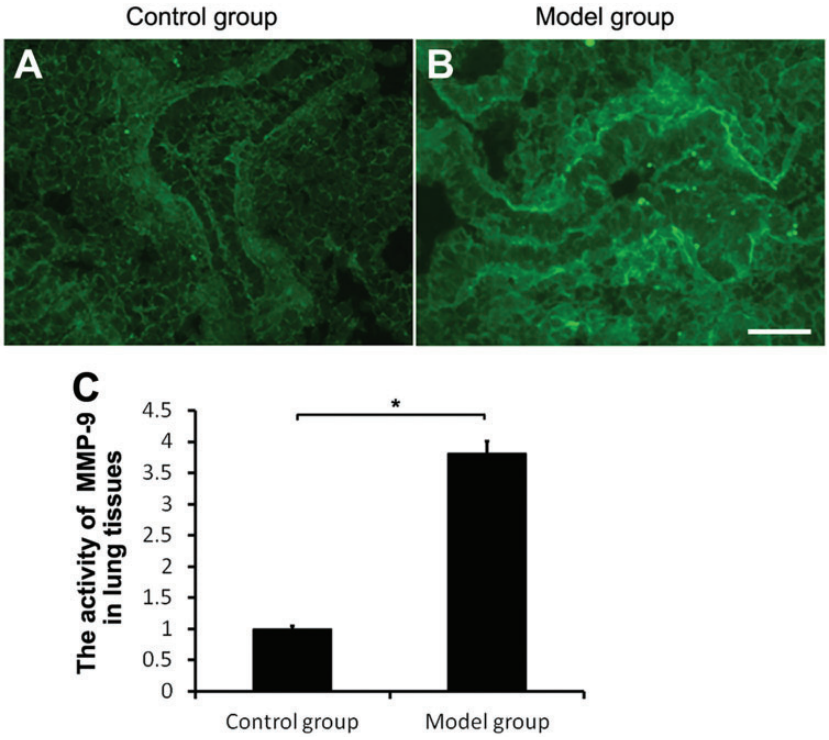

Figure 4. MMP-9 activity in lung tissue in the model and control groups. Fluorescence intensity of MMP-9 in the control (A) and model groups (B). (C) Graph showing MMP-9 activity in lung tissue. *$P < 0.05$ vs. the control group.
MMP-9, matrix metalloproteinase-9.

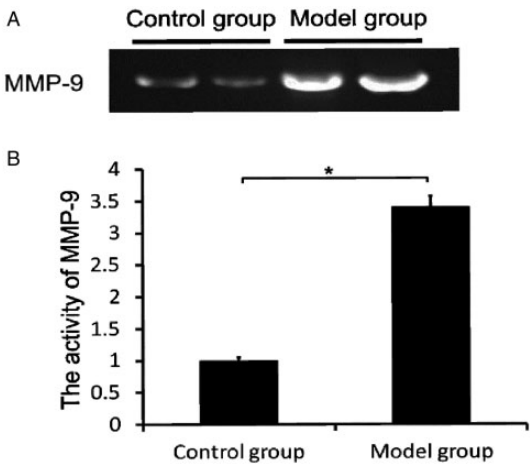

Figure 5. MMP-9 activity in transfected human lung adenocarcinoma cells. (A) Western blot showing MMP-9 activity in the control and model groups. (B) Graph showing MMP-9 activity in the control and model groups. *$P < 0.05$ vs. the control group.
MMP-9, matrix metalloproteinase-9.

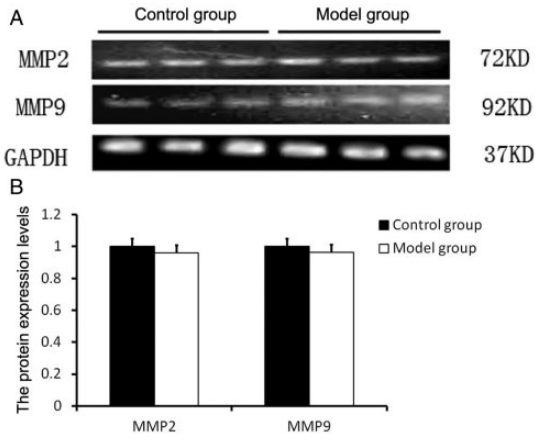

Figure 6. Protein expression levels of MMP-2 and MMP-9 in transfected human lung adenocarcinoma cells. (A) Western blot showing MMP-9, MMP-2, and GAPDH protein expression levels in the control and model groups. (B) Graph showing protein expression levels of MMP-2 and MMP-9 in the control and model groups ($P > 0.05$). MMP, matrix metalloproteinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

There are several limitations in this study. We did not observe any differences in protein expression of MMPs between the two groups in the current study. More cell lines should be included in further studies. Although we demonstrated a relationship between KLF5 and MMP-9, there might be more pathways involved in CCAML. Further investigations are required to clarify whether KLF5 can regulate other transcription factors and other types of cells in the pulmonary airways.

Conclusion

The present study suggests that high KLF5 expression plays a pivotal role in the pathogenesis of CCAML, partly through regulating the activity of MMP-9. Further investigations on the fetus regarding intrauterine death caused by CCAML are required.

Declaration of conflicting interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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