MicroRNA-30a-5p promotes proliferation and inhibits apoptosis of human pulmonary artery endothelial cells under hypoxia by targeting YKL-40

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Abstract. Pulmonary arterial hypertension (PAH) is a fatal and currently incurable cardiopulmonary disease. Numerous microRNAs (miRNAs) serve important roles in the development of PAH. While the expression of miR-30a-5p was downregulated in the lung tissue of rats in a pulmonary hypertension rat model, the expression pattern and function of miR-30a-5p in human PAH remain unclear. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was used to examine miR-30a-5p and chitinase-3-like protein 1 (YKL-40) mRNA expression levels. The expression levels of YKL-40 and apoptosis-associated proteins were measured by western blot analysis. Cell proliferation assays and flow cytometry analysis were performed to examine cell proliferation and apoptosis, respectively. The association between miR-30a-5p and YKL-40 was determined by a luciferase reporter assay, RT-qPCR and western blot analysis. The relative expression levels of miR-30a-5p in plasma were increased in patients with PAH [median=13.23 (25th percentile=6.388, 75th percentile=21.91)] compared with normal controls [median=2.25 (25th percentile=1.4, 75th percentile=3.7)]. The expression of miR-30a-5p was significantly upregulated while the protein expression of YKL-40 was significantly downregulated when the protein expression of YKL-40 was significantly upregulated in hypoxia-induced human pulmonary artery endothelial cells (HPAECs) when compared with the hypoxia-induced group at 0 h. miR-30a-5p overexpression promoted HPAEC growth and inhibited apoptosis of HPAECs under hypoxia. A miR-30a-5p mimic decreased the luciferase activity of a luciferase reporter construct containing YKL-40 3′-untranslated region and also decreased YKL-40 protein expression. YKL-40 overexpression partly alleviated the effects of miR-30a-5p upregulation on proliferation and apoptosis of HPAECs under hypoxia. In conclusion, the data indicated that miR-30a-5p promoted cell growth and inhibited apoptosis of HPAECs under hypoxia by targeting YKL-40. Therefore, the miR-30a-5p/YKL-40 axis may provide a potential target for the development of novel PAH therapies.

Introduction

Pulmonary arterial hypertension (PAH) is a fatal and currently incurable cardiopulmonary disease (1), characterized by pulmonary vascular remodeling and vasoconstriction that primarily manifests as a progressive elevation in pulmonary artery pressure and vascular resistance (1). Although notable improvements in the therapeutic strategy for PAH have been achieved, the prognosis of PAH remains unsatisfactory (2). In the early pathogenesis of PAH, pulmonary artery endothelial dysfunction serves a vital role; hypoxia is known to target pulmonary artery endothelial cells (PAECs) and exacerbate their dysfunction (3,4). However, the mechanisms by which hypoxia affects PAECs and regulates PAEC proliferation and apoptosis remain unclear.

Accumulating evidence has indicated that microRNAs (miRNAs), a class of evolutionarily conserved small non-coding RNAs, are essential modulators of a variety of genes and cellular processes including cell proliferation and apoptosis (5). A number of aberrantly expressed miRNAs serve a role in the development of PAH (6-9). miR-30a-5p is downregulated in various types of cancer and serves as a tumor suppressor by inhibiting cancer cell proliferation (10). In addition, miR-30a-5p expression was downregulated in the lung tissue of a PAH rat model (8). However, its function in PAH remains unknown.

Chitinase-3-like protein 1 (YKL-40), also known as human cartilage glycoprotein 39, is a 40 kDa secreted glycoprotein that was first identified in the secretome of a human osteosarcoma cell line (11). Under normal physiological conditions, YKL-40 expression may be observed in different cell types,
including vascular smooth muscle cells and chondrocytes (12). In cervical cancer, YKL-40 overexpression has been demonstrated to promote cell proliferation (13). In addition, YKL-40 is involved in endothelial dysfunction as a response to endothelial injury (14). As a marker of inflammation and tissue remodeling, elevated YKL-40 has been associated with disease severity and mortality in a number of cardiovascular diseases (15,16). Furthermore, YKL-40 levels are increased in idiopathic PAH (17), but the precise regulatory mechanism of aberrant YKL-40 expression is unknown. We hypothesized that miR-30a-5p may serve an important role in regulating aberrant YKL-40 expression in PAH. In the present study, miR-30a-5p and YKL-40 expression levels were assessed in PAECs in hypoxia and the role of miR-30a-5p overexpression in regulating cell proliferation and apoptosis in PAECs under hypoxic conditions was evaluated. Finally, the regulatory association between miR-30a-5p and YKL-40 was investigated.

Materials and methods

Clinical samples. A total of 30 patients with PAH (age, 36.70±10.31 years; 26 female and four male patients), including those with idiopathic or congenital heart disease- or connective tissue-associated PAH, were enrolled consecutively from January 2016 to March 2018 at Guangdong Provincial People's Hospital (Guangdong, China), none of whom received treatment during their enrollment. A total of 15 healthy normal controls (age, 33.02±8.52 years; 14 female patients and one male patient) were enrolled during the same time frame for the physical examination portion of the study. All participants involved in this study received oral and written information about the objectives of the study and provided written informed consent, as per the guidelines established in the Declaration of Helsinki. This study was approved by the Research Ethics Committee of Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences [approval no. GDREC20172979(HRI)].

Cell culture. Human PAECs (HPAECs) were obtained from ScienCell Research Laboratories, Inc. HPAECs were cultured in endothelial cell medium (ScienCell Research Laboratories, Inc.), consisting of 500 ml basal medium, 25 ml FBS (cat. no. 0025), 5 ml endothelial cell growth supplement (ECGS; cat. no. 1052), and 5 ml penicillin/streptomycin solution (cat. no. 0503), all purchased from ScienCell Research Laboratories, Inc. The 293T cells were obtained from the Cell Bank of the Chinese Academy of Sciences and maintained in Dulbecco's modified Eagle's medium (Hyclone; GE Healthcare Life Sciences) supplemented with 10% FBS (Hyclone; GE Healthcare Life Sciences) supplemented with 10% FBS (Hyclone; GE Healthcare Life Sciences). All cells were maintained at 37°C in a humidified incubator under normoxic (21% O2 and 5% CO2) or hypoxic (1% O2 and 5% CO2) conditions, as described previously (18,19).

Cell transfection and hypoxic treatment. The miR-30a-5p mimic (5'-UGUAAACAUCUCUGACUGGAAG-3') and negative control (NC) miRNA (miR-NC) (5'-UUCUCGGA CGUUGACGUTT-3') were purchased from Guangzhou Ribobio Co., Ltd. The full coding sequence of YKL-40 was cloned into pcDNA3.1 plasmids (Invitrogen; Thermo Fisher Scientific, Inc.) to construct the YKL-40-overexpressing plasmid (pcDNA-YKL-40). The primers used for YKL-40 overexpression-plasmid construction were as follows: BamHI-forward 5'-cgc gga tcct gcc acc ATG GGT GTG AAG and XhoI-reverse 5'-ccgctcgagCT GTTGCACGCGTGCATCCTGTG-3'. After transfection with 50 nM miR-30a-5p mimic, 50 nM miR-NC or 50 nM miR-30a-5p mimic, plus 0.5 µg pcDNA3.1 or 0.5 µg pcDNA-YKL-40 plasmid using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) for 24 h, HPAECs were exposed to hypoxic conditions (1% O2 and 5% CO2) at 37°C.

RNA extraction and reverse transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from HPAECs or plasma using TRIzol® reagent (Thermo Fisher Scientific, Inc.). To analyze miR-30a-5p expression, RT-qPCR was performed with specific stem-loop RT primers using the Mir-X™ miRNA First Strand Synthesis kit (Takara Biotechnology Co., Ltd) according to the manufacturer's protocol, following which qPCR was performed using the Mir-X™ mirRNA qRT-PCR SYBR® kit (Takara Biotechnology Co., Ltd.). U6 was used as an internal control. To analyze YKL-40 mRNA expression, cDNA was synthesized using the PrimeScript™ RT reagent kit (Takara Biotechnology Co., Ltd.). YKL-40 mRNA expression was measured using the SYBR® Premix Ex Taq™ (Takara Biotechnology Co., Ltd.) in an ABI PRISM® 7500 real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). 18S rRNA served as an internal control. The qPCR conditions were as follows: 95°C for 5 min; 40 cycles at 95°C for 15 sec and 60°C for 32 sec. The relative fold changes in the transcripts were calculated using the 2−ΔΔCq method (20). The sequences of the primers used were as follows: miR-30a-5p forward, 5'-ACACTCCAGCTGGTGTTGAACATCTCGACTG-3'; miR-30a-5p reverse, 5'-CTCAACTGGTGTGCCTGGGA-3'; U6 forward, 5'-CTCGGT TCCGACGACA-3'; U6 reverse, 5'-AAGCCTTCAGAATT TGCGT-3'; YKL-40 forward, 5'-CTCTACGGCATGCTC AACAC-3'; YKL-40 reverse, 5'-GGGCGTGACTGATTGA TGA-3'; and 18S rRNA forward, 5'-CCTGGATACCCAGC TGAGA-3'; 18S rRNA reverse, 5'-GCCGCGCAATACGAA TGCCCC-3'. All reactions were performed in triplicate.

Western blot analysis. Cellular protein was extracted with ice-cold radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology) containing a protease inhibitor. The concentration of protein was determined using a BCA protein assay kit (Jiangsu Kaiji Biotechnology Co., Ltd.). A total of 30 µg total protein from each sample was separated using 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. Following blocking with 5% non-fat milk dissolved in PBS with 0.1% Tween-20 (PBST) at 25°C for 2 h, the membrane was incubated at 4°C overnight with the following primary antibodies: Anti-caspase-3 (1:1,000 dilution; cat. no. 9579); anti-caspase-9 (1:1,000 dilution; cat. no. 52873); anti-Bcl-2-associated X protein (Bax; 1:800 dilution; cat. no. 5023); anti-Bcl-2 (1:2,000 dilution; cat. no. 9579); and anti-GAPDH (1:5,000 dilution; cat. no. 5174), all purchased from Cell Signaling Technology, Inc. The membrane was washed with PBST three times and then incubated with the
The cells were transfect with 50 nM NC using Lipofectamine
plus 50 nM miR-30a-5p mimics, or 0.5 µg mut-YKL-40
plus with 50 nM NC, 0.5 µg mut-YKL-40 3'UTR plasmid
co-transfected with 0.5 µg wt-YKL-40 3'UTR plasmid

The potential miR-30a-5p binding sites in the YKL-40
3'UTR were predicted using TargetScan Bioinformatics prediction and luciferase reporter assays.

Flow cytometry analysis. The cell apoptosis assay was
performed using the Annexin V-fluorescein isothiocyanate
(FITC) Apoptosis Detection kit (Jiangsu Kaiji Biotechnology
Co., Ltd.). Cells (4x10^5) were plated in 6-well plates. A total
of 24 h after transfection, the cells were exposed to hypoxic conditions using the AnaeroPouch culture system at 37°C for 0, 24, 48 or 72 h. CellTiter 96® AQueous One Solution reagent (20 µl) was added to the medium at each time point and incubated for an additional 3 h at 37°C and 5% CO2 in the dark. The absorbance was read at 490 nm using a microplate absorbance reader (Multiskan™ MK3; Thermo Fisher Scientific, Inc.).

Statistical analysis. The statistical analysis was performed using IBM SPSS Statistics 19.0 software (IBM Corp.). miR-30a-5p expression levels in the plasma of patients with PAH and normal controls were analyzed using the Mann-Whitney U test and described using the median and the 25th and 75th percentiles. All data for experiments in cultured cells are expressed as mean ± standard deviation. Statistical differences between two groups were evaluated by an unpaired Student's t-test. Statistical differences between multiple groups were evaluated by one-way analysis of variance followed by the Least Significant Difference post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-30a-5p expression in plasma of patients with PAH. The relative expression levels of miR-30a-5p in plasma were increased in patients with PAH [median=13.23 (25th percentile=6.388, 75th percentile=21.91)] compared with normal controls [median=2.25 (25th percentile=1.4, 75th percentile=6.388)] (P<0.0001; Fig. 1). The increased level of miR-30a-5p in the plasma of patients with PAH indicated that miR-30a-5p may serve a role in the development and progression of PAH.

Hypoxia decreases miR-30a-5p expression and increases YKL-40 expression in HPAECs. To investigate miR-30a-5p and YKL-40 expression in response to hypoxia, HPAECs were cultured under hypoxic conditions for 24, 48 and 72 h. The RT-qPCR results indicated that the relative expression levels of miR-30a-5p under hypoxic conditions for 24, 48 and 72 h were 0.71±0.11, 0.39±0.10 and 0.37±0.09, respectively, indicating that hypoxia significantly decreased miR-30a-5p expression in HPAECs (Fig. 2A). Western blot analysis results
miR-30a-5p overexpression promotes proliferation and inhibits apoptosis of HPAECs under hypoxia. In the preliminary experiments, it was identified that the endogenous expression of miR-30a-5p is low in HPA, and the effect of miR-30a-5p inhibitor on miR-30a-5p level was not marked (data not shown). Therefore, only the experiments on miR-30a-5p upregulation were performed. Following transfection with the miR-30a-5p mimic or miR-NC for 24 h, HPAECs were exposed to a hypoxic environment for 48 h. The results of the RT-qPCR assay suggested that the expression levels of miR-30a-5p was increased 1987.9-fold in HPAECs transfected with the miR-30a-5p mimic compared with the NC group (Fig. 3A). miR-30a-5p overexpression significantly promoted the proliferation of HPAECs compared with the miR-NC group under hypoxia for 24, 48 and 72 h (Fig. 3B). miR-30a-5p overexpression significantly decreased the apoptosis rate in HPAECs compared with the miR-NC group under hypoxia for 48 h (Fig. 3C). In addition, western blot analysis data demonstrated that miR-30a-5p overexpression decreased the levels of cleaved caspase-3, cleaved caspase-9 and Bax, while increasing the expression of Bcl-2 in HPAECs under hypoxia for 48 h (Fig. 3D). These results indicated that miR-30a-5p overexpression promoted proliferation and inhibited apoptosis of HPAECs under hypoxia.

YKL-40 is a target of miR-30a-5p. miR-30a-5p has a potential binding site in the YKL-40 3'-UTR (Fig. 4A). The results of the luciferase reporter assay indicated that luciferase activity was significantly decreased in the 293T cells co-transfected with the wt-YKL-40-3'UTR vector and the miR-30a-5p mimic under hypoxia for 48 h. In contrast, luciferase activity was unaffected in cells co-transfected with the mut-YKL-40-3'UTR vector and miR-30a-5p mimic, relative to the NC group under hypoxia for 48 h (Fig. 4B). These data indicated that miR-30a-5p directly targeted YKL-40 by binding to its 3'-UTR. The RT-qPCR results demonstrated that miR-30a-5p overexpression had no effect on the mRNA expression levels of YKL-40 in HPAECs under hypoxia for 48 h (Fig. 4C). However, western blot analysis indicated that the protein expression of YKL-40 decreased 60.3% in the miR-30a-5p overexpression group compared with the NC group in HPAECs under hypoxia for 48 h (Fig. 4D). These results indicated that YKL-40 is a target of miR-30a-5p in HPAECs under hypoxia.

Overexpression of YKL-40 partly alleviates the effects of miR-30a-5p upregulation on cell proliferation and apoptosis of HPAECs under hypoxia. To additionally clarify the association between miR-30a-5p and YKL-40 in the regulation of cell proliferation and apoptosis of HPAECs under hypoxia, HPAECs were transfected with NC + pcDNA, miR-30a-5p + pcDNA, or miR-30a-5p + YKL-40-pcDNA. Following transfection for 24 h, cells were subjected to hypoxia for 48 h. Western blot analysis revealed that the protein expression level of YKL-40 in HPAECs transfected with miR-30a-5p + YKL-40-pcDNA was remarkably upregulated when compared with the cells transfected with miR-30a-5p + pcDNA under hypoxia for 48 h (Fig. 5A). The results of the MTS assay suggested that cell proliferation was significantly decreased in the miR-30a-5p + YKL-40-pcDNA group compared with that in the miR-30a-5p + pcDNA group, but remained increased compared with that in the NC + pcDNA group under hypoxia for 48 h (Fig. 5B). Additionally, apoptosis was significantly upregulated in the miR-30a-5p + YKL-40-pcDNA group compared with that in the NC + pcDNA group under hypoxia for 48 h (Fig. 5B). Cleaved caspase-3, cleaved caspase-9 and Bax expression levels were significantly increased in the miR-30a-5p + YKL-40-pcDNA group compared with the miR-30a-5p + pcDNA group, while the expression of BCL2 was significantly decreased in the miR-30a-5p + YKL-40-pcDNA group compared with the
miR-30a-5p + pcDNA group under hypoxia for 48 h (Fig. 5D). These results indicated that the overexpression of YKL-40 partly alleviated the effects of miR-30a-5p upregulation on cell proliferation and apoptosis of HPAECs under hypoxia.

Discussion

The development of PAH is primarily associated with the aberrant functioning of PAECs, pulmonary artery smooth muscle cells (PASMCs) and vascular fibroblasts (21). Despite the progress in elucidating the function of miRNA in the dysregulated PAECs and PASMCs and in the etiology of PAH, the cellular role and underlying molecular mechanisms remain largely unknown. To the best of our knowledge, the present study is the first to describe the role of miR-30a-5p on cell proliferation and apoptosis in HPAECs under hypoxia. The present study also provided a novel potential therapeutic target for PAH.

Increasing evidence has indicated that miRNAs regulate PAH (22,23). miR-30a-5p is significantly upregulated in colorectal cancer and suppresses proliferation and induces
miR-30a-5p and YKL-40 in HPAECs

Apoptosis of colon carcinoma cells (24). MiR-30a-5p is down-regulated in gastric cancer tissues and cell lines and inhibits the proliferation of gastric cancer cells (25). These data suggest that miR-30a-5p is involved in regulating cell proliferation and apoptosis. Although the role of miR-30a-5p has been demonstrated in several cancer types, its role in PAH is not clear. Hypoxia exacerbates pulmonary artery endothelial dysfunction in the early pathogenesis of PAH (3,4); therefore, the expression of miR-30a-5p was examined from 24 to 72 h in hypoxia-induced HPAECs in the present study. The gradually downregulated expression profile of miR-30a-5p under hypoxic conditions revealed that miR-30a-5p may serve a role in HPAECs. This prediction is supported by results indicating that miR-30a-5p overexpression promoted cell proliferation and inhibited apoptosis of HPAECs under hypoxia. Hypoxia-induced HPAEC proliferation and inhibition of HPAEC proliferation is a strategy to attenuate the pathogenesis of PAH (26,27). PAH may also induce apoptosis-resistance in endothelial cells (28). Therefore, we hypothesized that miR-30a-5p may be a suitable therapeutic target for PAH.

miRNAs are able to inhibit the translation or accelerate the degradation of target genes at the 3'-UTR by interacting with the mRNA (29). The identification of target genes is therefore important, in order to illuminate the underlying mechanism of miR-30a-5p in HPAECs under hypoxia. Pulmonary vascular remodeling and inflammation have been implicated in PAH. YKL-40 is considered to serve a role in the process of inflammation and tissue remodeling (30,31). Bioinformatics analysis predicted that miR-30a-5p had a potential binding site in the 3'-UTR of YKL-40. Therefore, YKL-40 may be a potential target of miR-30a-5p. The luciferase reporter assay additionally confirmed that miR-30a-5p directly binds to the 3'-UTR of YKL-40. In addition, it was demonstrated that hypoxia increased YKL-40 expression in HPAECs in the present study. MiR-30a-5p overexpression had no effect on the mRNA expression of YKL-40, but it decreased the protein expression of YKL-40 in HPAECs. These data demonstrated that miR-30a-5p may suppress the expression of YKL-40 at the post-transcriptional level and that miR-30a-5p may negatively mediate the protein expression of YKL-40.
in HPAECs. To determine whether YKL-40 serves a pivotal role in miR-30a-5p-induced alterations in cell proliferation and apoptosis, a rescue assay was conducted. It was identified that overexpression of YKL-40 partly alleviated the effects of miR-30a-5p upregulation on cell proliferation and apoptosis of HPAECs under hypoxia. These data suggest that miR-30a-5p may regulate HPAEC proliferation and apoptosis, at least in part, by the direct inhibition of YKL-40 expression. However, the signaling pathway involved in HPAEC proliferation and apoptosis is different. Additional studies are required to illuminate the mechanism of YKL-40 in regulating proliferation and apoptosis in HPAECs under hypoxia.

In HPAECs under hypoxia, the miR-30a-5p expression level was gradually downregulated, while YKL-40 was gradually upregulated, indicating a negative correlation between YKL-40 and miR-30a-5p. In addition, it was identified that miR-30a-5p expression levels were increased in the plasma of patients with PAH compared with normal controls. Its target YKL-40 also exhibited increased levels in the plasma of patients with PAH compared with that in normal controls (17). This may be explained by the hypothesis that, in order to regulate the abnormally high expression of YKL-40, the human body increases the expression level of its inhibitory factor miR-30a-5p, so as to restrain the excessive expression of YKL-40 to a certain extent. However, this hypothesis requires additional investigation.

There are certain limitations to the present study. Although the effects of miR-30a-5p on cell proliferation and apoptosis of HPAECs under hypoxia were analyzed, direct evidence of the participation of miR-30a-5p in PAH was lacking, requiring
verification in an animal model. Secondly, the dynamic expression of miR-30a-5p in the plasma of different groups of patients with PAH was not explored. The association between clinical characteristics and the prognosis of PAH requires additional studies. Finally, the effect of YKL-40 knockdown on cell proliferation and apoptosis of HPAECs under hypoxia was not investigated, and additional studies are required to confirm the role of YKL-40 in HPAECs under hypoxia. Despite these limitations, these data indicate the potential role of miR-30a-5p as a novel therapeutic target for PAH.

In summary, miR-30a-5p served pivotal roles in proliferation and apoptosis of HPAECs under hypoxia by targeting YKL-40. The results of the present study provide new insights into the regulatory mechanisms underlying PAH progression and indicate that the miR-30a-5p/YKL-40 axis may provide a potential target in the treatment of PAH.

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Availability of data and materials
The datasets used and/or analyzed during the present study are available from the corresponding author.

Authors' contributions
HT was involved in study design and preparation of the manuscript. YZ was involved in data collection and literature analysis. HT and YZ performed the experiments. ZL was involved in data collection, statistical analysis and data interpretation. GC was involved in data collection, and the analyses involving PAH patients and controls. SL contributed to data analysis and interpretation. HY was involved in literature analysis, revising the manuscript and performed the plasma miR-30a-5p expression level experiments. All of the authors read and approved the final manuscript.

Ethics approval and consent to participate
This study was approved by the Research Ethics Committee of Guangdong Provincial People’s Hospital, Guangdong Academy of Medical Sciences [approval no. GDREC2017297H (R1)]. All participants involved in this study received oral and written information about the objectives of the study and provided written informed consent.

Patient consent for publication
All participants provided written informed consent for the publication of associated data.

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

References

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