MicroRNA expression profile of pulmonary artery smooth muscle cells and the effect of let-7d in chronic thromboembolic pulmonary hypertension

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Abstract: Chronic thromboembolic pulmonary hypertension (CTEPH) is a life-threatening condition characterized by single or recurrent pulmonary thromboemboli, which promote pulmonary vascular remodeling. MicroRNA (miRNA), is a small, noncoding RNA that is involved in multiple cell processes and functions and may participate in the pathogenesis of CTEPH. Our aims were to identify the miRNA expression signature in pulmonary artery smooth muscle cells (PASMCs) of CTEPH patients and to study the role of let-7d in CTEPH pathogenesis. The miRNA expression profile was analyzed by microarray in PASMCs of CTEPH and control patients. Differentially expressed miRNAs were selectively validated by stem-loop quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR). The role of let-7d was identified by *in silico* analysis, and its effect on the proliferation of PASMCs was measured by methyl thiazolyl tetrazolium (MTT). Student's unpaired *t* test, the Fisher exact test, and the χ^2 test were used for statistical analysis. Eighteen miRNAs were differentially expressed in PASMCs from CTEPH patients, including 12 upregulated miRNAs and 6 downregulated miRNAs; among the latter, let-7d decreased 0.58-fold in CTEPH patients, as validated by qRT-PCR. It was found that let-7d could inhibit the proliferation of PASMCs through upregulation of p21. In conclusion, PASMCs in CTEPH patients have an aberrant miRNA profile and reduced let-7d, which could promote PASMC proliferation and may be involved in the pathogenesis of CTEPH.

Keywords: chronic thromboembolic pulmonary hypertension, let-7d, pulmonary artery smooth muscle cell, proliferation.

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INTRODUCTION

Chronic thromboembolic pulmonary hypertension (CTEPH) is one of the leading causes of severe pulmonary hypertension (PH). CTEPH is characterized by persistent pulmonary embolism, which can in turn increase pulmonary vascular resistance, resulting in PH and consequent right-side heart failure.[1] It has been known for many years that CTEPH cannot be explained simply by pulmonary vascular obliteration due to unresolved thromboemboli but that a pulmonary embolism could be the initiating event, while ensuing PH would result from progressive pulmonary vascular remodeling.[2,3] The process of pulmonary vascular remodeling involves all layers of the vessel wall and is complicated by cellular heterogeneity within each compartment of the pulmonary arterial wall.[4] Until recently, the pathogenesis of pulmonary vascular remodeling in CTEPH was largely unknown, but new genetic and pathophysiological studies have emphasized the involvement of a number of molecular mechanisms, including pathways involving growth factors, cytokines, metabolic signaling, elastases, and proteases.[5,6]

MicroRNAs (miRNAs) are small, noncoding RNAs (21–23 nucleotides) that participate in diverse aspects of biology, including developmental timing, differentiation, proliferation, cell death, and metabolism.[7,8] Recently, differential expression of miRNAs has been implicated in many cardiovascular diseases, including PH. Dysregulated expression of bone morphogenetic protein receptor type II (BMPR2), which is a pathogenetic hallmark of PH, is regulated by miR-17/92.[9] MiR-204 plays an important role in the decrease of proliferation, vascular remodeling, and pulmonary artery blood pressure in PH and could be a new therapeutic target for PH.[10] MiR-21 also plays a significant role in hypoxia-induced pulmonary vascular smooth muscle cell (VSMC) proliferation and migration.[11]

Altered expression of miRNAs has now been found in some kinds of PH by microarray analysis, which is an established, high-throughput technique. Researchers who screened lung miRNA profiles in a longitudinal, crossover design during the development of PH caused by chronic hypoxia or monocrotaline in rats found that distinct miRNAs were regulated during the development of PH in rats.[12] However, the miRNA expression profile and its possible role in CTEPH patients remain unresolved. Recently, it has been reported that the expression of the fibrinogen alpha gene regulated by miR-759 was associated with susceptibility to CTEPH.[13]

Let-7 microRNAs form a conserved microRNA family consisting of 12 genes (let-7a to let-7i) encoding different miRNAs.[14] Because they are downregulated in various cancers and also can target oncogenes, they are usually known as tumor suppressors, and in lung cancer, low levels of let-7 was associated with shortened postoperative survival.[15] It was also reported that let-7 could repress cell proliferation via cell cycle regulation.[16] So alternately expressed let-7 may participate in pulmonary vascular remodeling in CTEPH. Of the let-7 family, let-7d could influence VSMC proliferation.[17] How its expression changes and how it affects CTEPH are still unknown.

In this study, we performed microarray analysis to determine whether any miRNAs differentially expressed and investigated the role of some alternately expressed miRNAs (e.g., let-7d) in the pathogenesis of CTEPH.

MATERIAL AND METHODS

Subjects

The study was approved by the Research Ethics Committee of Beijing Chao-Yang Hospital of Capital Medical University. Written informed consent was obtained from all patients before the procedure. The microarray cohort was composed of 5 CTEPH patients and 3 control subjects.

Isolation and culture of primary pulmonary artery smooth muscle cells (PASMCs)

PASMCs were isolated from tissues of CTEPH patients and control subjects. The PASMCs of CTEPH patients were carefully isolated from the endothelium and the thin layer of media covering the endarterectomy tissues (selected as far as possible from the downstream branches). The isolated cells were all spindle shaped, and almost all of the cells isolated from pulmonary endarterectomy (PEA) tissues were positive for α-smooth muscle actin and major histocompatibility complex, which are both smooth muscle cell–specific markers (data not shown). The selected

tissues were cut into small pieces, incubated in fresh Hank's balanced salt solution (HBSS) containing 2.5 mg/mL collagenase (Worthington Biochemical, Lakewood, NJ) and 1.0 mg/mL bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO) for 30 min at 37°C, centrifuged at 300 *g* for 5 min, resuspended in Medium 231 (Gibco, Grand Island, NE) supplemented with smooth muscle growth supplement, 200 μg/mL of penicillin, and 200 IU/mL streptomycin, seeded in dishes, and incubated in a humidified 5% CO_2 atmosphere at 37°C.

MiRNA microarray analysis

A miRCURY LNA Array (ver. 16.0; Exiqon, Vedbaek, Denmark) analysis was performed on RNA extracted from PASMC RNA from 5 CTEPH patients and 3 control subjects to identify the differential miRNA expression profile of CTEPH. Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA) and the miRNeasy mini kit (Qiagen, Valencia, CA) according to manufacturer's instructions. After RNA isolation, the miRCURY Hy3/Hy5 Power labeling kit (Exiqon) was used according to the manufacturer's guideline for miRNA labeling. The labeled samples were hybridized on the miRCURY LNA Array according to the array manual. The slides were scanned using the Axon GenePix 4000B microarray scanner (Axon Instruments, Foster City, CA), and the scanned images were then imported into GenePix Pro 6.0 software (Axon Instruments) for grid alignment and data extraction.

Stem-loop quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) assays

Real-time PCR was performed using an Applied Biosystems 7500 Sequence Detection system. The 20-μL PCR reaction included 2 μL reverse-transcription product, 9 μL SYBR Green Mix, 2 μL miRNA forward primer, 2 μL miRNA reverse primer, and 5 μL RNase-free H_2O. The reactions were heated to 95°C for 20 s, followed by 40 cycles of 95°C for 10 s, 60°C for 20 s, and 70°C for 6 s. The relative amount of miRNAs was normalized against U6 snRNA (small nuclear RNA).

Gene ontology (GO) analysis

GO analysis was applied to analyze the main function of target genes of differentially expressed miRNAs. GO is a key functional classification of the National Center for Biotechnology Information.[18] Fisher's exact test and a χ^2 test were used to classify the GO category, and the false discovery rate (FDR) was calculated to correct the *P* value (the smaller the FDR, the smaller the error in judging the *P* value). We chose only GOs that had a *P* value <0.001 and an FDR <0.05.

Pathway analysis

Pathway analysis was used to discover the significant pathways of the target genes of differentially expressed miRNAs according to the Kyoto Encyclopedia of Genes and Genomes (KEGG), Biocarta, and Reatome.[19,20] Fisher's exact test and a χ^2 test were used to select the significant pathway, and the threshold of significance was defined by a *P* value <0.001 and an FDR <0.05.

miRNA-gene network

The relationships between the miRNAs and target genes were calculated on the basis of their differing expression values and according to the interactions of miRNAs and target genes in the Sanger miRNA database to build the miRNA-gene network.[21,22] The adjacency matrix of miRNAs and target genes, $\mathbf{A} = [ai, j]$, is made by the attribute relationships among target genes and miRNAs, and $[ai, j]$ represents the relation weight of target gene i and miRNA j. In the miRNA-gene network, circles represent genes and squares represent miRNAs, and their relationship is represented by an edge (see Fig. S1, available online). The center of the network is represented by degree, that is, the contribution of one miRNA to the target genes around it or the contribution of one target gene to the miRNAs around it. The key miRNA and gene in the network always have the highest degrees.

Transfection of miRNA

Transient transfection of let-7d mimics (GenePharma, Shanghai) was carried out with Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's procedure. Briefly, the PASMCs were washed with a serum-free medium and cultured in the serum-free medium without antibiotics. The transfection complex (let-7d and the transfection reagent mixture) were added to the medium in a drop-wise manner and mixed gently by rocking the medium

back and forth. After 4–6 hours, the cell culture medium was transferred to a medium containing serum and antibiotics and incubated at 37°C for 48 hours before the proliferation assay, Western blot analysis, or PCR experiments were conducted.

Cell growth assay

We used methyl thiazolyl tetrazolium (MTT; Sigma-Aldrich) to determine the viability of cells. Cells were plated in 96-well plates at 5,000 cells/well and incubated for 48 hours after transfection of let-7d mimics or negative controls. Following this incubation, MTT (5 mg/mL) reagent was added to each well and the cells were incubated for an additional 4 hours, at which time the supernatant was removed and 150 μL/well of dimethyl sulfoxide (DMSO; Sigma-Aldrich) was added to solubilize the formazan salt crystals. Solubilized formazan products were quantified by spectrophotometry at 490 nm using an enzyme-linked immunosorbent assay reader (Bio-Rad, Hercules, CA).

Western blot analysis

PASMC proteins were separated on an SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) gel and subsequently blotted onto a nitrocellulose membrane (Millipore, Billerica, MA). After blocking for 1 hour with 5% nonfat dry milk dissolved in phosphate-buffered saline, the membrane was incubated with anti-p21 primary antibody (1 : 1,000; Cell Signaling Technology, Beverly, MA) and anti-β-actin primary antibody (1 : 1,000; Sigma-Aldrich) overnight at 4°C. The primary antibody-labeled membranes were then treated with IRDyeTM800 (green) or IRDyeTM700 (red) conjugated, affinity-purified antirabbit immunoglobulin G for 1 hour. The positive protein bands were visualized by LI-COR Odyssey infrared double-fluorescence imaging system (LI-COR, Lincoln, NE). The value of the relative density of the target protein band was normalized to the density of the β-actin band.

Statistical analysis

Data were presented as means ± standard error. Comparison between the groups of data was evaluated with Student's unpaired *t*-test. A P value <0.05 was considered statistically significant. Fisher's exact test and the χ^2 test were used in GO and pathway analysis.

RESULTS

Global miRNA profile of PASMCs in CTEPH patients

To analyze the miRNA expression profile, PASMCs were isolated from 5 CTEPH patients' endarterectomy tissues (Fig. 1) and 3 control patients' tissues from lobectomy for pneumothorax or lung transplantation in Beijing Chao-Yang Hospital. The clinical characteristics of the CTEPH patients are displayed in Table 1.

The miRCURY LNA Array (v.16.0) system was then employed to analyze RNA isolated from PASMCs of the 5 CTEPH patients and 3 controls. From the 1,891 capture probes, we found 12 miRNAs that were upregulated and 6 that were downregulated (each with a 2-fold change) in CTEPH patients relative to those in controls, as shown in Table 2. The hierarchical cluster of differentially expressed miRNAs in CTEPH patients versus controls is shown in Figure 2.

In silico analysis revealed the role of let-7d in CTEPH pathogenesis

In silico analysis was used to analyze and identify differentially expressed miRNAs that could play a role in the pathogenesis of CTEPH. First, a list of messenger RNA (mRNA) targets was identified by screening the 3′ untranslated regions of mRNAs for seed sequences of the 18 differentially expressed miRNAs. Because reliable miRNA target information is still limited, most of the target prediction methods used generate a large number of predicted targets, many of which are presumed to be false. We therefore integrated miRNA targets from both Targetscan and Miranda databases to predict the potential miRNA targets in our study, and we obtained 1,830 target genes. The functions of these target genes were then analyzed according to GO, and their target pathways were determined according to KEGG, Biocarta, and Reatome (data not shown). According to the threshold of significant GOs and pathways ($P < 0.001$ and FDR < 0.05), 308 target genes were identified.

To elaborate the relationship between the 18 differentially expressed miRNAs and the 308 target genes

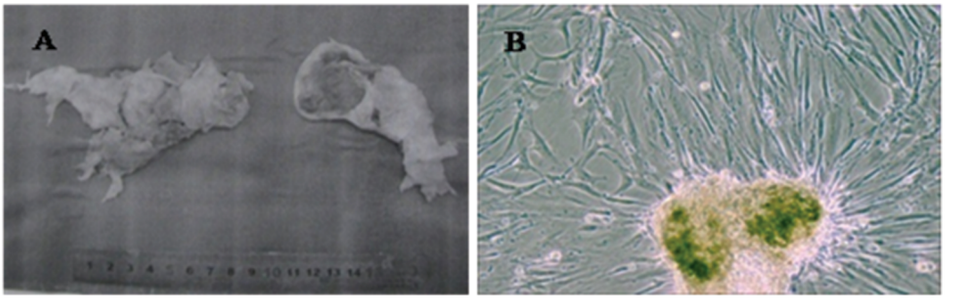

Figure 1. *A*, Thromboembolic tissue removed from the pulmonary arteries during pulmonary endarterectomy (PEA). The PEA tissues are unresolved thromboemboli covered by a thin layer of media and fibrotic intima. *B*, Light microscopy image of isolated pulmonary artery smooth muscle cells of chronic thromboembolic pulmonary hypertension patients. The spindle-shaped cells are growing from the adherent tissue pieces at the first passage.

and to find the major differentially expressed miRNAs in CTEPH, the miRNA–gene regulatory network (18 miRNAs and 308 target genes) was established, as shown in Figure S1. As explained in "Material and methods," the term "degree" describes the contribution of one miRNA to the genes around it or the contribution of one gene to the miRNAs around it, so the key miRNA and gene in the network will have the highest degree. The 18 differentially expressed miRNAs were ranked according to degree, and let-7c, miR-27b, and let-7d were the highest-ranked miRNAs, as shown in Table 3. Meanwhile, the top 10 target

Table 1. Clinical and hemodynamics data of the 5 CTEPH patients

	CTEPH-1	CTEPH-2	CTEPH-3	CTEPH-4	CTEPH-5
Demographics					
Age, years	62	37	44	41	49
Sex	Male	Male	Male	Male	Male
Weight, kg	80	63	68	69	75
Height, cm	182	173	170	172	174
NYHA functional class	III	IV	III	II	II
6-MWD, m	340	129	384	324	321
Hemodynamics data					
mPAP, mmHg	55	89	49	41	65
CI, L/min/m^2	3.1	1.5	1.8	1.44	1.3
PVR, dyn/s/cm^5	645	2,463	925	1,186	1,830
mRAP, mmHg	8	15	11	12	3
PWP, mmHg	6.2	12	12	2	8
Risk factors					
Previous VTE	No	No	No	Yes	Yes
Recurrent VTE	No	No	No	Yes	No
Malignancy	No	No	No	No	No
Ventriculo-atrial shunt	No	No	No	No	No
Thyroid hormone replacement	No	No	No	No	No
Pacemaker	No	No	No	No	No
Infected pacemaker	No	No	No	No	No
Splenectomy	No	No	No	No	No

Note: CTEPH: chronic thromboembolic pulmonary hypertension; NYHA functional class: New York Heart Association functional classification of the World Health Organization; 6-MWD: 6-minute walk distance; mPAP: mean pulmonary artery pressure; CI: cardiac index; PVR: pulmonary vascular resistance; mRAP: mean right atrial pressure; PWP: pulmonary wedge pressure; VTE: venous thromboembolism.

Table 2. Differentially expressed miRNAs identified in cells of CTEPH patients

miRNA	Accession	Control mean	CTEPH patient mean	Fold change
Upregulated miRNAs				
hsa-miR-320c	MIMAT0000087	0.3848122	0.7791244	2.0246874
hsa-miR-149	MIMAT0004609	0.0388231	0.0848726	2.1861367
hsa-miR-4288	MIMAT0016918	2.4845110	5.4681362	2.2008903
hsa-miR-191	MIMAT0000440	0.6079675	1.3494137	2.2195490
hsa-miR-425	MIMAT0003393	0.0393036	0.0880385	2.2399576
hsa-miR-151-5p	MIMAT0004697	0.1679546	0.3951892	2.3529527
hsa-miR-29a	MIMAT0000086	5.8436868	14.850401	2.5412725
hsa-miR-361-5p	MIMAT0000703	0.1938334	0.4987414	2.5730411
hsa-miR-3676	MIMAT0022734	0.1243556	0.3460382	2.7826502
hsa-miR-1246	MIMAT0005898	6.9651019	20.463536	2.9380096
hsa-miR-155	MIMAT0000646	0.2615062	0.8869655	3.3917565
hsa-miR-1290	MIMAT0005880	1.1478704	5.2392336	4.5643078
Downregulated miRNAs				
hsa-miR-30a	MIMAT0000087	0.7497516	0.2369417	0.3160270
hsa-let-7c	MIMAT0000064	0.6185536	0.2692877	0.4353506
hsa-miR-140-5p	MIMAT0000431	0.6613641	0.3037604	0.4592938
hsa-miR-27b	MIMAT0000419	2.0069687	0.9541719	0.4754294
hsa-let-7d	MIMAT0000065	0.2752446	0.1325376	0.4815267
hsa-miR-152	MIMAT0000438	0.1579967	0.0763367	0.4831536

Note: miRNA: microRNA; CTEPH: chronic thromboembolic pulmonary hypertension.

genes in the network are shown in Table 4; interestingly, the first three are all genes of the let-7 family.

Let-7d repressed the proliferation of PASMCs by upregulating p21

To confirm the findings obtained from the miRNA profile, we validated let-7c and let-7d expression levels in PASMCs from CTEPH patients, using stem-loop qRT-PCR, and found that only let-7d was decreased in our study (Fig. 3). It has been reported that the let-7 family can act as tumor suppressors and key regulators of cell proliferation pathways.[23] As a result, it is possible that let-7d, which was downregulated in CTEPH patients, may play an important role in repressing the proliferation of PASMCs. Therefore, the role of let-7d in the proliferation of PASMCs was investigated. As shown in Figure 4, overexpressing let-7d (Fig. 4*A*) could significantly repress the proliferation of PASMCs (Fig. 4*B*). To elucidate the mechanisms, the protein expression levels of its target genes, TGFBR1 and p21, were examined (Fig. 4*C*). It was observed that both of these proteins were downregulated in PASMCs of CTEPH patients.

After a detailed literature review, we discovered that let-7 could reduce cell cycle progression, thereby repressing cell proliferation,[23] and that p21 is a potent cyclin-dependent kinase inhibitor, which can arrest the cells at the G1 phase of the cell cycle. We therefore investigated the effect of let-7d on p21 expression and observed that let-7d significantly upregulated the expression of p21 (Fig. 4*D*). Thus, let-7d may act as a tumor suppressor and may repress the proliferation of PASMCs by promoting p21-mediated cell cycle arrest.

DISCUSSION

The role of miRNAs in the progression of several diseases, including PH, is becoming increasingly im-

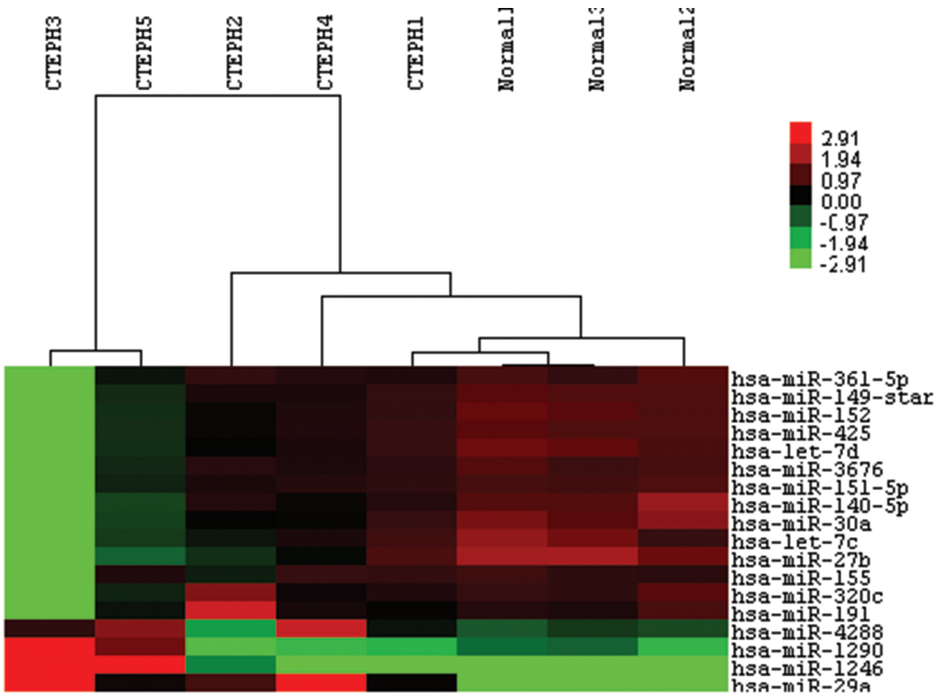

Figure 2. Heat maps of the 18 microRNAs (miRNAs) expressed differently between 5 chronic thromboembolic pulmonary hypertension (CTEPH) patients and 3 control patients. Rows represent miRNAs, and columns represent patients. A color change from red to green indicates that expression was downregulated. In contrast, a color change from green to red indicates that expression was upregulated. Color brightness represents the signal values of miRNAs in the microarray chip.

portant. In this study, we profiled the miRNA signature in PASMCs of CTEPH patients and found 18 differentially expressed miRNAs, 12 upregulated and 6 downregulated. One miRNA, let-7d, was decreased 0.58-fold and was found to inhibit the proliferation of PASMCs by upregulating p21.

In CTEPH patients, vascular remodeling in the distal pulmonary vascular bed has been shown to be an important component in the development of PH,[24,25] and excessive proliferation of smooth muscle cells contributes to vascular-wall thickening and remodeling in the pulmonary arteries. In addition, it has been reported that many pathways involving growth factors, cytokines, metabolic signaling, elastases, and proteases also participate in vascular remodeling. These include but are not limited to iron channels, BMPR2, 5-hydroxytryptamine (5-HT), angiopoietin-1, and endothelin-1.[26] The molecular mechanisms responsible for pulmonary artery remodeling are incredibly complex, and further clarification is required.

MiRNAs are highly conserved, single-stranded, noncoding, small RNAs that control cellular functions. Many miRNAs are highly expressed in the vasculature, and their expression is altered in vascular diseases. Several miRNAs have been found to be critical modulators of vascular pathologies, such as atherosclerosis, lipoprotein metabolism, inflammation, arterial remodeling, angiogenesis, smooth muscle cell regeneration, hypertension, apoptosis, and neointimal hyperplasia.[27] The role of miRNAs in PH has previously been investigated, and it has

Table 3. Degree of differentially expressed microRNAs

MicroRNA	MicroRNA style	Degree
hsa-let-7c	Downregulated	38
hsa-miR-27b	Downregulated	34
hsa-let-7d	Downregulated	30
hsa-miR-149*	Upregulated	29
hsa-miR-152	Downregulated	26
hsa-miR-29a	Upregulated	25
hsa-miR-30a	Downregulated	16
hsa-miR-320c	Upregulated	15
hsa-miR-140-5p	Downregulated	7
hsa-miR-1290	Upregulated	4
hsa-miR-155	Upregulated	4
hsa-miR-1246	Upregulated	3
hsa-miR-361-5p	Upregulated	3
hsa-miR-425	Upregulated	1

Table 4. Top 10 target genes according to degree

Gene symbol	Description	Degree
TGFBR1	Transforming growth factor, beta receptor 1	4
TSC1	Tuberous sclerosis 1	4
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1) c	3
CPEB1	Cytoplasmic polyadenylation element binding protein 1	3
GDF6	Growth differentiation factor 6	3
HTR4	5-hydroxytryptamine (serotonin) receptor 4	2
BCL2L1	BCL2-like 1	2
CTNND1	Catenin (cadherin-associated protein), delta 1	2
CCR7	Chemokine (C-C motif) receptor 7	2

been shown that inappropriate STAT3 (signal transducer and activator of transcription 3) activation in PH is linked to aberrant miR-204 expression. Furthermore, downregulation of miR-204 activates Src kinase and nuclear factor of activated T cells, which sustains PH-PASMC proliferation and resistance to apoptosis.[10] Alterations in the surface expression of BMPR2 have also been described in several forms of PH,[28,29] and it has been reported that interleukin 6 (IL6)–dependent STAT3 activation leads to the expression of miRNA cluster 17/92, which results in BMPR2 downregulation.[9] Hypoxia and oxygen sensing have a strong impact in PH pathogenesis, as several miRNAs have shown to be inducible by hypoxia; miR-34a was increased in whole lung of mice after 2 weeks under hypoxia,[30] and miR-210 is also a predominant hypoxia-sensitive miRNA.[31] MiR-21 is downregulated in the MCT (monocrotaline) experimental model of PH[12] and plays a significant role in hypoxia-induced pulmonary VSMC proliferation and migration by regulating multiple gene targets (PDCD4, SPRY2, and PPARα).[11] In our study, we profiled the miRNA signature in PASMCs of CTEPH patients and found that miR-320c, miR-149*, miR-4288, miR-191, miR-425, miR-151-5p, miR-29a, miR-361-5p, miR-3676, miR-1246, miR-155, and miR-1290 were upregulated and miR-30a, let-7c, miR-140-5p, miR-27b, let-7d, and miR-152 were downregulated. Among these 18 differentially expressed miRNAs, we discovered through *in silico* analysis that let-7d may be involved in the pathogenesis of CTEPH. Caruso et al.[12] profiled miRNA signatures in rat hypoxic and MCT models of PH and found that let-7f was downregulated in both hypoxic and MCT models, while let-7a was significantly reduced only in MCT-treated rats.

The let-7 family consists of 11 very closely related genes and is conserved among invertebrates and vertebrates, including humans.[32] Let-7 is widely viewed as a tumor suppressor miRNA, and consistent with this idea, the expression of let-7 family members is downregulated in many cancer types when compared to normal tissue and during tumor progression. For some forms of cancer, most or all let-7 family members appear to be downregulated.[15,33] In our study, let-7d was downregulated in CTEPH patients, and

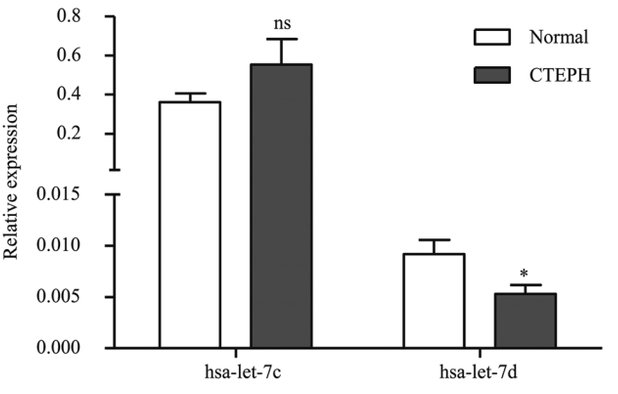

Figure 3. Expression of let-7c and let-7d in pulmonary artery smooth muscle cells of chronic thromboembolic pulmonary hypertension (CTEPH) patients and control ("normal") patients. The relative let-7c and let-7d expressions were determined by stem-loop quantitative real-time reverse-transcription polymerase chain reaction. Data shown are means + standard error; asterisk indicates $P < 0.05$ versus controls.

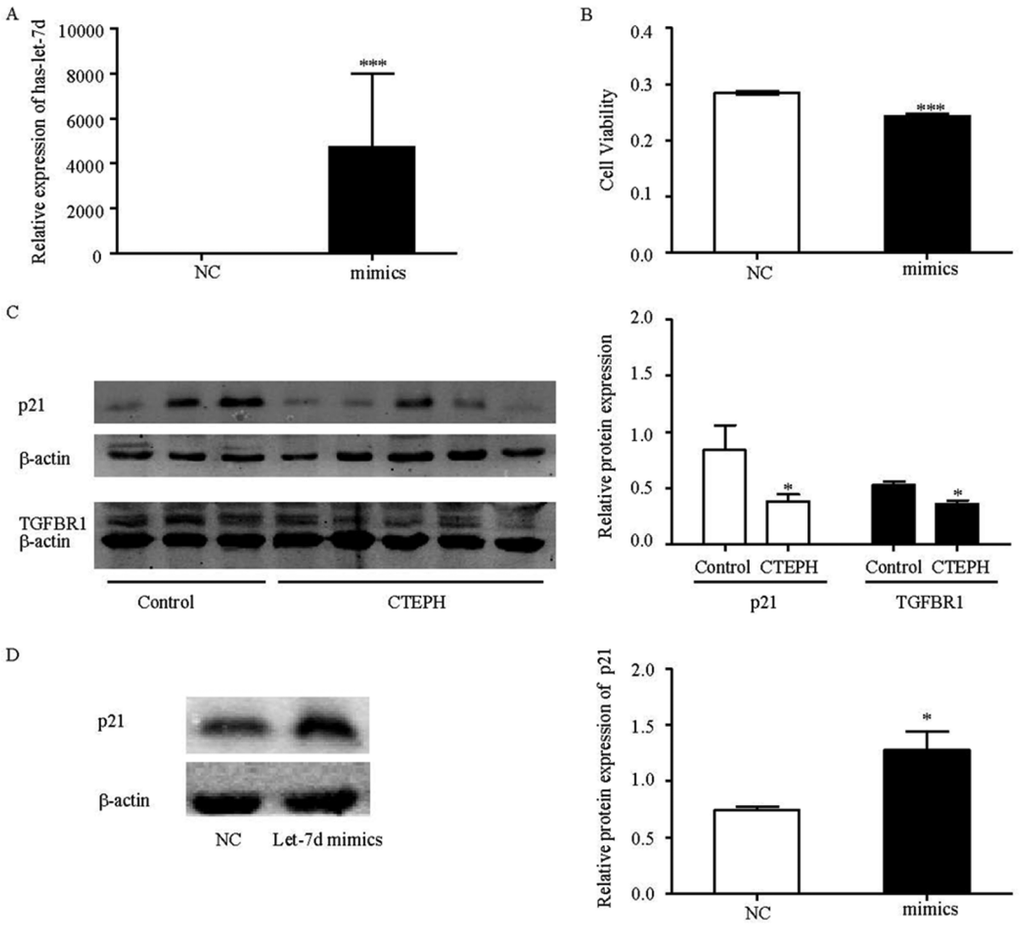

Figure 4. Let-7d repressed pulmonary artery smooth muscle cell (PASMC) proliferation by upregulating p21. *A*, Expression of let-7d after transfection of let-7d mimics and negative control (NC). *B*, Proliferation of PASMCs when treated with let-7d mimics. *C*, Protein expression levels of p21 (21 kDa), TGFBR1 (52 kDa; see Table 4 for definition), and β-actin (43 kDa) in PASMCs from chronic thromboembolic pulmonary hypertension (CTEPH; $n = 5$) and control ($n = 3$) patients. *D*, Protein expression levels of p21 in PASMCs of CTEPH patients after transfection of let-7d mimics. The results are presented as means + standard error; a single asterisk indicates $P < 0.05$, and three asterisks indicate $P < 0.001$, both versus NCs.

it repressed the proliferation of PASMCs by upregulating p21, which could arrest the cells at the G1 phase of the cell cycle. Consistent with our results, it has been reported that let-7 acts as a tumor suppressor and can repress cell proliferation by regulating multiple genes involved in cell cycle and cell division.[23] Let-7 plays a critical role in cell cycle control, as exogenous addition of pre-let-7 in primary human fibroblasts results in a decrease in cell number and an increased fraction of cells in the G2/M cell cycle phase.[16] In addition let-7g can restrict cellular proliferation and induce cell death by triggering a significant shift in cell cycle distribution, with an accumulation of G0/G1- and G2/M-phase cells and a corresponding reduction of S-phase cells.[34] Studies have also shown that let-7d reduces cell growth and leads to a greater number of cells in the G1 phase than in the G2/M phases in VSMCs.[17] But the role of let-7 in regulation of p21 is not fully clear; one paper showed that overexpression of let-7a caused an enhancement of p21 via let-7a-mediated suppression of NIRF (Np95 ICBP90 ring finger), through which let-7a played a growth-inhibitory effect.[35] In papillary thyroid carcinoma (PTC), let-7f was capable of reducing TPC-1 cell (a human PTC cell line) growth by increasing p21 cell cycle inhibitor mRNA, but potential mechanisms were not discussed.[36] Therefore, further research is necessary to reveal the possible mechanisms through which let-7d regulates p21.

In conclusion, to the best of our knowledge we are the first to report the global miRNA profile in PASMCs of CTEPH patients and to demonstrate that let-7d could repress the proliferation of PASMCs by upregulating p21. These results provide a potential therapeutic treatment (reestablishing the let-7d level) for CTEPH and suggest that further studies into miRNA expression would prove beneficial in understanding the pathogenesis of CTEPH.

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