Expression and analyses of the HIF-1 pathway in the lungs of humans with pulmonary arterial hypertension

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Abstract. Pulmonary arterial hypertension (PAH) is characterized by endothelial dysfunction and structural remodeling of the pulmonary vasculature, mediated initially by reduced oxygen availability in the lungs. Hypoxia inducible factor (HIF), consisting of the functional subunit, HIF-1α, and the constitutively expressed HIF-1β, is involved in the pathological processes associated with hypoxia. In the current study, the sequences of cDNAs and amino acids of HIF were characterized and analyzed using online bioinformatics tools. To further evaluate whether HIF accounts for the occurrence of PAH, the present study determine the expression and phosphorylation levels of HIF and its associated pathways, including extracellular signal-regulated kinase (Erk)1/2 and phosphoinositide 3-kinase (PI3K)/Akt, in the lungs of patients with PAH by reverse transcription-quantitative polymerase chain reaction and western blotting. The mRNA expression levels of PI3K, Erk2, and HIF-1α in the patients with PAH were significantly higher, compared with those in the control group, by 3.6-fold (P<0.01), 4.06-fold and 2.64-fold (P<0.05), respectively. No significant differences were found in the mRNA and protein levels of Akt between the two groups (P>0.05). The protein levels of phosphorylated (p-) Akt, Erk1/2, p-Erk1/2, HIF-1α and HIF-1β were significantly increased by 5.89-, 0.5-, 0.59-, 1.46- and 0.92-fold, respectively, in the patients with PAH, compared with those in the controls group (P<0.01 for p-Akt, Erk1/2; P<0.05 for p-Erk1/2, HIF-1α and HIF-1β). These findings suggested that the mitogen-activated protein kinase and PI3K/Akt signaling pathways, and HIF-1 may perform a specific function in the pathogenesis of PAH.

Introduction

Pulmonary arterial hypertension (PAH), defined as a mean pulmonary arterial pressure (mPAP) ≥25 mm Hg at rest, is a clinical syndrome of heart-lung circulation disorder, and can ultimately result in right heart failure with higher morbidity and mortality rates (1,2). Various types of PAH may affect up to 100,000,000 individuals worldwide (3). The estimated prevalence of PAH is ~15/1,000,000 individuals, with a mean age of 50±15 years, and women constitute 75% of those diagnosed (4,5). The average duration between the onset of symptoms and diagnosis is >2 years (5), and the 5-year mortality rate has reached 34% (6), reinforcing the importance of diagnosis, treatment and prognosis of PAH, which depends on investigations of the pathogenesis and etiology of the disease.

PHA is characterized by endothelial dysfunction and structural remodeling of the pulmonary vasculature, mediated initially by reduced oxygen availability in the lungs (7,8). Cell sensing and rapid response to oxygen deprivation are essential for survival of the organisms, in which the regulation of oxygen homeostasis becomes an important physiological system (9). As a result of evolution, adaptation to hypoxia involves a number of genes, in which hypoxia inducible factor (HIF) is considered to be a core regulator (10).

The first HIF, HIF-1, is a highly conserved transcription factor in almost all cells, and is involved in pathological processes associated with hypoxia, including pulmonary and systemic hypertension, cancer and ischemic myocardial injury (11-14). HIF-1 is a heterodimeric protein comprised of an oxygen-regulated HIF-1α subunit and a constitutively expressed HIF-1β subunit, also termed aryl hydrocarbon receptor nuclear translocater (15,16). HIF-1α is a master

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regulator of transcription in hypoxic cells and forms a dimer with HIF-1β, further activating genes involved in energy metabolism, cell proliferation and extracellular matrix reorganization (17,18). It has been reported that hypoxia mediates vascular remodeling through the induction of HIF-1α. In particular, HIF-1α in smooth muscle cells was demonstrated to be important in hypoxia-induced PAH in mice (19-21). However, the upstream signaling events responsible for hypoxia, and its effects on the proliferation of vascular smooth muscular and endothelial cells, remain to be fully elucidated.

Certain reports have shown that the expression and activity of HIF-1α are regulated by several protein kinase signaling pathways, in which extracellular signal-regulated kinase (ERK) and the serine/threonine kinase, Akt, have been identified as potent modulators of the expression of HIF-1α (22-25). ERK is a subfamily member of the mitogen-activated protein kinase (MAPK) family, and its pathway has been recognized to mediate cell growth, proliferation and survival (26,27). Li *et al* (28) found that the activation of ERK signaling induces the expression of HIF-1α and stimulates its transcriptional activity in the developing rat brain following hypoxia-ischemia, and an increase in the phosphorylation of ERK1/2 has been observed in retinal neovascularization and vein occlusion (29). In addition, Akt is activated by the phosphoinositide 3-kinase (PI3K)-dependent pathway, which is crucial in cell differentiation, proliferation and survival (30,31). Numerous studies have revealed the PI3K/Akt pathway to be critical for ischemia and angiogenesis (32,33), for example, the PI3K/Akt pathway is required for the upregulation of HIF-1α in a rat model of focal cerebral ischemia (34,35). However, whether these two pathways account for the occurrence of PAH induced by hypoxic conditions remains to be elucidated.

In the present study, the genes coding HIF-1α proteins were cloned from the lung tissues of human patients with PAH, and then were investigated by immunofluorescent techniques and bioinformatic methods. In addition, the expression and phosphorylation levels of the HIF-1α pathway components, including PI3K, Akt, ERK1/2 and HIF-1β, were examined using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analyses, and the association between target genes and the development of PAH were examined. The present clinical study aimed to contribute to the elucidation of the role of HIF-1α and its intracellular pathway in the occurrence of PAH, and provide a reference for further functional investigations of the pathogenesis of PAH.

Materials and methods

Collection of clinical samples. Human lung tissues were collected from participants during palliative surgery at the Affiliated Hospital of Guangdong Medical College (Guangdong, China). The participants comprised patients with PAH (mPAP >30 mmHg; n=5) and a control group of individuals with mPAP ≤20 mmHg (n=4). A total of 9 patients including 4 male and 5 female patients aged 15-53 years old (mean, 33.1±15.9 years old) were recruited. According to the updated clinical classification of pulmonary hypertension,

and the guidelines of the American College of Cardiology and American Heart Association, PAH was diagnosed using right heart catheterization (36). The lung tissues collected from the inferior lobes of left lungs were stored at -80˚C for further manipulation. All clinical protocols and experimental procedures were approved by the ethics committee of the Affiliated Hospital of Guangdong Medical College, and a written informed consent form was obtained from each individual participant.

Gene cloning. The cDNA fragments of HIF-1α and HIF-1β of patients with PAH were amplified using the Takara RNA LA PCR kit (AMV). PCR amplification was conducted at 94°C for 4 min, followed by 35 cycles at 94°C for 40 s, at 60°C for 50 s, at 72°C for 3 min, and a final extension at 72°C for 10 min. The primer sequences of human HIF-1α were 5'-CGAACGACAAGAAAAAGATAAG-3' (sense) and 5'-CCACAGAAGATGTTTATTTGATG-3' (antisense), and HIF-1β were 5'-CCGAAATGACATCAGATGTAC-3' (sense) and 5'-GTTAGATCAGGGAATTCTTCATTG-3' (antisense). The PCR products were sequenced by Invitrogen (Thermo Fisher Scientific, Inc., Shanghai, China). The sequencing results were used as queries in the BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Bioinformatic analyses. The sequences containing the complete coding regions of the human HIF-1α and HIF-1β genes, and the corresponding amino acid sequences were obtained from the GenBank (http://www.ncbi.nlm.nih.gov/genbank) and GenPept (http://www.ncbi.nlm.nih.gov/protein) databases (HIF-1α, GenBank accession no. U22431; GenPept accession no. AAC50152; HIF-1β, GenBank accession no. M69238; GenPept accession no. AAH60838 (37,38).

Comparative bioinformatics analyses of HIF-1α and HIF-1β were performed online (http://www.ncbi.nlm.nih.gov and http://www.expasy.org). The protein physical and chemical parameters were circulated using the Protparam tool (http://web.expasy.org/protparam) (39). The motifs and structural domains were searched in the amino acid sequences using the NCBI conserved domain database (CDD; http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) (40-43) and the secondary structures were predicted using the self-optimized prediction method (SOPMA; https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html) (44).

RT-qPCR analysis. The lung tissues were homogenized on ice with a Teflon-pestle homogenizer in TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and total RNAs were isolated following the manufacturer's instructions. A 1 μg sample of total RNA was reverse-transcribed into cDNA using AMV Reverse Transcriptase XL (Takara Bio, Inc., Otsu, Japan) and olido (dT) primers at 42˚C for 1 h. The primers for RT-qPCR are listed in Table I; GAPDH was selected as the internal control gene for normalization. The qPCR analysis was performed using 2 μl of cDNA in a total volume of 20 μl containing 10 μl 2X SYBR Premix Ex Taq II (Takara Bio, Inc.,), 0.8 μl forward primer (10 μM) and 0.8 μl reverse primer (10 μM), in a LightCycler® 480 System Real-Time PCR system (Roche Diagnostics GmbH, Mannheim, Germany) using the following thermal cycling profile: 95˚C for 30 sec, followed by

Table I. Sequences of primers.

Gene	Primer sequence	Size (bp)
GAPDH	Forward 5'-GGCACAGTCAAGGCTGAGAATG-3' Reward 5'-ATGGTGGTGAAGACGCCAGTA-3'	143
PIK3CA	Forward 5'-TCTGTCTCCTCTAAACCCTG-3 Reward 5'-TTCTCCCAATTCAACCAC-3'	103
Akt1	Forward 5'-TCTTTGCCGGTATCGTGT-3' Reward 5'-TGTCATCTTGGTCAGGTGGT-3'	150
Erk1	Forward 5'-GGGGAGGTGGAGATGGTGA-3' Reward 5'-GCTGGCAGTAGGTCTGATGTT-3'	175
Erk2	Forward 5'-TGTTCCCAAATGCTGACT-3' Reward 5'-AACTTGAATGGTGCTTCG-3'	160
HIF-1α	Forward 5'-GCTCATCAGTTGCCACTTCCAC-3 Reward 5'-CATCTGTGCTTTCATGTCATCTTC-3'	144
HIF-1β	Forward 5'-TGTGGACCCAGTTTCTGTGA-3 Reward 5'-GACCACCACGAAGTGAGGTT-3'	100

PIK3CA, phosphatidylinositol- 3-kinase catalytic subunit α; Erk, exrtracellular signal-regulated kinase; HIF, hypoxia inducible factor.

40 cycles of amplification (95°C for 5 sec and 60°C for 20 sec). The qPCR reactions were performed in triplicate. Fluorescence was detected during annealing and extension, and melting curve analysis was performed immediately following the PCR cycling. The relative transcript levels were analyzed using the $2^{-\Delta\Delta Cq}$ method (45).

Western blot analysis. The lung tissues were homogenized in ice-cold cell lysis buffer for western blot analysis and IP (Beyotime Institute of Biotechnology, Shanghai, China), and were centrifuged at 10,000 x g for 5 min at 4°C. The supernatants were used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis. The concentrations of the proteins in the supernatants were detected using an Enhanced BCA Protein Assay kit (Beyotime Institute of Biotechnology). The protein (~50 µg) was separated using 10% SDS-PAGE and transferred onto PVDF membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% fat-free milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T) and probed with the following primary antibodies: Mouse monoclonal HIF-1α (610958; 1:500; BD Biosciences, San Jose, CA, USA), rabbit polyclonal HIF-1β (bs-1407R; 1:500, BIOSS, Beijing, China), rabbit monoclonal ERK (#4695; 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit monoclonal phosphorylated (p)-ERK (Thr202/Tyr204; #4370; 1:2,000; Cell Signaling Technology, Inc.), rabbit monoclonal Akt (#4691; 1:1,000; Cell Signaling Technology, Inc.) and p-Akt (ser473; #4060; 1:2,000; Cell Signaling Technology, Inc.) in TBS-T containing 5% bovine serum albumin (Beyotime Institute of Biotechnology) overnight at 4°C. Following rinsing in TBS-T three times, the membranes were incubated with goat anti-mouse (#7076) and goat anti-rabbit (#7074) horseradish-peroxidase-coupled secondary antibodies (Cell Signaling Technology, Inc.) for 1 h at room temperature. Immunodetection was performed using BeyoECL Plus (Beyotime Institute of Biotechnology). Bands were visualized using the Bio-Rad ChemiDoc MP system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and analyzed using Quantity One software (Bio-Rad Laboratories, Inc.).

Statistical analysis. Data were analyzed using an independent-samples *t*-test with SPSS 20.0 software (IBM SPSS, Armonk, NY, USA. The data are presented as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Results

Bioinformatics analysis of HIF-1α and HIF-1β. The cDNA sequences of human HIF-1α and HIF-1β were aligned using the Basic Local Alignment Search Tool (BLAST) in the nucleotide database, and the results showed that they were 100% homologous with *homo sapiens* HIF-1α and HIF-1β mRNAs, respectively. The biochemical properties and molecular structures of human HIF-1α and HIF-1β were analyzed using the online tools, ProtParam and SOPMA, the results of which are listed in the Table II. As the dimer of these two subunits, the HIF protein was found to consist of 1,244 amino acids with a molecular weight of 138.592 Da; the most frequent residues were Leu and Ser. The functional domains were scanned in the CDD database (Fig. 1), following which three motifs were obtained, including a basic-helix-loop-helix (bHLH) region and two PAS repeat profiles, which have been previously demonstrated to be transcriptional activators of HIF-1α and HIF-1β in mammals (46,47).

Expression of the PI3K/Akt pathway. The relative mRNA expression level of PI3K was significantly elevated (2.6-fold) in the PAH group, compared with that in the control group (P<0.01; Fig. 2). No significant differences in the mRNA or

Table II. Biochemical properties and molecular structures of HIF-1α and HIF-1β.

Index	HIF-1α	HIF-1β
Amino acids (n)	826	416
Molecular weight (Da)	92,670.4	45,921.6
Theoretical isoelectric point	5.17	5.79
Formula	C4027H6410N1108O1309S43	C1963H3146N584O637S25
Atoms (n)	12,897	6,355
Extinction coefficients	50,155	20,690
Estimated half-life (h)	30	30
Instability index	55.97	52.65
Aliphatic index	74.96	71.44
Grand average hydropathicity	-0.573	-0.508
Charged amino acids (%)	31.72	31.97
Acidic amino acids (%)	14.29	13.46
Basic amino acids (%)	10.29	11.78
Polar amino acids (%)	31.60	29.09
Hydrophobic amino acids (%)	27.60	28.13
Major amino acids (%)		
	Leu 10.05	Ser 9.86
	Ser 9.44	Leu 7.69
	Thr 7.99	Asp 7.69
Secondary structure (%)		
α-helix	30.87	30.29
Extended strand	18.28	19.71
Random coil	43.83	41.11

HIF, hypoxia inducible factor.

protein levels of Akt were found between the two groups (P>0.05), however, the level of p-Akt in the PAH group was significantly increased (5.89-fold), compared with that in the control group, indicating that Akt was activated though phosphorylation by PI3K (P<0.01; Figs. 2 and 3).

Expression of the Erk1/2 pathway. The mRNA level of Erk2 in the PAH group was 3.06-fold higher, compared with that of control group (P<0.05), however, no significant difference in the mRNA level of Erk1 was observed between the two groups (P>0.05; Fig. 2). The results of the western blot analysis showed that the protein levels of Erk1/2 and p-Erk1/2 in the PAH group were significantly upregulated, compared with those in the control group (P<0.01 and P<0.05, respectively; Fig. 3).

Expression levels of HIF-1α and HIF-1β. The mRNA and protein levels of HIF-1α in the PAH group were respectively increased by 1.64- and 1.46-fold, compared with the control group (P<0.01 and P<0.05, respectively), suggesting that a higher mRNA level of HIF-1α increased synthesis of the HIF-1α protein (Figs. 2 and 3). No significant difference in the mRNA level of HIF-1β was found between the two groups, however, the protein level of HIF-1β was significantly elevated (by 92%) in the PAH group, compared with that in the control group (P<0.05; Figs. 2 and 3).

Discussion

Pulmonary vascular remodeling, including hyperplasia of pulmonary artery endothelial cells and pulmonary artery smooth muscle cells is the major pathological change in PAH. Multiple cytokines, including platelet-derived growth factor, vascular endothelial growth factor and transforming growth factor-β can promote cell proliferation and migration in the physiopathological processes of PAH (48-50).

The MAPK family comprises key factors for regulating the proliferation, differentiation and apoptosis of cells in response to certain environmental stresses and cytokines (51,52). MAPKs usually exist in forms of non-phosphorylated proteins in mammalian cells. As a member of the MAPK family, Erk1/2 can be activated though phosphorylation of the Thr185 and Tyr187 residues to produce a dimer, which is then translocated into the cell nucleus to activate various transcription factors (53). In the present study, it was found that Erk1/2 and p-Erk1/2 were upregulated in the patients with PAH, suggesting that Erk1/2 signaling pathway may be important for pulmonary vascular remodeling in PAH.

Similar to the MAPK signaling pathway, PI3K/Akt also induces cell growth, triggered by certain growth factors (54). Activated PI3K drives the production of
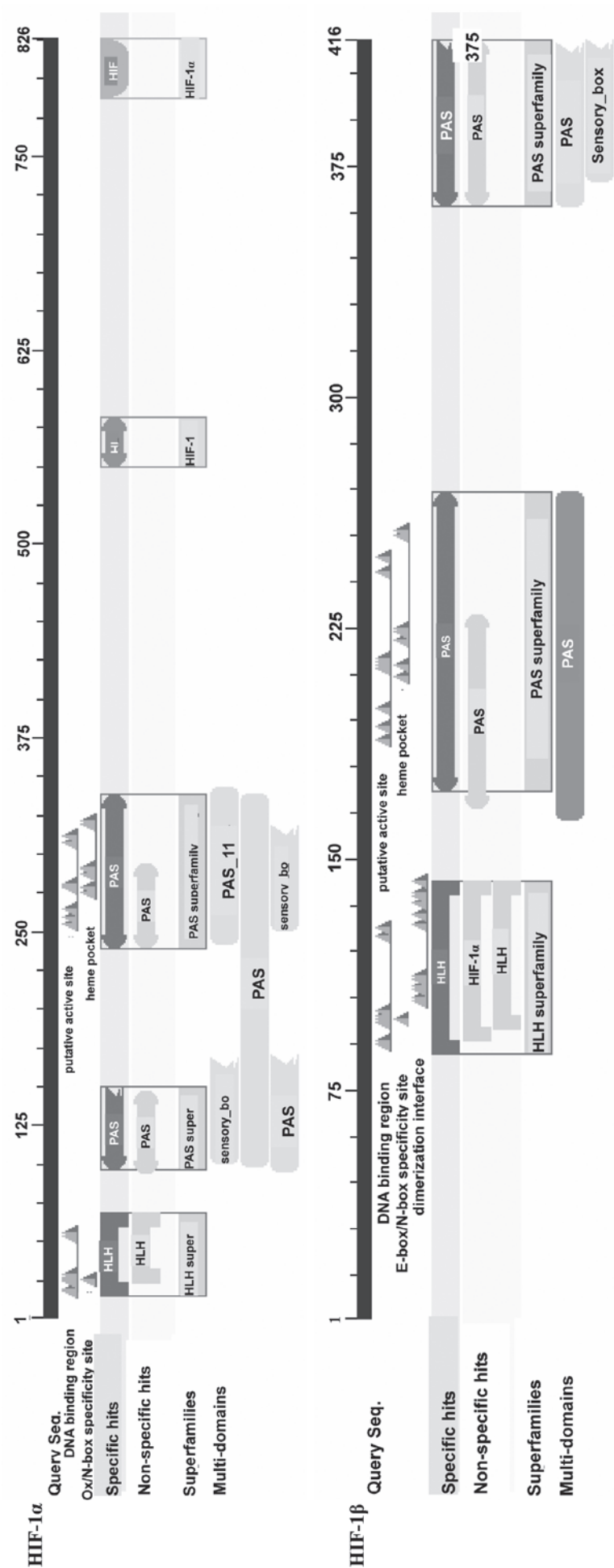
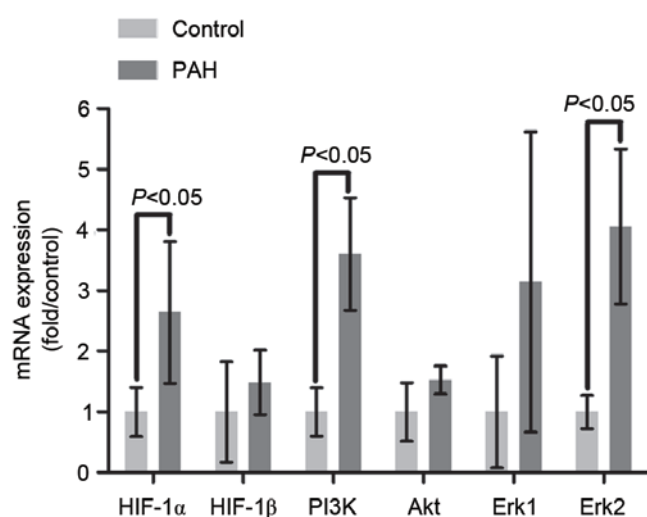Figure 1. Conserved domains of the HIF-1α and HIF-1β proteins. HIF, hypoxia inducible factor.

Figure 2. mRNA expression levels of HIF-1α, HIF-1β, PI3K, Akt, Erk1 and Erk2 in patients with PAH and control individuals. GAPDH was used as an internal control. Fold changes in the expression, compared with the control were calculated using the $2^{-\Delta\Delta Cq}$ method. Data are expressed as the mean ± standard deviation. PAH, pulmonary arterial hypertension; HIF, hypoxia inducible factor; PI3K, phosphoinositide 3-kinase; Erk, extracellular signal-regulated kinase.

Figure 3. (A) Representative images of western blot analysis. (B) Relative protein levels of HIF-1α, HIF-1β, Akt, p-Akt, Erk1/2 and p-Erk1/2 in the patients with PAH and the control individuals were determined using wetern blot analysis. β-actin was used as an internal control. Values are expressed as the mean fold change ± standard deviation relative to the control. PAH, pulmonary arterial hypertension; HIF, hypoxia inducible factor; PI3K, phosphoinositide 3-kinase; Erk, extracellular signal-regulated kinase; p-phosphorylated.

phosphatidyl-inositol-3,4,5-trisphosphate, which can bind to pleckstrin homology domains of Akt, and promote Akt phosphorylation at Thr308 and Ser473 residues, which induces the translocation of Akt into the nucleus to provide signals for cell survival (55). In addition, the second messenger PIP3 interacts with several cytoskeletal proteins, including paxilin, profilin, vinculin and filamin, to promote the polymerization of actin filaments, which can affect cell morphosis and migration (56-59). The present study showed that the levels of PI3K and phosphorylated Akt were markedly elevated in the patients with PAH, suggesting that the PI3K/Akt pathway may be involved in the pathological lesion of PAH by regulating cell proliferation, migration and adhesion, and even vascular stability.

HIF-1α and HIF-1β belong to the bHLH/PAS protein family, functioning as modulators in cell proliferation and differentiation (60). As HIF-1α lacks a transmembrane domain, HIF-1β is recruited to dimerize with HIF-1α for nuclear translocation. The degradation of the HIF-1α is suppressed by hypoxia, whereas the expression of HIF-1β in cells is commonly considered to be oxygen-independent. However, Wolff *et al* (61) found that the regulation of HIF-1β is more complex, and showed that the protein levels of HIF-1β are affected by hypoxia and hypoxia mimetics (61). In addition to HIF-1α, the present study found that the protein levels of HIF-1β were also elevated in the lungs of patients with PAH, suggesting that these two molecules may be involved in the pathogenesis of PAH. Therefore, the present study hypothesized that HIF-1β may exhibit corresponding responses to the changes of HIF-1α.

The present study demonstrated for the first time, to the best of our knowledge, changes in the expression levels of ERK1/2, PI3K, Akt and HIF-1 in the lungs of patients with
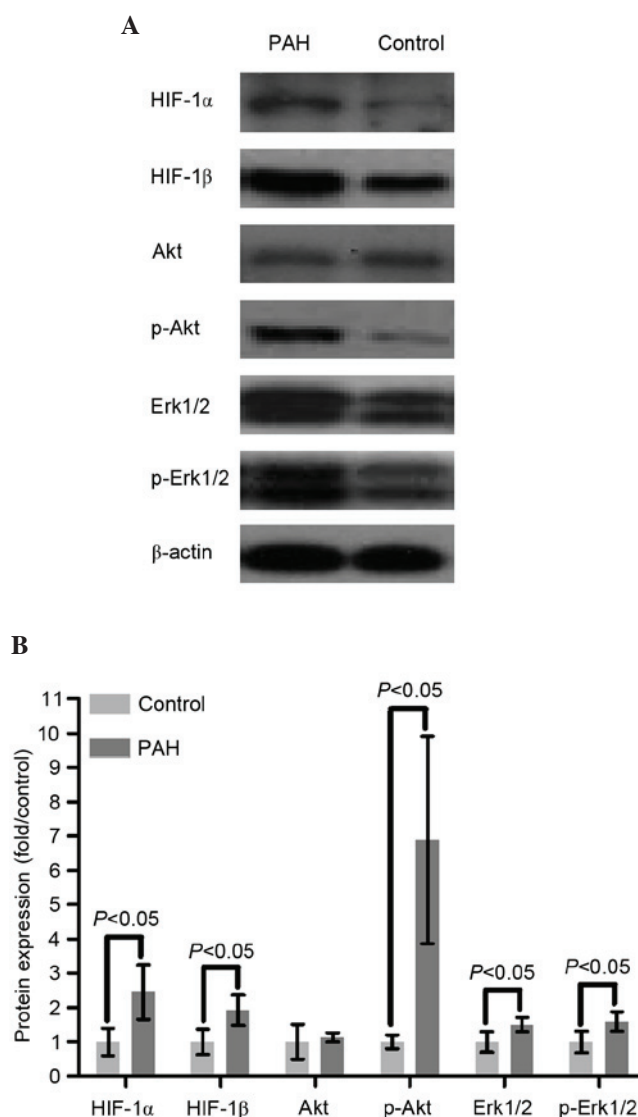
PAH. However, the roles of these signaling molecules in the pathogenesis of PAH and the associations among these signaling molecules require further investigations in the future.

Acknowledgements

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