Microarray profiling of long non-coding RNAs associated with idiopathic pulmonary arterial hypertension

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Abstract. Idiopathic pulmonary arterial hypertension (IPAH) is a fatal disease with a poor prognosis and the molecular pathways underlying the pathogenesis of IPAH are not fully understood. In the present study, the long non-coding RNA (lncRNA) and mRNA expression profiles of lymphocytes obtained from 12 IPAH patients and 12 healthy controls were analyzed using Arraystar Human lncRNA Microarray v2.0, and their roles in the pathogenesis of IPAH were characterized using comprehensive bioinformatic tools. A total of 2,511 lncRNAs (2,004 upregulated and 507 downregulated) and 1,169 mRNAs (609 upregulated and 560 downregulated) were aberrantly expressed in IPAH patients with a fold-change of >2.0. Gene ontology analysis indicated that the coexpressed lncRNAs and mRNAs were involved in the process of translation, while pathway analysis indicated that the coexpressed RNAs were enriched during the process of oxidative phosphorylation and in the ribosome. It was concluded that dysregulated lncRNAs are potentially associated with IPAH, and aberrant lncRNA expression in blood cells may serve as a diagnostic marker of IPAH.

Introduction

Pulmonary arterial hypertension (PAH) is a fatal disease that is difficult to diagnose. The pathogenesis of PAH involves the obstruction and constriction of pulmonary arteries, and increased pulmonary vascular resistance, ultimately leading to right ventricular hypertrophy and failure (1). Chronic obstructive pulmonary disease and prolonged exposure to hypoxic conditions are two major causes of PAH (2). It has been established that the hallmarks of PAH include pulmonary vascular endothelial dysfunction leading to vascular remodeling, pulmonary artery smooth muscle cell (PASMC) proliferation and migration, medial hypertrophy, inflammation and thrombosis in situ leading to the formation of plexiform lesions (3,4).

Idiopathic PAH (IPAH) patients are essentially patients with PAH; however they do not harbor the known risk factors, including drug exposure, genetic variants, related pathologies, of PAH. Patients with IPAH are characterized according to the following measures: Mean pulmonary artery pressure (mPAP) of ≥25 mmHg, pulmonary capillary wedge pressure (PCWP), left atrial pressure or left ventricular end-diastolic pressure of ≥15 mmHg, and pulmonary vascular resistance (PVR) ≥3 Wood Units (5).

To date, numerous studies have investigated the pathogenic mechanisms of IPAH, with implications that cytokines, including phosphodiesterase 2 (6), nitric oxide (7) and transforming growth factor-β (TGF-β) (8), are involved in development of the disease. However, the molecular pathways underlying the pathogenesis of IPAH remain largely unknown.

Long non-coding RNAs (lncRNAs) are loosely defined as endogenous cellular RNAs of >200 base pairs (bp) that lack protein-coding capacity (9). The Encyclopedia of DNA Elements project reported that there are 49,500 independent lncRNA genes in the human genome, which collectively produce 415,500 transcripts (10). Previous studies have demonstrated that lncRNAs are involved in a variety of biological processes, including cell-cycle control, chromatin remodeling, differentiation and epigenetic regulation (11,12). The dysregulation of lncRNAs is also implicated in the pathogenesis of various diseases, including colorectal cancer (13) and schizophrenia (14). However, the dysregulation of lncRNAs in IPAH has not been investigated.

Therefore, the present study aimed to determine the possible roles of lncRNAs in the pathogenesis of IPAH, via a microarray analysis of potentially dysregulated lncRNAs and mRNAs in the peripheral blood of IPAH patients.

Materials and methods

Patients. From July to December 2013, 12 consecutive, well-characterized IPAH patients (5 males and 7 females, aged 52.0±10.2 years) were admitted to Qilu Hospital, a tertiary teaching hospital affiliated with Shandong University (Shandong, China). Pulmonary hypertension was defined as...
a mPAP of ≥25 mmHg, a PCWP of ≤15 mmHg at rest, as assessed by right heart catheterization (RHC), and a PVR of >3 Wood Units, also measured by RHC. IPAH patients were diagnosed according to the 2009 diagnostic algorithm developed by the European Society of Cardiology and the European Respiratory Society (5). Therefore, no patients had a family history of PAH. PAH patients with other known causes were excluded from the current study on the basis of clinical characteristics, echocardiography, high-resolution computed tomography, RHC, computed tomographic pulmonary angiography, ventilation/perfusion lung scan, and/or pulmonary angiography. Patients with ≥1 of the following conditions were excluded: i) Other types of pulmonary hypertension, including familial pulmonary hypertension; ii) heart diseases, including known left ventricular diseases and acute heart failure; iii) chronic respiratory disorders, including chronic obstructive pulmonary disease; iv) diabetes mellitus; and v) prior targeted therapy. No patients had received medical treatment (bosentan, treprostinil, nifedipine or iloprost) prior to sample collection.

A total of 12 healthy controls (5 males and 7 females, aged 49.2±11.8 years) were recruited from local communities in Shandong, China in the current study. The inclusion criteria for healthy controls were that subjects must be age- and sex-matched with patients and absent of any diseases when enrolled. The patients' clinical features are summarized in Tables I and II. The experimental protocols in the present study were approved by the Ethics Committee of Qilu hospital (protocol no. 2014-B-046). The recruited subjects provided written informed consent prior to participation in the study.

**RNA extraction.** A total of 5 ml peripheral blood from each subject was collected in PAXgene RNA stabilization tubes (PreAnalytiX GmbH, Hombrechtikon, Switzerland). Following the removal of red blood cells according to the manufacturer's protocol (Qiagen, Inc., Valencia, CA, USA), which involved the addition of sufficient buffer BG1 and BG2 in order, vortexing for 5 sec, centrifugation for 3 min and subsequent discarding of the supernatant, total RNA was extracted from peripheral blood leukocytes using PAXgene RNA collection tubes (Qiagen, Inc.) according to the manufacturer's guidelines. The kit included all reagents and protocols for extraction and purification. The whole leukocyte fraction consisted of T and B lymphocytes, natural killer cells, monocytes, neutrophils, basophils and eosinophils. The quality and concentration of the RNA samples were assessed at absorbance ratios of A260/A280 and A260/230 using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE, USA) and denaturing agarose gel electrophoresis (2% gel; 3 µl RNA samples and 1.5 µl loading buffer (3X) per lane).

**RNA labeling and array hybridization.** The expression levels of IncRNAs and mRNAs in each sample were determined using Arraystar Human IncRNA Microarray v2.0 (CapitalBio Corporation, Beijing, China). Sample labeling and array hybridization were performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technologies, Inc., Santa Clara, USA). mRNA was purified from total RNA following removal of rRNA using an mRNA-ONLY™ Eukaryotic mRNA Isolation kit (Epicientre Biotechnologies, Madison, WI, USA). Each sample was then transcribed into fluorescent cRNA along the entire length of the transcripts without 3' bias using the QuantScript RT kit (TIANGEN, China) on a Bio-Rad CF-96X platform (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using the following procedure: 42°C for 2 h, 16°C for 1 h and 40°C for >2 h. Following purification with an RNeasy Mini kit (Qiagen GmbH, Hilden, Germany), the labeled cRNAs were hybridized with the specific probes on the Human IncRNA Array v2.0. According to the manufacturer's instructions, the hybridized arrays were washed with washing buffer, fixed and scanned at 5 mm/pixel resolution with an Agilent DNA Microarray Scanner G2505C (Agilent Technologies, Inc.) equipped with GenePix Pro 6.0 software (Molecular Devices, LLC- Sunnyvale, CA, USA).

**Microarray data analysis.** Scanned images (TIFF format) were imported into Agilent Feature Extraction software (version 11.0.1.1; Agilent Technologies, Inc.) for grid alignment and expression data analysis. Expression data were normalized by a quantile normalization and a Robust Multichip Average algorithm included in the Agilent software. Probe-level files, including IncRNAs and mRNAs, were generated following normalization. Final results were generated after combining the probe-level files and gene-level files using Agilent GeneSpring GX software (version 11.5.1; Agilent Technologies, Inc.). Following fold change (FC) analysis (FC>2.0 or FC<0.5) and false discovery rate (FDR) analysis (FDR<0.05), differentially expressed IncRNAs and mRNAs were identified through FC filtering according to the predetermined P-value threshold (P<0.05).

**Functional group analysis.** The Database for Annotation, Visualization and Integrated Discovery (http://david.abcc.ncifcrf.gov/), which utilizes Gene Ontology (GO) to identify the molecular functions of gene profiles (15,16), was applied in the present study to determine the functions of the differentially expressed coding genes identified by microarray analysis. Pathway analysis was used to place differentially expressed coding genes according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway from the Biocarta and Reactome database (http://www.genome.jp/kegg/). The FDR-corrected P-value threshold was set at 0.05.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted as stated previously and reverse transcribed using a PrimeScript RT reagent kit with gDNA Eraser (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocol. RT-qPCR was performed using a SYBR-Green PCR kit (Takara Biotechnology Co., Ltd.) on a CFX96 Real-Time PCR Detection System. The PCR conditions included an initial step at 95°C for 5 min, followed by 40 cycles of amplification and quantification (95°C for 15 s, 60°C for 30 s). Each cDNA sample was obtained in triplicate in a final volume of 25 µl containing 1 µl CDNA and 400 nM of forward and reverse gene-specific primers. The primer sequences are provided in Table III.

Relative gene expression level was quantified using the ΔΔCt method (17), in which GAPDH was used as an internal control. For quantitative results, expression of each gene was
Table I. Clinical characteristics of IPAH patients.

| ID    | Gender | Age (years) | Onset of symptoms (mo) | WHO functional class | 6MWT (min) | mPAP (mmHg) | CI (l/min/m²) | PVR (WU) | VR |
|-------|--------|-------------|------------------------|---------------------|------------|-------------|---------------|----------|----|
| IPAH 1 | F      | 45          | 29                     | III                 | 255        | 48          | 1.8           | 12.5     | No |
| IPAH 2 | M      | 52          | 36                     | II                  | 554        | 38          | 2.8           | 13.6     | No |
| IPAH 3 | F      | 34          | 18                     | III                 | 382        | 35          | 2.2           | 11.0     | Yes|
| IPAH 4 | F      | 58          | 22                     | III                 | 460        | 48          | 1.7           | 12.4     | Yes|
| IPAH 5 | F      | 38          | 41                     | II                  | 350        | 40          | 3.0           | 8.5      | No |
| IPAH 6 | M      | 63          | 33                     | IV                  | 273        | 53          | 2.1           | 7.5      | No |
| IPAH 7 | M      | 64          | 29                     | I                   | 334        | 50          | 1.8           | 8.6      | No |
| IPAH 8 | M      | 60          | 32                     | III                 | 320        | 31          | 1.7           | 11.8     | No |
| IPAH 9 | F      | 48          | 16                     | II                  | 534        | 49          | 2.5           | 13.1     | No |
| IPAH 10| F      | 55          | 26                     | III                 | 281        | 59          | 1.9           | 6.1      | No |
| IPAH 11| M      | 44          | 27                     | III                 | 449        | 49          | 2.2           | 9.3      | No |
| IPAH 12| F      | 63          | 39                     | IV                  | 391        | 51          | 2.7           | 10.8     | No |

n=12. WHO, World Health Organization; 6MWT, 6-minute walk test; mPAP, mean pulmonary arterial pressure; CI, cardiac index; PVR, pulmonary vascular resistance; WU, Wood units; VR, vascular reactivity; IPAH, idiopathic pulmonary arterial hypertension; mo, months; M, male; F, female.

Table II. Demographics of IPAH patients and matched healthy controls.

| Item             | IPAH patients | Controls |
|------------------|---------------|----------|
| Number (n)       | 12            | 12       |
| Age (years, mean ± SD) | 52.0±10.2   | 49.2±11.8 |
| Males (n, %)     | 5 (41.7%)     | 3 (41.7%) |
| Onset of symptoms (mo, mean ± SD) | 29.0±7.3     | n/a      |
| WHO functional class (I/II/III/IV) | 1/3/6/2     | n/a      |
| 6MWT (min, mean ± SD) | 381±499.4   | n/a      |
| mPAP (mmHg, mean ± SD) | 45.9±26.1   | n/a      |
| CI (l/min/m², mean ± SD) | 2.2±0.45    | n/a      |
| PVR (WU, mean ± SD) | 10.4±2.40   | n/a      |

SD, standard deviation; 6MWT, 6-minute walk test; PAP, pulmonary arterial pressure; CI, cardiac index; PVR, pulmonary vascular resistance; WU, Wood units; IPAH, idiopathic pulmonary arterial hypertension; WHO, World Health Organization.

represented as a FC using the following mathematical model: FC = (Etarget)ΔCq(target)−(Eref)ΔCq(ref). In this model, Etarget and Eref were the PCR efficiency of target gene transcription and reference gene transcription, respectively, ΔCq(target) was the Cq deviation of control-sample of the target gene transcript and ΔCq(ref) was the Cq deviation of control-sample of the reference gene transcript.

Statistical analysis. The statistical significance of microarray data was analyzed in terms of FC using the Student's t-test and FDR was calculated to correct the P-value. The Mann Whitney test was also applied to compare the patient and control groups using GraphPad Prism 5.0 software (GraphPad software, Inc., La Jolla, CA, USA) and Microsoft Office Excel 2010 software (Microsoft Corporation, Redmond, WA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

IncRNA profile changes in IPAH patients. A total of 7,249 IncRNAs were detected by Arraystar Human LncRNA Microarray v2.0 (data not shown). Hierarchical clustering was applied in order to group IncRNAs based on their expression levels among samples. As depicted in Fig. 1A, differentially expressed IncRNAs were observed between the IPAH patient and control groups.

When a threshold of FC>2.0, P<0.05 and FDR<0.05 was set, it was found that 2,511 IncRNAs were differentially expressed, including 2,004 upregulated and 507 downregulated IncRNAs. When the P-value was set at P<0.01, a total of 1,722 IncRNAs were dysregulated, including 1409 upregulated and 313 downregulated IncRNAs. Table IV provides details of the 10 most upregulated (ENST00000424119.1, ENST00000530600.1, ENST00000587759.1, ENST0000062495.1, ENST00000414407.1, ENST00000452477.1, TCONS_00003801, TCONS_00005167, TCONS_00017343 and ENST00000602863.1) and 10 most downregulated (uc001nxj.1, ENST00000376482.3, TCONS_00008036, ENST00000445107.1, NR_024412.1, ENST00000421013.1, ENST00000569048.1, XR_159116.1, TCONS_00017669 and TCONS_00017647) IncRNAs in the patient group compared with the control group.

In addition, the classification and length distribution of dysregulated IncRNAs was summarized. Among the dysregulated IncRNAs, there were 1,362 intergenic, 572 antisense, 357 intronic and 220 divergent IncRNAs (Fig. 1B). These differentially expressed IncRNAs ranged from 61 bp to
Table III. Reverse transcription-quantitative polymerase chain reaction primers for randomly selected IncRNAs and mRNAs.

| Transcript ID | Forward primer (5'-3') | Reverse primer (5'-3') |
|---------------|------------------------|----------------------|
| TCONS_00023744 | TGGAGGAAGGTGCGTGCTCAAAG | GACACAACTCCACCTCAACAAGA |
| TCONS_00004224 | GTTCTGTGTGATGTTGGGATTGG | AAGTGTCTTTATATGTAGCTGAG |
| ENST00000552663.1 | GCTCTCTCCTGCTGGCTC | CTTTTTTGTGTATTTTCTCCGTC |
| ENST00000417305.1 | TTCTTCTGCCCTGTTTTG | TGTTTTCTCCTTTCAGCCTCA |
| TCONS_00018111 | ACTCTGATTTTGAGAAGTTGAAGA | TAGGATACCCCTGAAATAGAGC |
| ENST00000532124.1 | ATGGAGGAGGGTGTTGAG | TTGGGAGGCTGAGGCGCA |
| ENST00000419223.1 | CACAGAAGTGGAAGAGACAAA | TGGGAGGCAAGACCTCCGAG |
| ENST00000507856.1 | AACGGGATGTACACGGTGAAGA | AGAGAGGTGGGAGGGCCAG |
| TCONS_00012819 | TTCCAACCCCAACAGC | CAACGTGAAACGTCCCGATC |
| ENST00000566645.1 | GGAGAAAAGTGCCAGACGAGA | TGGTACATTCCTTCCATTGCAG |
| LOC100287651 | GAAAGAAATCCCAAGGTGATC | CATATCCGAGAATACACAGG |
| TSL | AAAGTTATCCTGTTGAAATAGTG | TCATTCTGAGAGGGTGGC |
| TRIM9 | CGCCGATGATCTCTTCTG | CCGGCTGCTGCCTTGTG |
| RRH | ACCAGGAGGTGTCCAGTGGT | CCAGAATGTCGTTGAGTGT |
| BAG1 | CGTCTGACGACCTCATTGCAAAGA | AATCCCTGGGAGAAACCAAC |
| PDGFD | GTATGATTCTGAGAAGTTGGAGA | CTACCTGGCAGGCTGAG |
| LY96 | ATCTGATGAGGTATTACTTTTG | CAACACATTTGAATCTTCTTAG |
| HOPX | ACCGTCGCTCTCCTATCGG | CGGCGCGACTTTGGCA |
| ATP5I | GCAGGTCCTCTCGCTGAT | TCTCTGGAATTCCTTTCA |
| RAP2A | AGCCTCGTCACAACAGCAG | CTTCACATTTCCAGGTCCACT |

IncRNA, long non-coding RNA.
GO and pathway analysis for differentially expressed lncRNAs. Existing evidence implies that the majority of lncRNAs are cis-acting and regulate the expression of adjacent genes (18). The present study identified protein-coding genes within 100 kb of dysregulated lncRNA on chromosomal DNA. GO and KEGG pathway analyses subsequently indicated the functions of the potential target genes of lncRNAs. These coding genes were found to be predominantly involved in
Table IV. The 10 most upregulated and 10 most downregulated lncRNAs in IPAH patients relative to matched controls.

| IncRNA ID          | FC (abs) | Regulation | FDR    | Chromosome | Strand | Start\(^a\) | End\(^b\) | Class      | Database       |
|-------------------|----------|-----------|--------|------------|--------|-------------|-----------|------------|----------------|
| ENST0000424119.1  | 13.41    | Up        | 1.50E-12 | 2          | -      | 64565200    | 64568781  | Intergenic  | ENSEMBL        |
| ENST0000530600.1  | 15.33    | Up        | 9.15E-08 | 8          | -      | 144624142   | 144631899 | Divergent   | ENSEMBL        |
| ENST0000587759.1  | 15.56    | Up        | 1.84E-11 | 19         | +      | 6067963     | 6077130   | Intronic    | ENSEMBL        |
| ENST0000602495.1  | 18.83    | Up        | 4.58E-06 | X          | -      | 73048972    | 73053596  | Intergenic  | ENSEMBL        |
| ENST0000414407.1  | 18.52    | Up        | 1.28E-02 | 13         | +      | 31377342    | 31384782  | Intergenic  | ENSEMBL        |
| ENST0000452477.1  | 21.75    | Up        | 3.27E-11 | 5          | -      | 46359241    | 46380257  | Intergenic  | ENSEMBL        |
| TCONS_00003801    | 26.25    | Up        | 2.02E-06 | 2          | +      | 102661124   | 102674449 | Intergenic  | Human LincRNA Catalog |
| TCONS_00005167    | 35.20    | Up        | 1.22E-08 | 2          | -      | 64530720    | 64565467  | Intergenic  | Human LincRNA Catalog |
| TCONS_00017343    | 80.27    | Up        | 4.20E-06 | 3          | -      | 73048922    | 73061505  | Intergenic  | Human LincRNA Catalog |
| ENST0000602863.1  | 313.67   | Up        | 2.27E-06 | 11         | -      | 63391555    | 63395325  | Intergenic  | UCSC           |
| uc001nxj.1        | 12.23    | Down      | 1.07E-03 | 11         | +      | 99728586    | 99738062  | Intergenic  | ENSEMBL        |
| ENST0000376482.3  | 13.17    | Down      | 1.77E-10 | 7          | +      | 99728586    | 99738062  | Intergenic  | ENSEMBL        |
| TCONS_00008036    | 14.36    | Down      | 0.00159  | 4          | +      | 33517098    | 33522181  | Intergenic  | Human LincRNA Catalog |
| ENST0000445107.1  | 14.40    | Down      | 7.32E-09 | X          | -      | 27767800    | 2778101   | Intronic    | ENSEMBL        |
| NR_024412.1       | 14.59    | Down      | 0.0191   | 7          | -      | 11275672    | 11275863  | Intergenic  | RefSeq         |
| ENST0000421013.1  | 16.37    | Down      | 4.56E-16 | 1          | +      | 101491409   | 101552819 | Divergent   | ENSEMBL        |
| ENST0000569048.1  | 16.63    | Down      | 4.01E-05 | 16         | -      | 18027043    | 18066399  | Intergenic  | ENSEMBL        |
| XR_159116.1       | 20.31    | Down      | 5.33E-05 | 1          | +      | 224219240   | 224221571 | Intergenic  | RefSeq         |
| TCONS_00017669    | 33.01    | Down      | 5.66E-03 | 10         | +      | 6622386     | 6627323   | Divergent   | Human LincRNA Catalog |
| TCONS_00017647    | 49.40    | Down      | 9.31E-13 | Y          | -      | 14774285    | 14775639  | Intergenic  | Human LincRNA Catalog |

*Chromosomal positions based on the human reference genome GRCh38. FC, fold-change; FDR, false discover rate; +, sense strand; -, antisense strand; up, upregulated; down, downregulated.*
Figure 2. Unsupervised hierarchical clustering of partially differentially expressed mRNAs in IPAH relative to matched controls. High relative expression levels (red), and low relative expression levels (green) were observed.

Figure 3. RT-qPCR validation of dysregulated lncRNAs and mRNA identified by microarray analysis. (A) A total of 10 lncRNAs were randomly selected for RT-qPCR validation. (B) A total of 10 mRNAs were chosen randomly for RT-qPCR validation. Fold changes were calculated by the $\Delta\Delta C_q$ method. Data shown are representative of 12 IPAH patients and 12 matched controls. Error bars indicate the mean ± standard error of the mean. *P<0.05 vs. microarray. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; lncRNA, long non-coding RNA.
the following processes and functions (P<0.05, with -log10(P)>1.30): i) Regulation of DNA replication, translational elongation, cell division and organelle fission (biological processes; Fig. 5A); ii) the hemoglobin complex, intracellular organelle compartment, cytoplasmic compartment and intr-organelle lumen compartment (cellular component, Fig. 5B); and iii) protein homodimerization activity, rRNA binding, gamma-tubulin binding (molecular function; Fig. 5C). KEGG pathway analysis also found the neighbouring genes of the dysregulated lncRNAs to be principally involved in the following (P<0.05): i) Splicesomal pathways; ii) the cell cycle; iii) apoptosis; iv) the p53 signaling pathway; v) oocyte meiosis; vi) Huntington's disease; and vii) prostate cancer (Fig. 5D).

Discussion

In the present study, dysregulated lncRNAs in IPAH were analyzed, via comparison of the peripheral blood transcriptome profiles of IPAH patients and healthy volunteers. A total of 7,429 lncRNAs and 8,110 mRNAs were measured. In terms of dysregulated RNAs, it was found that in IPAH, 2,004 lncRNAs were upregulated and 507 were downregulated, while
609 mRNAs were upregulated and 560 were downregulated. These RNAs were subsequently evaluated by microarray, GO and KEGG pathways analyses to determine their general characteristics and functional annotations. Collectively, the data suggests a potentially novel association of RNA profiles, particularly for IncRNA, with IPAH. The description of dysregulated mRNAs and IncRNAs obtained may provide insight into the pathogenesis and development of IPAH. Furthermore, the results of the present study may enable the development of novel therapeutic targets, along with diagnostic and prognostic markers for IPAH.

The RNA profiling analysis between IPAH and healthy subjects utilized lymphocytes as the RNA source. This was due to the difficulty in obtaining the involved tissues, namely the human pulmonary vasculature, from live individuals. Though pulmonary vasculature can be taken from healthy individuals at autopsy, RNAs may be degraded at varying rates, resulting in a discrepancy in the expression profiling relative to other studies (19). An additional objective of the present study was to identify a non-invasive diagnostic biomarker of IPAH, and thus, lymphocytes were more advantageous (for example, easy access) than other source tissues, such as alveolus tissue and vascular tissue (20). Furthermore, peripheral lymphocytes have been implicated in IPAH pathogenesis. Austin et al (21) found that, relative to controls, circulating T cell subsets, particularly cluster of differentiation 8 (CD8)+T and CD4+ T (regulatory) lymphocytes, were markedly increased in IPAH. In addition, the number of circulating monocyte-derived dendritic cells was lower in IPAH patients than in controls (22). Hautefort et al (23) also identified T helper 17 cell immune-polarization in PAH patients that was absent in controls. Collectively these alternations in peripheral lymphocytes may contribute to alterations in RNA expression profiles.

The dysregulation of IncRNA expression in IPAH is not well established. However, a number of proteomic and transcriptome profiling studies for IPAH have been performed, in which several dysregulated genes have been identified (24-26). In particular, four previous profiling studies of IPAH have used peripheral lymphocytes as the RNA source. This was due to the difficulty in obtaining the involved tissues, namely the human pulmonary vasculature, from live individuals. Though pulmonary vasculature can be taken from healthy individuals at autopsy, RNAs may be degraded at varying rates, resulting in a discrepancy in the expression profiling relative to other studies (19). An additional objective of the present study was to identify a non-invasive diagnostic biomarker of IPAH, and thus, lymphocytes were more advantageous (for example, easy access) than other source tissues, such as alveolus tissue and vascular tissue (20). Furthermore, peripheral lymphocytes have been implicated in IPAH pathogenesis. Austin et al (21) found that, relative to controls, circulating T cell subsets, particularly cluster of differentiation 8 (CD8)+T and CD4+ T (regulatory) lymphocytes, were markedly increased in IPAH. In addition, the number of circulating monocyte-derived dendritic cells was lower in IPAH patients than in controls (22). Hautefort et al (23) also identified T helper 17 cell immune-polarization in PAH patients that was absent in controls. Collectively these alternations in peripheral lymphocytes may contribute to alterations in RNA expression profiles.

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Discrepancies between the mRNA expression profiles of the current study and previous studies are likely due to a number of limitations. For example, the IPAH RNA and protein profiles fluctuate markedly during disease progression (31,32), leading to variable RNA profiles. In addition, different detection platforms may have substantial impact on the profiling results and thus, subsequent experimental verification is necessary. The majority of previous studies have used an Affymetrix platform for mRNA profiling analysis, while the present study used Arraystar Human LncRNA Microarray v2.0 for the specific analysis of IncRNAs.

The gene signature identified in IPAH patients was characterized by an increased expression of genes, gene sets, functions and networks related to fibroblast and PASMC proliferation. Therefore, the cause of IPAH may be aberrant proliferation of fibroblasts and PASMCs. In the present study, numerous genes with potential effects on cell proliferation were identified, including erythroblast transformation-specific-related gene, JUN proto-oncogene and mouse double minute 2 homolog. Furthermore, several members of the matrix metalloproteinase (MMP) family, including MMP1, MMP11 and MMP15, showed dysregulated expression in the IPAH group. It has been reported that MMP1 upregulation is involved in fibroblast proliferation and migration, along with extracellular matrix accumulation (33), while MMP11 may cause basal membrane disruption and stimulate the adventitial thickening of pulmonary vessels (34). Collectively, myofibroblast proliferation and basal membrane disruption results in progressive parenchymal fibrosis, alveolar damage and incorporation of connective tissue around the pulmonary vessels, which may also lead to the development of IPAH.

Previous pathway analysis has suggested that numerous genes are involved in IPAH. In particular, genes involved in cellular growth/proliferation and cell cycle regulation, and signaling pathway genes including mitotic activators, polo-like kinase and ataxia telangiectasia mutated, are activated in IPAH (26). Similarly, the present study found that genes dysregulated in IPAH were involved in translational elongation, cell cycle phases and ribosomal pathways, suggesting that transcriptional and translational processes are disrupted in IPAH. This would result in disruption to protein expression, with potentially far-reaching effects on normal physiological processes, including impairment of mitochondrial translation and energy generation (35,36). Analogous to these results, PASMC proliferation is established to be a driving factor in PAH (3).

To conclude, the IncRNA profile of IPAH patients was previously unknown. The present study has identified a number of dysregulated IncRNAs that are potentially implicated in IPAH, which may provide insight into the pathogenesis and mechanisms of the disease. Therefore, further study is warranted into the functions of these IncRNAs, as potential therapeutic targets for the treatment of IPAH.

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