Article

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Transcriptome Sequencing of lncRNAs, circRNAs, miRNAs, mRNAs, and Interaction Network Constructing in Acute Exacerbation of Idiopathic Pulmonary Fibrosis

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Background: Idiopathic pulmonary fibrosis (IPF) patients who suffer from acute exacerbation of idiopathic pulmonary fibrosis (AE-IPF) are at increased risk of respiratory deterioration and death. Non-coding RNAs (ncRNAs) play a vital role in AE-IPF, but studies of crosstalk between transcripts of IPF based on Traditional Chinese Medicine (TCM) syndrome type are relatively few. The construction of long non-coding RNAs (lncRNA)/circular RNAs (circRNA)-microRNAs (miRNA)-mRNA interaction networks can promote understanding RNA interaction in different syndrome types of AE-IPF. The study aimed to identify the difference in RNA transcription expression between IPF patients with "lung heat and collateral stasis (LHCS)" and "lung deficiency with collateral stasis (LDCS)" syndromes, further to construct the potential RNA networks.
Methods: Five IPF patients with LHCS and five IPF patients with LDCS were recruited in this study to perform RNA sequencing and miRNA sequencing. Further analysis was carried out on the differential expression profiles of lncRNAs, circRNAs, miRNAs, and mRNAs among patients with LHCS and LDCS. Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed. The lncRNA/circRNA-miRNA-mRNA competing endogenous RNAs (ceRNAs) network was constructed, and the key regulatory molecules were analyzed.
Results: For LHCS and LDCS, we identified 69 lncRNAs, 150 circRNAs, 27 miRNAs, and 56 mRNAs. Differential expression analysis through GO and KEGG highlights that differentially expressed mRNAs have significant associations with pathways such as tight junction and Hepatitis C. Within the ceRNA network, all nodes have a direct or indirect association with LHCS progression. The *hsa-miR-150-5p* core sub-network is composed of 1 lncRNA, 6 circRNAs, 1 miRNA, and 5 mRNAs. From the ceRNA sub-network analysis, *NR_120628/hsa-miR-150-5p/E2F3* and *hsa-circ-0053515/hsa-miR-150-5p/E2F3* emerged as the pivotal ceRNA pairs.
Conclusions: This study highlights that the *NR_120628/hsa-miR-150-5p/E2F3* and *hsa-circ-0053515/hsa-miR-150-5p/E2F3* axes could be central in the regulation of LHCS, providing valuable insights into potential directions for subsequent research on LHCS.
Trial registration: Chinese clinical trial registry (CHiCTR23007405). Registered on July 27, 2023. https://www.chictr.org.cn/.

Keywords: IPF; lung heat and collateral stasis; lung deficiency with collateral stasis; transcriptome sequencing; competing endogenous RNA

Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic and progressive interstitial lung disease with an obscure etiology [1]. The prognosis of IPF patients is poor, with a median survival time of 2–3 years [2]. Acute exacerbation of idiopathic pulmonary fibrosis (AE-IPF) is a clinically significant acute respiratory decline within 1 month, and it is frequently the leading cause of death in IPF patients [3]. Beyond fibrosis, AE-IPF histopathological examinations show diffuse alveolar damage. Those diagnosed with AE-IPF have a median overall survival of 2.2 months from disease onset; a staggering 50% succumb during hospital stays and another 40% in Intensive Care Unit (ICU) (with mortality rates exceeding 90%) [4].

Treatment for AE-IPF predominantly involves systemic corticosteroids, sometimes coupled with immunosuppressants. However, the efficacy of this approach has not been validated in randomized control trials. Integrating four

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diagnostic methods, as well as the symptoms and signs of pulmonary fibrosis, modern physicians classify pulmonary fibrosis under the umbrella of pulmonary collateral disease. This classification is based on syndrome differentiation, considering both disease cause and location. By combining modern insights on pulmonary fibrosis progression and collateral disease theory, the disease mechanism of pulmonary fibrosis is divided into acute outbreak (dominated by lung heat and collateral stasis (LHCS)) and remission (primarily lung deficiency with collateral stasis (LDCS)) phases.

Non-coding RNA has emerged as a key player in studying Traditional Chinese Medicine (TCM) syndrome types [5]. Non-coding RNAs (ncRNAs) are classified as long non-coding RNAs (lncRNAs), circular RNAs (circRNAs), microRNAs (miRNAs), and other structural, size, and functional ncRNAs. miRNAs are small, endogenous ncRNAs consisting of 19–25 nucleotides that bind to the 3′-untranslated region (UTR) of the target mRNAs to suppress gene expression. Studies show a notable correlation between plasma miRNAs and AE-IPF [6]. lncRNAs lack open reading frames, don't exceed 200 nucleotides, and are non-coding. There is increasing evidence that ncRNAs and their interactions may play a role in pulmonary fibrosis; however, the underlying mechanisms are not fully understood. Acting as miRNA sponges, lncRNAs directly bind with miRNA, mitigating inhibitory effects on mRNA expression, which are known as competing endogenous RNAs (ceRNAs) [7]. For example, Small nucleolar RNA host gene 16 (*SNHG16*) promotes pulmonary fibrosis by regulating Notch2 expression via *miR-455-3p* sequestration [8], while Zinc finger E-box Binding Homeobox 1-Antisense 1 (*ZEB1-AS1*) which is a novel profibrotic molecule modulates Zinc finger E-box Binding Homeobox 1 (*ZEB1*) expression through *miR-141-3p* in the same context [9]. circRNAs represent a unique class of ncRNAs. Some studies have revealed dysregulation of circRNAs in plasma, with ceRNAs manifested not only in lncRNAs but also circRNAs [10,11]. Notably, Li *et al*. [12] reported *circ-TADA2A*'s role in inhibiting the activation and proliferation of lung fibroblasts via *miR-526b/Cav1* and *miR-203/Cav2*, which in turn inhibits extracellular matrix (ECM) overdeposition. Similarly, *circHIPK3* can enhance Forkhead box K2 (*FOXK2*) expression through sponge 3p, thus promoting fibroblast glycolysis and activation in pulmonary fibrosis [13]. Previous investigations by our team have unveiled differential miRNA and mRNA expression between healthy individuals and IPF patients, constituting a gene regulatory network tied to pulmonary collateral diseases. Based on our previous research, this study employed high-throughput sequencing to analyze LHCS and LDCS syndrome IPF cases, aiming to pinpoint differentially expressed (DE) lncRNA, miRNA, and mRNA. The study's goal was to craft a gene interaction-based regulatory network, shedding light on the intrinsic quality of TCM syndromes in IPF at the transcriptome level. As a result, this study spotlighted DE genes and core RNAs in IPF pathogenesis, identifying pivotal DeRNA mechanisms in distinct TCM syndromes of IPF based on ceRNA theory. Moreover, further clarity is sought on the genetic basis and influential biomolecules in IPF LHCS syndrome, and this study endeavors to elucidate this syndrome's mechanism, crafting its core network.

Using peripheral blood samples from IPF patients with both LHCS and LDCS, we identified lncRNAs, circRNAs, miRNAs, and mRNAs. We employed high-throughput RNA sequencing (RNA-Seq) and miRNA sequencing (miRNA-Seq). The identified DemRNAs were subject to enrichment analyses. Following this, we assembled the ceRNA network, mapping potential DeRNA interactions. Subsequently, we built the core ceRNA subnetwork for IPF LHCS to identify pivotal RNAs and pathways. Our findings suggested the *NR_120628/hsa-miR-150-5p/E2F3* and *hsa-circ-0053515/hsa-miR-150-5p/E2F3* axes might be integral to IPF LHCS progression. This study deepens our understanding of IPF LHCS at the transcriptome level, potentially uncovering the key ceRNA axes in IPF LHCS and opening doors to novel therapeutic avenues for IPF LHCS.

Methods

Recruitment of Participants and Collection of Clinical Samples

The study is in line with the Helsinki Declaration and has been approved by the Ethics Committee of the Affiliated Hospital of Liaoning University of Traditional Chinese Medicine under approval No 20200055FS (KT)-028-02. Before participating in the study, all participants gave written informed consent.

For sequencing, ten participants (five IPF patients with LDCS and five IPF patients with LHCS were recruited from the Affiliated Hospital of Liaoning University of Traditional Chinese Medicine (Shenyang, China)). The ages of all subjects were between 45 and 80 years. The diagnosis of IPF LHCS syndrome is consistent with the diagnostic criteria of AE-IPF, which was confirmed by clinical symptoms of significant exacerbation of acute dyspnea chest within a month and high-resolution computed tomography of new Ground-glass opacity (GGO) and/or consolidation shadows in both lungs on the background of Usual Interstitial Pneumonia (UIP) type changes. All IPF LHCS patients met the international working group criteria for AE-IPF [2]. The diagnosis of IPF LDCS syndrome is consistent with the diagnostic criteria of IPF, which was mainly according to UIP of High Resolution CT (HRCT) mentioned by the American Thoracic Society/European Respiratory Society/Japanese Respiratory Society/Asociación Latinoamericana de Tórax (ATS/ERS/JRS/ALAT) IPF criteria [1]. This study excluded individuals with other respiratory or rheumatic immune diseases.

Fresh whole blood samples from peripheral blood (4–8 mL) were drawn from each subject and centrifuged to obtain plasma. The samples were frozen using liquid nitrogen, and then transported in frozen conditions using dry ice.

RNA Extraction

According to the manufacturer's instructions, plasma RNA was extracted using a TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA concentration and purity were measured using a NanoDrop 1000 spectrophotometer (A51119500CThermo Fisher Scientific, MA, USA) to ensure the absorption 260 nm/280 nm ratio was above 1.8.

Microarray Analysis of lncRNA, circRNA, and mRNA

Microarray hybridization was conducted according to previously reported protocols [14]. Purification of total RNA using the RNeasy Mini Kit (Qiagen, 74134, Germany) guaranteed the probe labeling efficiency and microarray hybridization quality. From the purified sample, 250 ng was collected for labeling and amplification. Subsequently, the AffinityScript-RT kit (600188, Agilent technologies, Santa Clara, CA, USA) and Promoter Primer were used to reverse transcription the RNA into the first strand of cDNA. And the second strand of cDNA was synthesized using the Anti-sense Promoter. This cDNA strand was then used as a template to produce cRNA via T7 RNA polymerase, which was subsequently labeled with Cyanine-3-CTP (Cy3). The labeled cRNA was purified using the RNeasy Mini Kit (Qiagen,74134, Germany) and then hybridized to a human ceRNA array V1.0 (4 × 180 K, Design ID: 085202). A microarray scanner (Agilent technologies, Santa Clara, CA, USA) was used to scan slides after hybridization. Finally, Agilent Feature Extraction software (version 12.0.3.1, Agilent technologies, Santa Clara, CA, USA) was used to extract the features from the images. A Quantile algorithm was used to normalize the raw data. For further analysis, probes that were marked as 'detected' in at least 80% of the samples in any given group were considered. The fold change and T-test determined differential expression. A significance threshold of $|\log2 \text{ (fold change)}| > 1$ and a p-value of 0.05 were used. The microarrays were carried out according to the Agilent technology protocol of LC Sciences Corporation (Hangzhou, China).

Preparation and Sequencing of the miRNA Library

Preparation and sequencing of the miRNA library were performed as previously reported [15]. TruSeq Small RNA Sample Prep Kits (Illumina, San Diego, CA, USA) were employed to construct the miRNA library following the manufacturer's guidelines. The quality and quantity of the library were assessed using the BioAnalyzer 2100 in conjunction with the High Sensitivity DNA Chip Kit reagent (Agilent, CA, USA). Sequencing of the resulting libraries was done on an Illumina HiSeq 2500 instrument (Illumina, San Diego, CA, USA), which generated paired-end reads each 50 nucleotides in length. An in-house program was used to process the raw data, ACGT101-miR (LC Sciences, Houston, TX, USA) to remove adapter dimers, junk, low complexities, common RNA families (rRNA, tRNA, snRNA, snoRNA) (https://rfam.org/), repeats (http://www.girinst.org/repbase), and sequences <18 nt or >26 nt in length. Subsequently, the 18~26 nt length was mapped to the miRNA in miRBase 22.0 (University of Manchester, Manchester, UK) (http://www.mirbase.org/). Mapping of the pre-miRNA to the genome data of Homo sapiens was also carried out. Unique sequences aligned to known miRNA sequences in miRBase 22.0 were identified as known miRNAs. In miRBase 22.0, unique sequences aligned with known miRNA sequences have been identified as known miRNAs. Unique sequences (unannotated in miRBase 22.0) mapped to the other arm of a pre-miRNA sequence were considered miRNA candidates for 5p or 3p. The remaining unmapped sequences were matched to the Homo sapiens genome sequence to identify potential novel miRNAs.

GO Annotations and KEGG Pathway Analysis

The Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were analyzed to explore the role of all the DemRNAs [16]. In brief, GO analysis was used to elucidate the genetic regulatory networks of interest by forming hierarchical classifications on basis of the molecular functions, biological processes, and aspects of the cellular components of the DEgenes (http://www.geneontology.org). The –log10 (p-value) denotes enrichment scores representing the significance of GO term enrichment among DEgenes. KEGG pathway analysis was conducted to investigate the relevant pathways related to the DEgenes (http://www.genome.jp/kegg/). The –log10 (p-value) indicates an enrichment score for the significance of pathway correlations.

Construction of lncRNA/circRNA-miRNA-mRNA Network

The bioinformatics prediction tools, TargetScan 5.0 (Massachusetts Institute of Technology, MA, USA) and miRanda 3.3a (Computational Biology Center, Memorial Sloan-Kettering Cancer Center, New York, NY, United States of America), target genes for DemiRNAs, were predicted to construct the relationship between miRNA-mRNA-mRNA/lncRNA-miRNA/circRNA-miRNA. TargetScan or miRanda algorithm excluded target genes with a context percentile <90 or max energy >–20, respectively. Combining the predicted results of these two algorithms, the overlapping genes are considered potential target genes. Then, the cor function in R version 4.0.3 (New Zealand University of Auckland, New Zealand) calculated the Pearson correlation coefficient (R) in these interactions. Co-expression relations between DeRNAs were estimated with these correlation coefficients. Using

Cytoscape (v3.4.0, Institute for Systems Biology, Seattle, WA, USA), the strong interaction between DeRNAs (whose threshold value is $|R| > 0.99$) was chosen to construct a lncRNA/circRNA-miRNA-mRNA network.

Statistical Analysis

The default screening condition for RNA-Seq data was $|\log2\ (\text{fold change})| > 1$, and $p < 0.05$ between the two groups suggested statistical significance. Volcano plots and heat maps were produced using R (v3.4.3).

Results

Identification of circRNAs, miRNA, and mRNA in IPF LHCS with Differential Expression

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive lung disease of unknown cause. Its prognosis varies, with some patients demonstrating stable disease (IPF LDCS) and others showing rapid progression (IPF LHCS). Understanding the molecular mechanisms underlying these different prognostic pathways can illuminate potential therapeutic targets. By analyzing differential expressions of various RNA species, including lncRNAs, circRNAs, miRNAs, and mRNAs, this study aims to unveil potential biomarkers and molecular pathways involved in IPF disease progression. Therefore, in order to identify IPF LHCS-related RNAs, we used fresh peripheral blood samples from five IPF LHCS patients and five IPF LDCS patients for RNA-Seq. The mean ages of IPF LDCS and IPF LHCS groups were 62.0 ± 12.71 and 74.6 ± 6.62 (mean ± SD) years, respectively. Each group consisted of three men and two women. All participants met the international working group criteria for AE-IPF [2] or IPF [1], without any respiratory or rheumatic immune diseases history. Using the criteria of $|\log2\ (\text{fold change})| > 1$ and a p-value < 0.05 for RNA screening from the RNA-Seq and miRNA-Seq data, we detected DeRNA differences between the IPF patients of both groups. The results, depicted as heat maps and volcanic plots in Fig. 1, included lncRNAs (Fig. 1a,b), circRNAs (Fig. 1c,d), miRNAs (Fig. 1e,f), and mRNAs (Fig. 1g,h). Subsequent alignment with Gene Symbols resulted in the exclusion of any DeRNAs without a corresponding Gene Symbol. A total of 69 DelncRNAs, 150 DecircRNAs, 27 DemiRNAs, and 56 DemRNAs were extracted. Details of DeRNAs were shown in **Supplementary Table 1**.

This analysis yielded significant DeRNAs with potential roles in IPF LHCS and laid the foundation for a deeper functional examination of the mRNAs that exhibited differential expression.

Functional Analysis of mRNAs with Differential Expression

Understanding the intricate interplay between genes and their downstream effects is paramount in the context of disease pathology. Specifically, in conditions like IPF LHCS, the role of mRNAs and their related pathways can provide critical information of disease progression and potential therapeutic targets. Hence, beyond identifying differentially expressed mRNAs, we must delve into the functional implications of these changes, offering a broader pic-

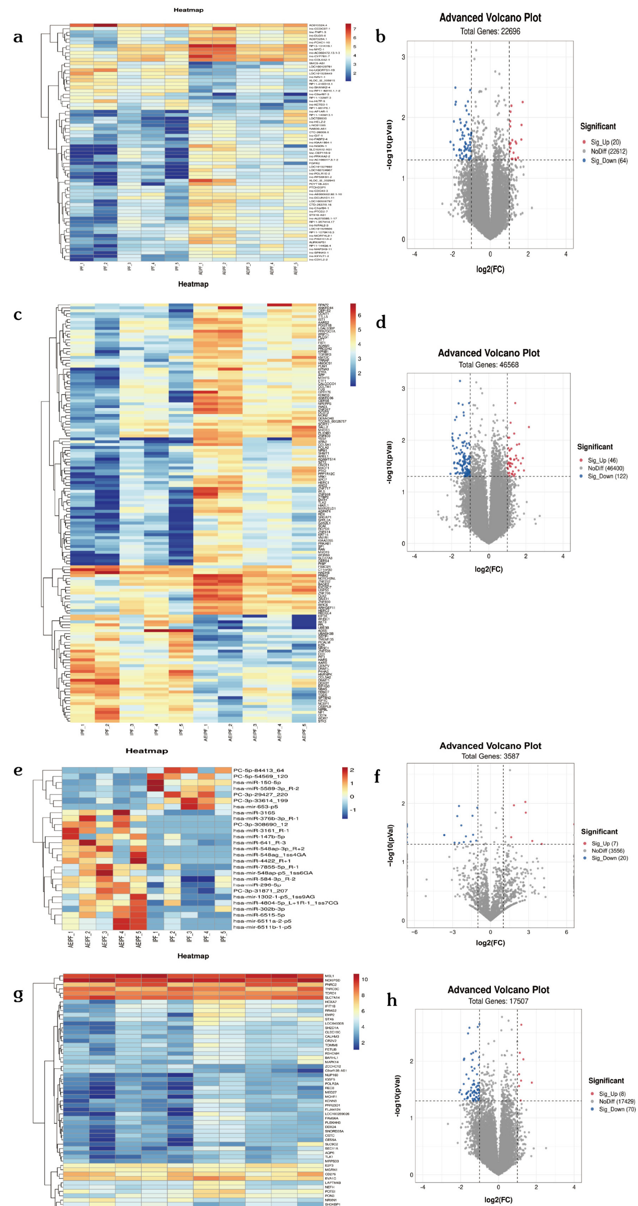

Fig. 1. The differentially expressed RNAs (DERs) between the acute exacerbation of idiopathic pulmonary fibrosis (AE-IPF) patients and idiopathic pulmonary fibrosis (IPF) control. (a,c,e,g) Hierarchical cluster heatmaps of differentially expressed long non-coding RNAs (lncRNAs), circular RNAs (circRNAs), microRNA (miRNA), and mRNA. The standardized expression level from blue to red represents the expression level from low to high. Each column represented a sample, and each row indicated an RNA. (b,d,f,h) Volcano plot of differentially expressed lncRNA, circRNAs, miRNA, and mRNA.

ture of the disease's molecular underpinnings. We undertook a comprehensive functional analysis to delve into the pathways and biological processes linked to IPF LHCS. We focused on those mRNAs showcasing differential expression using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) methodologies.

The GO enrichment analysis revealed significant involvement in 296 Biological Processes, 67 Cellular Components, and 80 Molecular Functions. Concurrently, our investigation identified 72 distinct KEGG pathways associated with these differentially expressed mRNAs. Details of the GO enrichment and the top 20 KEGG pathways are illustrated in Fig. 2. Upon closer inspection of the GO results, we observed that predominant biological processes for the DemRNAs encompassed activities like 'gene expression', 'transcription', 'DNA-templated' and 'transcription initiation from RNA polymerase II promoter'. Notably, the most enriched cellular components were the integral component of membrane, the nucleus, and the plasma membrane, and the most abundant molecular function was protein binding (Fig. 2a). Moreover, the KEGG analysis highlighted the correlation of DemRNAs with vital processes and conditions such as tight junctions, Hepatitis C, Cell adhesion molecules and Epstein-Barr virus (EBV) infection, among others (Fig. 2b).

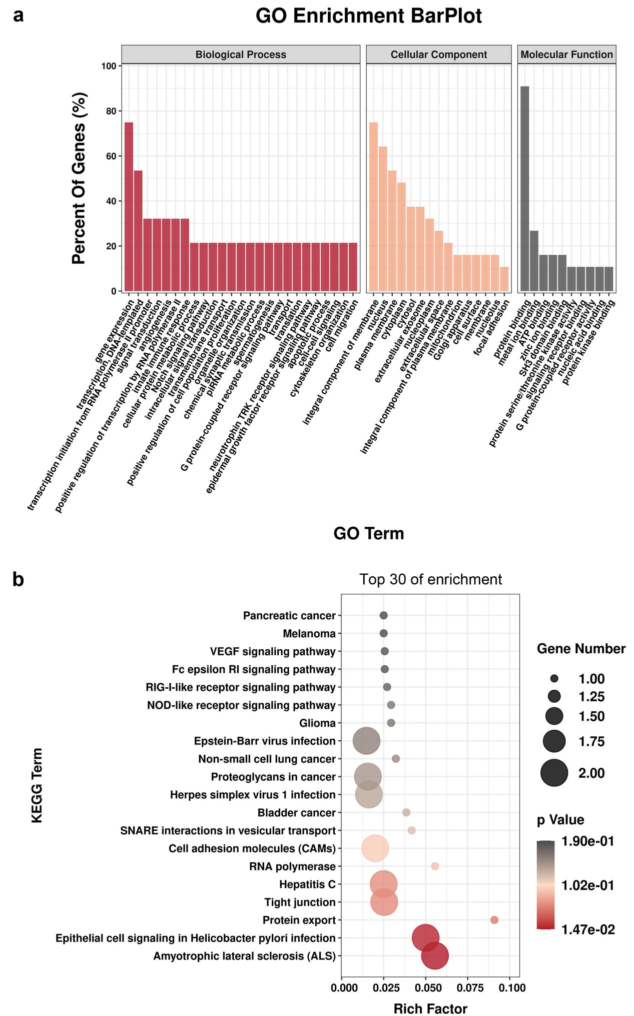

Fig. 2. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of differentially expressed mRNAs. (a) Top GO enrichment items of differentially expressed mRNAs. The GO enrichment items were sorted by significance separately in Biological Process, Cellular Component, and Molecular Function. (b) The top 20 enriched pathways in the KEGG pathway analysis. The size of the symbol represents the number of genes, and the colors represent the *p*-value.

Construction and Analysis of IPF LHCS, lncRNA-miRNA-mRNA, and circRNA-miRNA-mRNA ceRNA

After dissecting the functional implications of differentially expressed mRNAs, it became evident that understanding their interplay with non-coding RNAs could unlock a deeper layer of the disease's regulatory landscape. As lncRNAs and circRNAs have emerged as pivotal modulators in many diseases, their relationship with miRNAs and mRNAs in IPF LHCS could provide additional insights. To understand this layer of regulation, we aimed to illuminate the potential interactions between specific lncRNAs/circRNAs, miRNAs, and mRNAs. Our analysis identified 130 mRNA-miRNA, 99 lncRNA-miRNA, and 273 circRNA-miRNA interactions. These findings were integrated to construct a co-regulatory network among DeRNA. The interaction network was then visualized using Cytoscape, revealing a comprehensive ceRNA regulatory network. The details of the network composition and the expression patterns of its components are illustrated in Fig. 3a,b.

Key Genes in the CeRNA Sub-Network

Having unveiled the overarching interactions within the ceRNA network, we aimed to identify and understand the importance of the pivotal genes or nodes that may serve as central regulators or hubs within this complex landscape. To isolate these key genes, the nodes within the ceRNA network were thoroughly screened, and tropical analyses were performed. Using metrics like degree, betweenness, and closeness centrality, the genes (top 50%) were visualized through Venn diagrams (Fig. 4). The lncRNA-miRNA-mRNA ceRNA sub-network comprised 40 nodes, including 9 lncRNAs, 20 miRNAs, and 11 mRNAs, while the circRNA-miRNA-mRNA ceRNA sub-network encompassed 68 nodes, including 28 lncRNAs, 25 miRNAs, and 15 mRNAs.

Notably, when miRNAs were sorted based on their expression levels, *hsa-miR-150-5p* emerged as the most expressed, designating it as the core-regulated miRNA. We selected the hub *hsa-miR-150-5p* and its associated lncRNAs, circRNAs, and mRNAs, and then reconstructed the ceRNA subnetwork (Fig. 5). Bioinformatics prediction

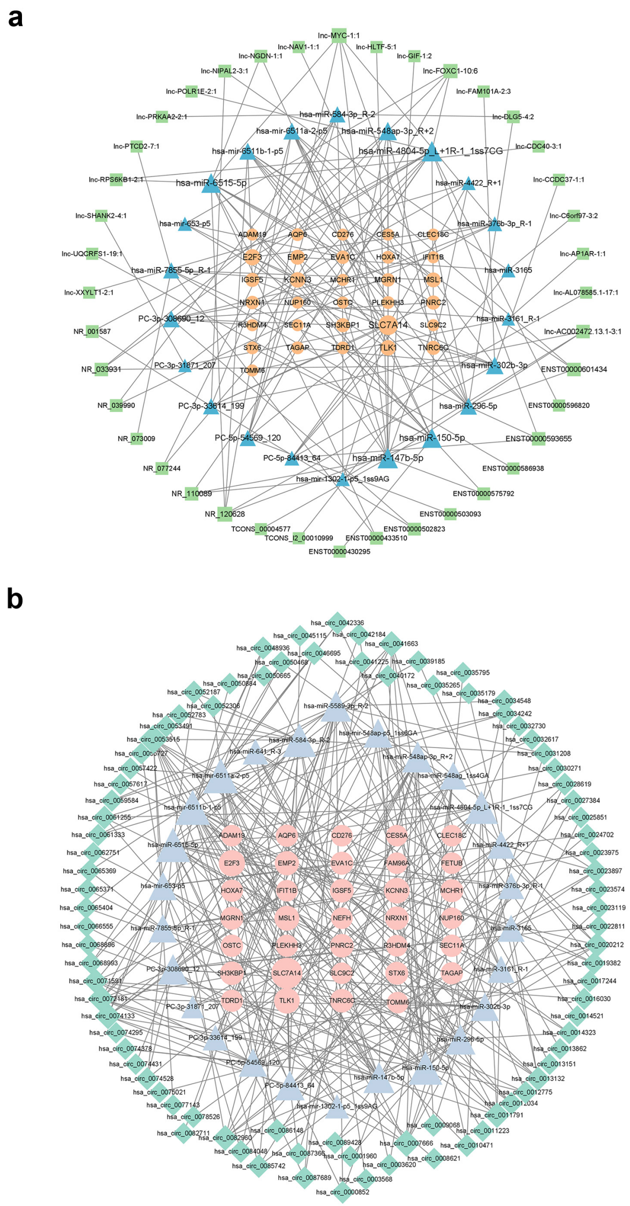

Fig. 3. lncRNA-miRNA-mRNA network and circRNA-miRNA-mRNA competing endogenous RNA (ceRNA) network in AE-IPF. (a) The lncRNA-miRNA-mRNA ceRNA network. The network was constructed with 93 nodes (40 lncRNAs, 22 miRNAs, and 31 mRNAs) and 316 edges. The green diamonds represent lncRNAs, blue triangles represent miRNAs, and orange ellipses represent mRNAs. (b) The circRNA-miRNA-mRNA ceRNA network. The network was constructed with 147 nodes (87 circRNAs, 26 miRNAs, and 34 mRNAs) and 328 edges. The green diamonds represent circRNAs, blue triangles represent miRNAs, and pink ellipses represent mRNAs.

tools, such as TargetScan and miRanda, identified 2716 potential target genes for *hsa-miR-150-5p*. Of these, five genes—Recombinant E2F Transcription Factor 3 (*E2F3*), trinucleotide repeat containing 6c (*TNRC6C*), Ca2+ activates K^+ channel protein gene 3(*KCNN3*), sapiens syntaxin 6 (*STX6*), Tousled-like Kinase 1 (*TLK1*)—integrated into our previously detailed lncRNA/circRNA-miRNA-mRNA network and were within the top 50% of mRNAs discerned from the centrality analyses intersection. Intriguingly, existing literature pinpoints *E2F3* as a critical player in pulmonary fibrosis in the elderly [17], anchoring our focus on the *hsa-miR-5p/E2F3* axis as a core miRNA-mRNA axis for further exploration. Further analyses identified specific lncRNAs and circRNAs associated with this core axis. Notably, lncRNA *NR_120628* and a series of circRNAs (*hsa-circ-0053515*, *hsa-circ-0011791*, *hsa-circ-0012775*, *hsa-circ-0028619*, *hsa-circ-0041663* and *hsa-circ-0074133*), with *hsa-circ-0053515* being the most highly expressed among them, were identified under the same filtering conditions. Consequently, we proposed that the *NR_120628/hsa-miR-150-5p/E2F3* and *hsa-circ-0053515/hsa-miR-150-5p/E2F3* axis could be instrumental in the progression of IPF LHCS.

Discussion

The observed short-term mortality of IPF LHCS remains notably high, and the exact causes for this remain elusive [18]. Furthermore, it is challenging to predict or preempt these cases [19]. IPF is a heterogeneous disease, occurring primarily in older adults. In the context of disease progression, DeRNA emerges as a potential biomarker, having links to numerous diseases [20,21]. The ceRNA network is a complex post-transcriptional regulation network that competes for miRNAs to bind to the miRNA response elements (MREs), thereby enabling interaction between mRNAs, lncRNAs/circRNAs, and miRNAs. Recent literatures emphasize the role of the ceRNA network in regulating various diseases; however, only a handful address its association with IPF patient prognosis.

Foundational mechanisms of pulmonary fibrosis indicate that LDCS plays a pivotal role during remission periods. This repeated cycle saps vital energy and leads to compounded effects such as lung-qi and lung-yin deficiencies. During acute-outbreak phases, LHCS emerges as the principal mechanism, characterized by the interplay of phlegm heat, toxin, and blood stasis. Contemporary studies correlate pulmonary phlegm syndrome with mechanisms like airway inflammatory infiltration and immune abnormalities [18]. Modern studies have confirmed that the syndrome of LHCS can lead to the infiltration of inflammatory cells in the alveolar wall, producing and releasing a large number of cytokines and inflammatory mediators. This is consistent with the understanding of the concept of phlegm heat in TCM. Further, the LHCS syndrome in IPF is classified under ‘toxin’ in Traditional Chinese Medicine (TCM) and aligns closely with the ‘extracellular matrix’ (ECM) concept. The increased synthesis and deposition of ECM disrupt normal lung tissue structures, accelerating inflammatory reactions. Therefore, ECM plays a key role in the de-

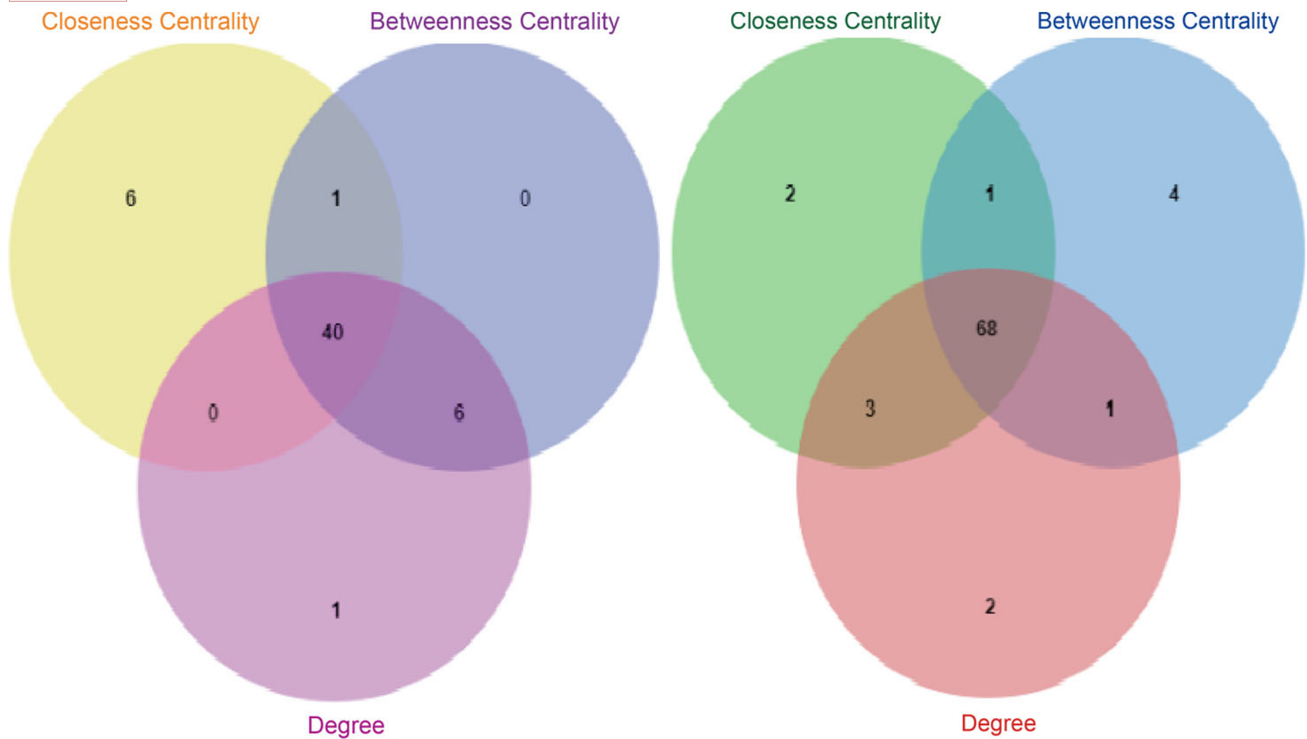

Fig. 4. Venn mapping of the top 50% genes in the ceRNA network. The left Venn diagram depicts the intersection of the lncRNA miRNA mRNA network nodes. Yellow represents Closeness Centrality, purple represents Betweenness Centrality, and pink represents the Degree. The right Venn diagram depicts the circRNA miRNA mRNA network nodes, with green indicating Closeness Centrality, blue indicating Betweenness Centrality, and red indicating the Degree.

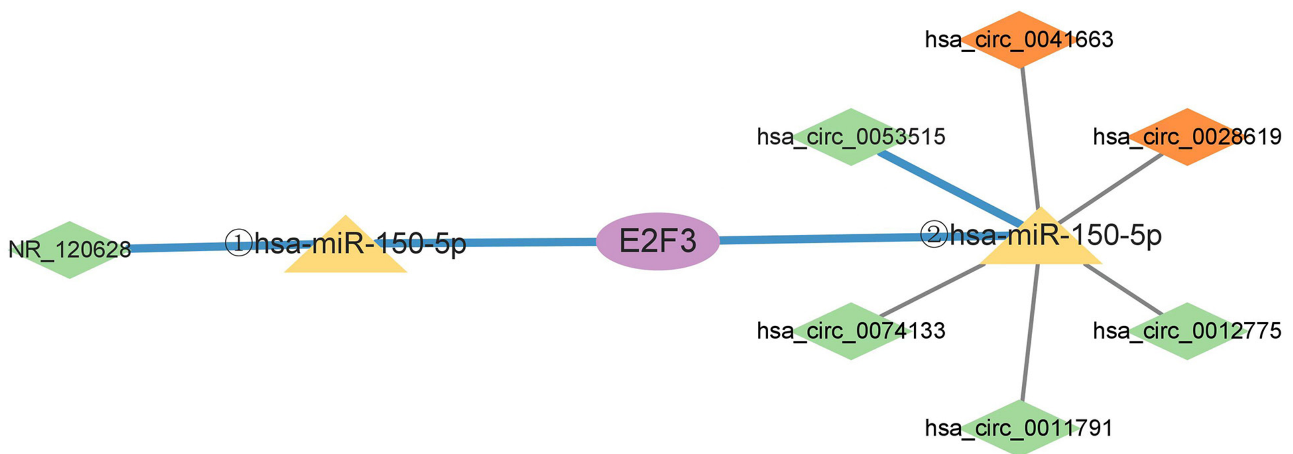

Fig. 5. Core ceRNA sub-network centered on *hsa-miR-150-5p*. The diamonds represent lncRNAs, arrows represent circRNAs, yellow triangles represent miRNAs, and purple ellipses represent mRNAs. Colors indicate regulation: green for downregulated and orange for upregulated.

velopment of LHCS. Heat, poison, and deficiency are intertwined. The deeper the deficiency, the more severe the poison, the more stagnant the evil, and the more injured the collaterals. Eventually, a large amount of accumulated internal toxin damages the qi-collaterals of the lung, causing damage to the qi-collateral structure, disturbance of air exchange, and even respiratory failure. This condition will be critical, and changes in symptoms will be numerous.

In our current study, RNA sequencing of blood samples from IPF patients (both LDCS and LHCS) allowed us to identify differential expressions of 69 lncRNAs, 150 circRNAs, 27 miRNAs, and 56 mRNAs specific to IPF LHCS. GO analysis highlighted critical biological processes associated with IPF LHCS, such as 'gene expression', 'transcription, DNA-templated', and 'transcription initiation from RNA polymerase II promoter'. KEGG anal-

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ysis shed light on potential pathological connections to tight junctions, Hepatitis C, cell adhesion molecules, and EBV infection in IPF. IPF is associated with tight junction protein in type II lung cell proliferation [22]. Zou *et al*. [22] performed immunohistochemical staining for occludin tight junction proteins, claudin-2, among others, and increased occludin and reduced claudin-18 expression were observed in pneumocytes adjacent to fibroblastic foci, as well as an increase in claudin-2 and a decrease in the pulmonary expression of claudin-4 in IPF patients. Two studies indicated a positive relationship between Hepatitis C virus (HCV) and IPF [23,24], whereas one in the United Kingdom did not show an association of HCV infection with IPF [25]. Wootton and colleagues found that 19 out of 43 patients with AE-IPF were infected, including 2 EBV [26]. While several factors, such as viral infections, can cause or worsen IPF, the underlying cause is still unclear and, therefore, needs further investigation.

A noteworthy discovery from our research is the centrality of *hsa-miR-150-5p* in ceRNA sub-networks. Prior studies have validated its potential as a prognostic marker for IPF LHCS [6], and its significant role across various cancers, including lung, gastric, and pancreatic cancer [27]. For instance, *miR-150-5p*, a cancer stem cell suppressor, inhibits Non-Small-Cell lung cancer (NSCLC) metastasis and recurrence by targeting High Mobility Group AT-hook 2 *(HMGA2)* and β-catenin signaling [28]. lncRNA *linc00673* regulates the proliferation, migration, invasion, and epithelial mesenchymal transformation of non-small cell lung cancer using *miR-150-5p* [29]. However, the biological role of *miR-150-5p* and its underlying mechanisms has been extensively studied in the AE-IPF study. There is a well-known association between IPF and lung cancer [30]. IPF has a high rate of co-morbidity with lung cancer, and there are many common molecular pathways between IPF and lung cancer. Recent studies show that in bleomycin (BLM)-induced pulmonary fibrosis rat model, ncRNA, *ZFAS1*, acts as a ceRNA via the *miR-150-5p/SLC38A1* axis to promote the transformation and ferroptosis of the lung [31]. In addition, the low expression of *miR-150-5p* in the blood indicates a reduction in survival in patients with Chronic Obstructive Pulmonary Disease (COPD) [32]. *miR-150-5p* is involved in the development of liver fibrosis and cardiac fibrosis [33,34]. As a critical miRNA in the ceRNA network, miR-150-5p is closely related to lung disease or fibrosis and may play an important role in IPF LHCS.

Furthermore, our analysis spotlighted five DemRNAs (*E2F3, TNRC6C, KCNN3, STX6,* and *TLK1*) as primary targets of *hsa-miR-150-5p*. E2F3, a crucial member of the E2F transcription factors family, is crucial in the pathogenesis of pulmonary fibrosis in the elderly. Its role, especially when modulated by *miR-34a*, is pivotal in influencing the alveolar epithelial dynamics and promoting fibrosis [17]. Ren *et al*. [35] showed that E2F3 was the direct target of *miR-449a* in lung cancer cells and that *miRNA-449a* targeted E2F3 to induce cell cycle arrest and aging. E2F3 has also been reported as a new prognostic marker for human lung cancer [36]. Furthermore, *Circ_0016760* acts as a sponge of *miR-4295*, which enhances the expression of E2F3 and promotes cell proliferation and glycolysis in non-small cell lung cancer, thus providing a new potential therapeutic target for NSCLC [37]. *TNRC6C* mutant developed cyanosis, shortness of breath, and progressive respiratory failure after the first day of life. The alveolar vesicles of these mutant mice appear to have structural defects associated with a reduction in the Transforming growth factor-β (*TGF-β*) gene [38]. Circular RNA *mmu_circ_0005019* inhibits fibrosis of cardiac fibroblasts by regulating the *miR-499-5p/KCNN3* axis [39]. We predicted that these mRNAs might play important roles in IPF LHCS and might be new targets for treatment.

In conclusion, our bioinformatics analysis introduced two unprecedented regulatory axes: *NR_120628/hsa-miR-150-5p/E2F3* and *hsa-circ-0053515/hsa-miR-150-5p/E2F3*. No literature has been reported on the role of *lncRNA NR_120628* or *hsa-circ-0053515* in any disease. Their potential involvement in IPF LHCS necessitates further research, promising pivotal insights into IPF LHCS dynamics.

While being the first to examine RNA differential expression in IPF LHCS, this study has limitations. The limited sample size, owing to the difficulty in obtaining IPF LHCS RNA samples, may constrain the breadth of our findings. Additionally, our ceRNA network predictions still require experimental validations. A deeper understanding of the specific roles of the identified RNAs and axes in IPF LHCS will undoubtedly benefit future studies.

Conclusions

In this study, we identified differentially expressed lncRNAs, circRNAs, miRNAs, and mRNAs between IPF LHCS and IPF LDCS groups using RNA-Seq. Furthermore, we constructed a ceRNA network for IPF LHCS for the first time. Our results suggest that the *NR_120628/hsa-miR-150-5p/E2F3* and *hsa-circ-0053515/hsa-miR-150-5p/E2F3* axis could play a pivotal role in regulating IPF LHCS. Overall, these findings enhance our understanding of the molecular relationship between IPF LHCS and IPF LDCS, offering potential avenues for predicting and therapeutically targeting IPF LHCS.

Abbreviations

AE-IPF, acute exacerbation of idiopathic pulmonary fibrosis; BLM, bleomycin; ceRNAs, competing endogenous RNAs; circRNAs, circular RNAs; DE, differentially expressed; EBV, Epstein-Barr virus; GO, Gene Ontology; HCV, Hepatitis C virus; IPF, idiopathic pulmonary fibrosis; KEGG, Kyoto Encyclopedia of Genes and Genomes; lncRNAs, long non-coding RNAs; miRNAs, microRNAs; miRNA-Seq, miRNA sequencing; MREs, miRNA re-

sponse elements; ncRNAs, non-coding RNAs; NSCLC, Non-Small-Cell lung cancer; RNA-Seq, RNA sequencing; LHCS, lung heat and collateral stasis; LDCS, lung deficiency with collateral stasis.

Availability of Data and Materials

The datasets generated and/or analyzed during the current study are available in the GEO website, but are currently private until 2024-01: GSE222065 dataset (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE222065, Enter token qpmjoscmvfurxqx into the box) and GSE221937 dataset (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE221937, Enter token knchaiykrjmzlqj into the box).

Author Contributions

NZZ, LJP and XDL conceived and designed the work. NZZ, JRW and JYW drafted the manuscript. NZZ, TTL and JXP provided study materials or patients. JRW, PL and YML participated in data analysis and interpretation. NZZ revised the manuscript and participated in discussion. LJP and XDL supervised the study. All authors contributed to data interpretation, manuscript review and editing. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study was conducted in accordance with the amended Declaration of Helsinki and the study was approved by the institutional ethics committee of the Affiliated Hospital of Liaoning University of Traditional Chinese Medicine, institutional review board approval number 20200055FS (KT)-028-02. The authors declare that written informed consent was obtained from all the patients for their participation in the study.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.24976/Discov.Med.202335178.84.

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