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In *Silico* Insight of *Endothelin* Signaling in *Idiopathic Pulmonary Fibrosis* (IPF)

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Abstract

Idiopathic pulmonary fibrosis (IPF), a form of interstitial lung disease (ILD), is characterized by progressive lung scarring with a poor prognosis. *Endothelin* signalling has emerged as a key player in lung fibrogenesis under various pathological conditions. However, its specific contribution to IPF pathogenesis remains poorly elucidated. We propose that leveraging in *silico* modelling approaches can provide valuable insights into the role of *endothelin* signalling in IPF, potentially paving the way for novel therapeutic and diagnostic strategies. We employed datasets curated from the Gene Expression Omnibus (GEO) database. Comprehensive data analyses from lung, bronchoalveolar lavage cells, blood and human primary fibroblast samples from both control and IPF patients were utilized to uncover the expression patterns and clinical correlations of *endothelin* genes. Furthermore, single-cell RNA-sequencing (*scRNA-seq*) was leveraged to explore the cellular heterogeneity and specific cell types harboring aberrant *endothelin* expression in the IPF lung microenvironment. Our analysis revealed a significant changes of *endothelin* genes expression pattern in IPF patient samples as compared to healthy control. Notably, IPF patients with upregulation of *endothelin-1*, demonstrated a statistically significant poorer survival. These findings implicate *endothelin* signalling as a novel and potentially targetable pathway in IPF. Further investigations are warranted to validate these findings and explore the therapeutic potential of modulating *endothelin* signalling in this lung disease.

Keywords**:** *Endothelin; Idiopathic Lung Fibrosis; In Silico Insight*

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive lung disease with unknown cause, characterized by inflammation, fibrosis, and destruction of lung architecture, leading to respiratory failure and death (Barratt et al., 2018; Koudstaal & Wijsenbeek, 2023; Lederer & Martinez, 2018; Wolters et al., 2018). It is the most common idiopathic interstitial pneumonia and is associated with significant morbidity and mortality. The prevalence of IPF fibrosis ranges from 4 to 725 cases per 100,000 people, with an increasing trend due to aging populations worldwide (Cottin et al., 2019; Ferrara et al., 2019; Harari et al., 2020; Kaul et al., 2022; Maher et al., 2021a, 2021b; Skvortsov et al., 2022). The pathogenesis of IPF involves altered wound healing in response to persistent lung injury (L. Zhang et al., 2018), genetic susceptibility (Michalski & Schwartz, 2021), cellular senescence (Yanagihara et al., 2019), profibrotic pathways (Yanagihara et al., 2019), microbial dysbiosis (Invernizzi & Molyneaux, 2019), metabolic dysregulation (Bargagli et al., 2020), and multiple signalling pathways such as inflammation, oxidative stress, and fibrosis (Lv et al., 2019). The *endothelin* system, a family of regulatory peptides, comprises three signalling molecules (*endothelin-1, endothelin-2, and endothelin-3*). These bind to specific receptors on target cells, *endothelin* receptor A and B, triggering downstream effects through G-proteins. Specialized enzymes, endothelin converting enzyme 1 and 2, activate the system by processing precursor molecules into mature endothelin peptides (Barton & Yanagisawa, 2019; Davenport et al., 2016; Haryono et al., 2022).

Endothelin's pro-fibrotic actions involve triggering the transformation of *endothelial* cells into scar-forming fibroblasts via *endothelial-to-mesenchymal* transition (Chang et al., 2018), actively altering blood vessel structure through vascular remodeling (Hartopo et al., 2018), inducing oxidative stress(Argentino et al., 2022) and acting as a pro-inflammatory mediator (Liu et al., 2018). Endothelin-1 also has been shown to induce lung *fibroblast* activation into *myofibroblast* via activation of the RhoA/ROCK (Rho-associated protein kinase) signalling cascade and Hippo/Yap pathway (Neubig, 2022; Sun et al., 2021; Tocci et al., 2021). Furthermore, animal models suggest that the profibrotic effects of endothelin-1 might primarily be mediated through the ETB receptor. Knocking down this receptor significantly attenuated fibroblast activation and collagen synthesis (Akashi et al., 2016). These diverse mechanisms underpin its crucial role in the development and progression of various fibrotic diseases.

Increased *endothelin-1* serum levels were found in patients with IPF and might be associated with ILD severity (Pulito-Cueto et al., 2023). Studies have shown that blocking *endothelin* signalling with endothelin receptor antagonist can attenuate lung fibrosis in animal models (Bellaye et al., 2018; Hartopo et al., 2018). However, a meta-analysis found that *endothelin* receptor antagonists did not significantly improve lung function in *idiopathic pulmonary fibrosis* patients compared to placebo (Li et al., 2022). As such, while the *endothelin* system is implicated in IPF, its precise involvement and underlying mechanisms remain unclear, demanding further exploration. In this study, we propose that by leveraging in *silico* modelling approaches can provide valuable insights into the role of *endothelin* signalling in IPF.

RESEARCH METHOD

1. Dataset data extraction

According to the GEO database (http://www.ncbi.nlm.nih.gov/geo/), 4 datasets were selected: from lung tissue samples [GSE47460 (Agilent) (Anathy et al., 2018)], from bronchoalveolar lavage fluids (BAL) samples [GSE70866 (Agilent) (Prasse et al., 2019)]; from blood samples [GSE93606 (Affymetrix) (Molyneaux et al., 2017)], and from human primary fibroblast [GSE40839 (Affymetrix)(Lindahl et al., 2013)]. Gene expression data for EDN1 (*endothelin-1*), EDN2 (*endothelin-2*), ENDRA (*endothelin* receptor A), and EDNRB (*endothelin* receptor B) were extracted from all four database by using GEO2R platform. Survival data was extracted from GSE93606 dataset (blood). Approval of the Ethics Committee was not required because the information of patients was obtained from the GEO database.

2. Analysis of *scRNA-seq* Data

Lung scRNA-seq data from both healthy control and IPF patients was extracted from a publicly available HLCA (Human Lung Cells Atlas) database (Sikkema et al., 2023) (https://cellxgene.cziscience.com/collections/6f6d381a-7701-4781-935c-db10d30de293). The raw and normalized counts, integrated embedding, cell type annotations and clinical and technical metadata were extracted for analysis. The computational analysis of *scRNA-seq* dataset (lung) was performed using CZ CELLxGENE Discover platform (Program et al., 2023). UMAP (uniform manifold approximation and projection) for dimension reduction was calculated dan cell types were identified based on markers of each cluster using CZ CELLxGENE Discover.

3. Survival analysis

The optimal cut-off value of genes was determined by using the median among each gene expressions. IPF patients were grouped according to low and high gene expression of EDN1, EDN2, EDNRA, and EDNRB. Kaplan–Meier analysis with statistical test was performed to compare survival among low and high gene expression groups.

4. Statistical analysis

A series of statistical analyses were performed using Graphpad Prism software version 10 (Graphpad Software, Inc). Differences between two groups were analyzed using student t-test. Numerical data were summarized as median and interquartile range. Survival data were summarized using the Kaplan-Meier method and differences between groups were analyzed using Gehan-Breslow-Wilcoxon test. $P < 0.05$ was considered statistically significant.

RESULT AND DISCUSSION

1. *Endothelin* genes expression in patients with *Idiopathic Pulmonary Fibrosis* (IPF)

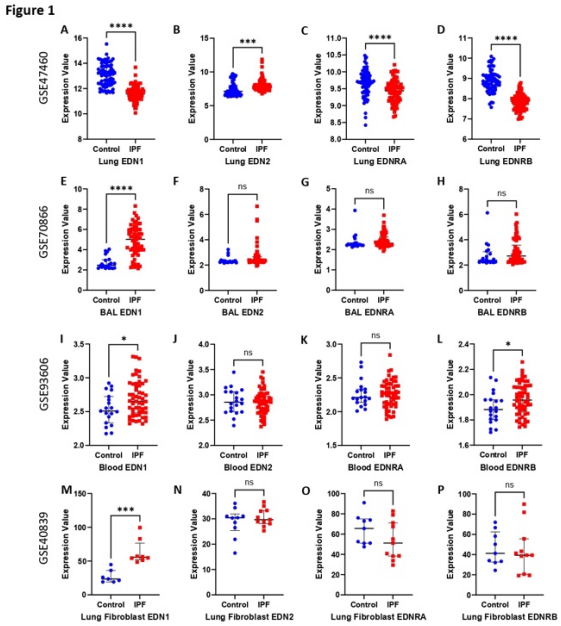

Figure 1. Expression of endothelin and its receptor in lung tissue, BAL cells, blood and human primary lung fibroblast in patients with IPF.
(A - D) Expression of EDN1, EDN2, EDNRA and EDNRB of lung tissue in IPF patients (n=75 for control and n=84 for IPF).
(E - H) Expression of EDN1, EDN2, EDNRA and EDNRB of BAL cells in IPF patients (n=20 for control and n=62 for IPF).
(I - L) Expression of EDN1, EDN2, EDNRA and EDNRB of blood cells in IPF patients (n=20 for control and n=57 for IPF).
(M - P) Expression of EDN1, EDN2, EDNRA and EDNRB of human primary lung fibroblasts in IPF patients (n=10 for control and n=11 for IPF).
Abbreviation used are; EDN1 = endothelin-1; EDN2 = endothelin-2; EDNRA = endothelin receptor A; EDNRB = endothelin receptor B. Data represent median and interquartile range. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001. ns, not significant. Statistical analyses were performed using student t-test.

Figure 1. Expression of *endothelin* and its receptor in lung tissue, BAL cells, blood and human primary lung fibroblast in patients with IPF, Data Processing, 2022 (edited)

In order to explore the *endothelin* genes (EDN1, EDN2, ENDRA, and EDRB) expressions, 4 datasets were extracted from the GEO database. In lung tissue, we observed significant downregulation of EDN1, EDNRA, and EDNRB, suggesting decreased pro-contractile signalling (Fig. 1A-D). Notably, EDN2 expression was significantly upregulated (Fig. 1C). In contrast, both BAL cells and human primary lung fibroblasts exhibited significant upregulation of EDN1 only (Fig. 1E-H, 1M-P), suggesting its specific role in these cell types. Interestingly, blood cells showed upregulation of both EDN1 and EDRB (Fig. 1I-L).

2. The *scRNA-seq* Analysis Revealed Elevated Expression of *EDNI* Mainly in *Endothelial* cells of IPF Lung

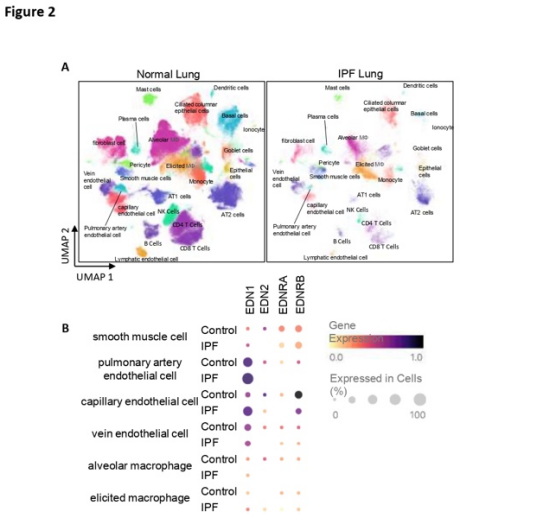

Figure 2. The scRNA-seq analysis in the HLCA dataset
(A) UMAPs of the immune, epithelial and endothelial/stromal cells of healthy control lung and IPF lung.
(B) Color dot plot of EDN1, EDN2, EDNRA, and EDNRB expressions across different types of cells
Abbreviation used are; EDN1 = endothelin-1; EDN2 = endothelin-2; EDNRA = endothelin receptor A; EDNRB = endothelin receptor B.

Figure 2. The *scRNA-seq* analysis in the HLCA Dataset, Data Processing, 2022 (edited)

Single-cell RNA sequencing (*scRNA-seq*) of lung tissue from healthy controls and IPF patients, retrieved from the HLCA database (Sikkema et al., 2023), revealed comparable cell types in both groups (Fig. 2A). However, IPF lungs exhibited a global decline in cell number. While EDN1 displayed widespread expression across cell types, particularly in pulmonary artery *endothelial* cells, capillary *endothelial* cells, and vein *endothelial* cells, with limited expression in smooth muscle cells and macrophages, its expression was upregulated in IPF lungs within these aforementioned cell types and elicited macrophages. Conversely, EDN2 expression was comparatively lower across cell types and primarily downregulated in IPF lungs, affecting smooth muscle cells, *endothelial* cells (pulmonary artery, capillary, and vein), and alveolar macrophages. Notably, both EDNRA and EDNRB expression was downregulated in all cell types within IPF lungs (Fig. 2B).

3. Elevated Blood *EDN1* Expression were Associated with Worse Survival in IPF Patients

Figure 3

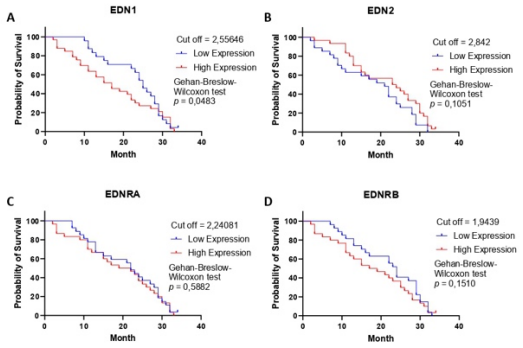

Figure 3. Kaplan-Meier analysis of EDN1, EDN2, EDNRA, and EDNRB expression in the blood for the survival of patients with IPF according to GSE93606 dataset.
(A) Survival of IPF patients with low and high EDN1 expression
(B) Survival of IPF patients with low and high EDN2 expression.
(C) Survival of IPF patients with low and high EDNRA expression
(D) Survival of IPF patients with low and high EDNRB expression.
Statistical analyses were performed using Gehan-Breslow-Wilcoxon test (A-D).

Figure 3. Kaplan-Meier Analysis of *EDN1, EDN2, EDNRA,* and *EDNRB* Expression in the Blood for the Survival of Patients with IPF According to GSE93606 Dataset, Data Processing, 2022 (edited)

Analyzing data from the GSE93606 dataset, we employed survival analysis to assess the impact of *endothelin* gene expression on IPF patient outcomes. Patients with IPF were divided into two groups based on the optimal cut-off value of genes as described in the methods section. IPF patients with high EDN1 expression exhibited significantly shorter survival times compared to those with low expression (Fig. 3A). Although statistically non-significant, a similar trend towards lower survival was observed in patients with high EDNRB expression (Fig. 3B). Interestingly, neither EDN2 nor EDNRA expression in blood cells significantly impacted IPF patient survival (Fig. 3C-D).

4. *Endothelin-1* as a Central Mediator and Prognostic Marker in IPF

IPF, a progressive scarring lung disease, remains shrouded in uncertainty. While inflammation and fibrosis are hallmarks, understanding the underlying mechanisms is crucial for therapeutic development. The *endothelin* system, known for its pro-fibrotic effects (Akashi et al., 2016; Argentino et al., 2022; Bellaye et al., 2018; Chang et al., 2018; Rodríguez-Pascual et al., 2014; Sun et al., 2021; Wermuth et

al., 2016; Wu et al., 2019), emerges as a potential player in this complex puzzle. Elevated *endothelin-1* levels in IPF patients suggest its involvement (Mishra et al., 2021; Pulito-Cueto et al., 2023; Remuzgo-Martínez et al., 2022). However, studies on *endothelin-1* and *endothelin* receptor blockage in IPF have yielded mixed results (Akashi et al., 2016; Bellaye et al., 2018; Hartopo et al., 2018; Li et al., 2022; Liu et al., 2018). This highlights the intricate interplay within the *endothelin* system, encompassing not only *endothelin-1* but also other endothelin peptides, receptors, and processing enzymes.

Our investigation of *endothelin* genes in IPF revealed a complex and intriguing picture. Notably, *endothelin-1* emerged as a central player, exhibiting distinct expression patterns across various tissues and cell types. In lung tissue, there were downregulation of EDN1, EDNRA, and EDNRB suggesting a weakened pro-contractile signalling pathway (Fig. 1A-D). This may contribute to the impaired vascular function observed in IPF. However, an upregulation of EDN1 was observed in BAL cells, human primary lung fibroblasts, and even blood cells (Fig. 1E-H, 1M-P, 1I-L). This suggests a cell-specific activation of EDN1 signalling in these compartments, potentially driving inflammation and fibrosis.

Single-cell RNA sequencing analysis further refined our understanding of EDN1 localization. While diverse cell types express EDN1 in healthy lungs, its upregulation in IPF specifically localizes to *endothelial* cells, including pulmonary artery, capillary, and vein *endothelial* cells, as well as elicited macrophages (Fig. 2B). This endothelial-specific upregulation of EDN1 suggests a critical role in the aberrant angiogenesis and vascular dysfunction associated with IPF. Indeed, previous studies has highlighted the upregulation of endothelin-1 in endothelial cells (Hartopo et al., 2018) that induced endothelial to mesenchymal transition (Wermuth et al., 2016), induction of fibroblasts to myofibroblast activation (Argentino et al., 2022; Shi-Wen et al., 2004) and *endothelin-1* related macrophages polarization and secretion of cytokines (Elisa et al., 2015; J. Zhang et al., 2021) that might contribute to lung inflammation and fibrosis.

Growing evidence underscores the intricate link between *endothelin-1* and IPF severity. Previous study showed elevation of serum *endothelin-1* levels in IPF patients compared to healthy controls, suggesting its potential role as a disease marker (Remuzgo-Martínez et al., 2022). Another study by Pulito-Cueto et al. (2023) demonstrated a positive correlation between circulating *endothelin-1* and worsened lung function in both IPF and rheumatoid arthritis-associated interstitial lung disease (RA-ILD) patients (Pulito-Cueto et al., 2023). In our study, high levels of EDN1 expression in blood cells were associated with significantly shorter survival times in IPF patients (Fig. 3A). This suggests that circulating *endothelin-1* may serve as a potential biomarker for disease progression and prognosis. While the trend towards poorer survival with high EDNRB expression requires further investigation, the lack of significance for EDN2 and EDNRA highlights the specific role of EDN1 in this context. By database data analysis, our study contributes for understanding *endothelin-1's* role in IPF. Additional research utilizing in vitro and *in vivo* models will be vital to refine our understanding and translate these findings into clinical applications.

CONCLUSION

Our findings pave the way for further exploration of EDN1's multifaceted role in IPF. Understanding the mechanisms underlying cell-specific EDN1 regulation and its contribution to various IPF pathologies could lead to novel therapeutic strategies. Targeting this key player in both the lung and peripheral circulation holds promise for improving outcomes in IPF patients.

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