ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
April 2023; 22(2):190-199.
DOI: 10.18502/ijaai.v22i2.12680

Insights into Overlapping of Fibrosis and Cancer: Exploring the Tumor-related Cardinal Genes in Idiopathic Pulmonary Fibrosis

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Received: 26 September 2022; Received in revised form: 8 December 2022; Accepted: 3 January 2023

ABSTRACT

The pathogenesis of idiopathic pulmonary fibrosis (IPF) is quite similar to that of cancer pathogenesis, and several pathways appear to be involved in both disorders. The mammalian target of the rapamycin (mTOR) pathway harbors several established oncogenes and tumor suppressors. The same signaling molecules and growth factors, such as *vascular endothelial growth factor* (VEGF), contributing to cancer development and progression play a part in fibroblast proliferation, myofibroblast differentiation, and the production of extracellular matrix in IPF development as well.

The expression of candidate genes acting upstream and downstream of *mTORC1*, as well as *Vegf* and *low-density lipoprotein receptor related protein 1(Lrp1)*, was assessed using specific primers and quantitative polymerase chain reaction (qPCR) within the lung tissues of bleomycin (BLM)-induced IPF mouse models. Lung fibrosis was evaluated by histological examinations and hydroxyproline colorimetric assay.

BLM-exposed mice developed lung injuries characterized by inflammatory manifestations and fibrotic features, along with higher levels of collagen and hydroxyproline. Gene expression analyses indicated a significant elevation of *regulatory associated protein of mTOR (Raptor)*, *Ras homolog enriched in brain* (*Rheb)*, *S6 kinase 1*, and *Eukaryotic translation initiation factor 4E-binding protein 1 (4Ebp1)*, as well as a significant reduction of *Vegfa*, *Tuberous sclerosis complex (Tsc2)*, and *Lrp1*; no changes were observed in the *Tsc1* mRNA level.

Our findings support the elevation of *S6K1* and *4EBP1* in response to the TSC/*RHEB*/mTORC1 axis, which profoundly encourages the development and establishment of IPF and cancer. In addition, this study suggests a possible preventive role for VEGF-A and LRP1 in the development of IPF.

Keywords: Cancer; Idiopathic pulmonary fibrosis; Lrp1; mTOR; Rheb1; Rptor; S6k1; Tsc1; Tsc2; Vegf; 4E-bp1

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INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is the most common, progressive, and fatal interstitial lung disease with complex pathogenesis; this illness has been widely represented with the bleomycin-induced IPF mouse model.[1,2] IPF has a very poor prognosis, limited treatment options, and an unknown etiology. Chronic damage to the alveolar epithelial cells, persistent molecular cross-talk between these epithelial cells and fibroblasts, and activation of myofibroblasts leading to dysregulated production of extracellular matrix (ECM) constitute the main events of the pathogenesis of this disease.[3] Although IPF is known primarily as a fibroproliferative disorder, it resembles cancer in several aspects, including pathological pathways and signaling patterns.[4] Despite the diverse therapeutic strategies against cancer, no treatments are approved to address the pathological mechanisms involved in IPF.[5] Given the cancer-like nature of IPF, a more comprehensive understanding of its pathogenesis could be learned from approaches already utilized in cancer treatment. For instance, lung fibrosis is associated with ECM stiffness, which is also significantly involved in tumor progression and invasion.[6] Therefore, targeting the collagen deposition and signaling pathways that result in the abnormal accumulation of ECM offers a potential approach to hinder both tumorigenesis and fibrogenesis.

The pathogenesis of IPF has been shown to assimilate pathways previously known to be involved in the tumorigenic process.[4,7] Recently, a common signature of gene expression has been identified between lung cancer and IPF.[8] The mechanistic target of the rapamycin (mTOR) signaling complex is situated at the crossroads of several key pathways modulating various cellular processes such as proliferation, autophagy, and protein synthesis, which could contribute to the pathogenesis of both cancer and fibrosis.[9,10,11,12,13]. Therefore, regulators of mTOR signaling have recently attracted considerable attention to explore novel therapeutic targets.

Vascular endothelial growth factor (VEGF) is a potent angiogenic factor that plays a substantial part in tumor development and progression. Therefore, VEGF has been considered an important therapeutic target for cancer therapy, and anti-VEGF–based antiangiogenic drugs are widely used for the treatment of patients with different types of cancer.[14] On the other hand, VEGF has also been linked to the pathogenesis of pulmonary fibrosis; however, the relationship between VEGF and IPF remains to be clarified.[15] Another signaling protein involved in various biological processes is low-density lipoprotein receptor related protein 1 (LRP1), which has been implicated in cancer pathogenesis.[16] Given the role of LRP1 in various biological processes, such as cell growth, differentiation, migration, and apoptosis, it might also participate in lung fibrosis.[17]

Accordingly, we also sought to investigate the aforementioned tumor-associated genes and pathways in IPF pathogenesis. For this purpose, we examined the expression of oncogenic and tumor suppressor genes acting upstream and downstream of mTORC1 (including Tuberous sclerosis complex1,2 (*TSC1*, *TSC2)*, Ras homolog enriched in brain (*RHEB)*, Eukaryotic translation initiation factor 4E-binding protein 1 (*4EBP1)*, and S6 kinase (*S6K*)) as well as two tumor progression-related factors (*VEGF-A* and *LRP1*) to figure out whether the expression of these genes is affected by changes resulting from bleomycin-induced pulmonary fibrosis.

MATERIALS AND METHODS

Animals and Experimental IPF Model

Six- to eight-week-old male C57BL/6 mice (from the Center for Experimental Studies, Iran University of Medical Sciences, Tehran, Iran) were used in this experiment. Mice were maintained in standard cages at room temperature, ambient humidity and a 12-hour light-dark cycle, allowing them to feed freely.

On day 1, mice were first anesthetized with intraperitoneal administration of ketamine and xylazine; then, the IPF model was induced by surgical intratracheal instillation of bleomycin (BLM; Nippon Kayaku, Tokyo, Japan) at a dose of 5 mg/kg in phosphate-buffered saline (PBS) at a final volume of 70 µL. Control animals only received PBS in the same manner. On day 21, all animals were anesthetized by intraperitoneal administration of ketamine (75 mg/kg) and xylazine (5 mg/kg) and sacrificed to remove their lungs.[18]

Histological Assessments

The lung segments were fixed in 10% formalin for 24 hours and embedded in paraffin. Paraffin-embedded tissue was sliced into 4-μm sections and stained with hematoxylin and eosin (H&E) and Masson's trichrome according to standard histological procedures. Then,

stained sections were observed under a light microscope and histologically evaluated by an experienced pathologist to evaluate the extent of fibrotic changes. Moreover, the blue color in Masson-stained sections, which reflects collagen fibers accumulated within the tissue, was semi-quantified by ImageJ software and was reported as the percentage of fibrosis area.

Hydroxyproline Measurement

The lung segments were snap-frozen and stored at −80°C to measure the amount of hydroxyproline in the lung tissue. Hydroxyproline expresses about 13.5% of the amino acid content of collagen and is thus considered an indicator of the level of collagen subsisting within the tissue.[19] A portion of each isolated lung tissue was weighed, and HCL 6N was added proportionally to each sample. Acid hydrolyzation took place overnight at 110°C. Then 10 µL of each acid-hydrolyzed sample was transferred to a microplate. The color resulting from an enzymatic reaction between chloramine-T and hydroxyproline residues in collagen fibers was measured by an Enzyme-linked immunosorbent assay (ELISA) plate reader during a colorimetric assay (Sigma Aldrich, St. Louis, MO, USA). The hydroxyproline values per mg of lung tissue were identified by comparing the absorbance (560 nm wavelength) of the samples with a standard curve plotted by measuring the known standard concentrations of hydroxyproline.

RNA Extraction, Reverse Transcription, and Real-time Quantitative PCR Assay

The lung tissue specimens kept in RNALater solution were cut into small fragments, then homogenized in RNXPLUS reagent (Sinacolon, Iran) using a homogenizer. The total RNA of tissue homogenates was extracted using the manufacturer's instructions. After evaluating the quantity and quality of the isolated RNA, cDNA was prepared by the RevertAid First Strand cDNA Synthesis Kit (Fermentase, USA). The cDNA amplification was performed using SYBR green master mix (Amplicon, England) and specific primer sets against target genes in a Rotor-Gene Q PCR instrument. Each run was comprised of 40 cycles with an annealing-extension temperature of 60°C for 40 seconds. The relative mRNA expression was analyzed using the $2^{-\Delta\Delta Ct}$ method and normalized to the reference gene, *Gapdh*; the results are expressed as fold change. Primer sequences used in this study are listed in Supplementary Table 1.

Statistical Analysis

All statistical analyses were performed and plotted using GraphPad Prism software version 8.0. Comparative analysis among the study groups was made by student t-test, and the data are presented as mean ± SD. p values less than 0.05 were considered statistically significant.

RESULTS

Fibrotic Changes in Lung Tissue

To verify fibrotic changes, the histological characteristics of lung tissue from each experimental group were assessed on day 21 by H&E staining and Masson's trichrome staining of the tissue sections. Furthermore, enzymatic hydroxyproline measurement was performed to determine the collagen content in lung segments indirectly.

While H&E-stained lung sections from the control group showed normal alveolar structure without inflammatory injury, those from the BLM-challenged group represented evident inflammation and inflammatory cell infiltration, plus collapsed alveoli and destructed lung architecture (Figure 1A).

Masson's trichrome staining of lung tissue sections from the control group represented normal epithelium and alveolar structure with a few connective tissue fibers (blue-stained area) next to the large vessels, bronchi, and bronchioles as normal supporting connective tissue. In contrast, those from the BLM-challenged group showed inflammatory cell infiltration, hyperemia, and epithelial injury. In addition to the fibers supporting normal connective tissue, some foci of collagen deposition (blue area) within the parenchyma and destructed alveolar structures were identified (Figure 1B).

Furthermore, the extent of the blue area that revealed collagen fibers was significantly higher in the IPF group (p<0.05) compared to the control (Figure 2A).

Consistent with the histological observations, the hydroxyproline assay of lung segments showed a significantly increased hydroxyproline amount in the IPF group (p<0.0001) compared to the control group (Figure 2B).

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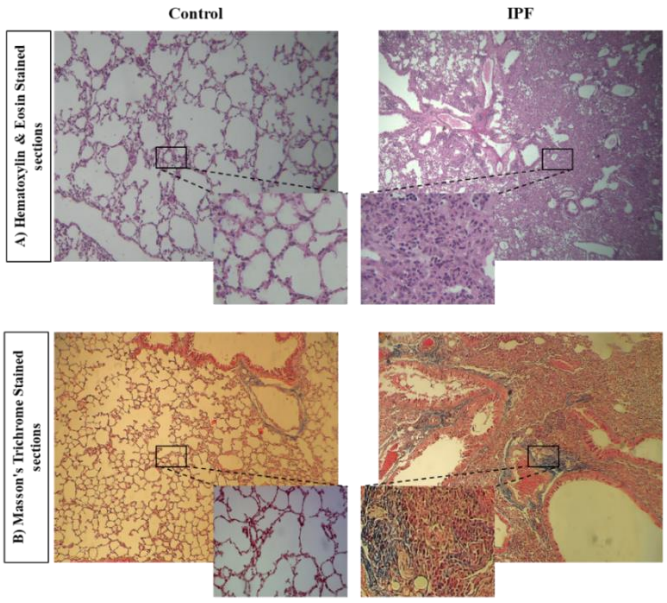

Figure 1. Histological features and fibrotic changes in the lung tissue sections from the experimental groups. A) Hematoxylin and eosin staining of lung sections represented inflammation, cell infiltration, and reduction of alveolar spaces (×100 and ×200 magnification). B) Masson's trichrome staining of lung sections represented collagen deposition (blue color) and destruction of alveoli (×100 and ×200 magnification).

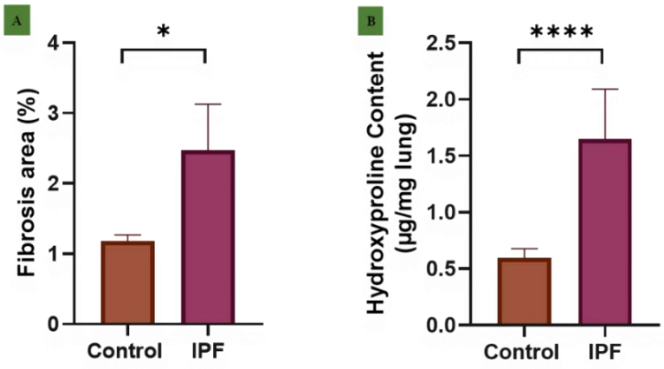

Figure 2. Examination of collagen deposition in the lung tissues. A) Fibrotic area of Masson-stained sections, semiquantified using ImageJ software. B) The hydroxyproline content of the lung segments from the control and IPF groups was measured by colorimetric assay, and values were expressed per mg of lung tissue. n=7 per group, *$p < 0.05$, ****$p<0.0001$.

Gene Expression Changes in Fibrotic Lung Tissue

To explore the mTOR signaling in lung fibrosis, we evaluated the mRNA expression of *Tsc1*, *Tsc2*, and *Rheb*, which are considered upstream regulators of *mTORC1*, through regulating *Raptor*, the specific subunit of the *mTORC1* complex, responsible for its activation. Gene expression analyses showed significantly increased expression of *Rheb* and *Raptor* within the lung tissues of the IPF group ($p<0.0001$ and $p<0.001$, respectively) compared to controls (Figure 3C, 3D). The mRNA level of *Tsc1* was slightly increased in the IPF group; however, it was not statistically significant in comparison with the control group (Figure 3A). In contrast, the mRNA expression of *Tsc2* was significantly reduced in the IPF group ($p<0.0001$) compared to the control (Figure 1B).

We further evaluated two main targets of mTORC1, namely *4Ebp1* and *S6k1*, involved in protein synthesis and the production of ECM components. It was found that the mRNA expression of both *4Ebp1* and *S6k1* was

significantly increased within the lung tissues of the IPF group (p<0.0001) compared to the control (Figures 3E and 3F).

Along with histologic changes in lung tissues, we also investigated the expression of *Vegfa* and *Lrp1*. We found significantly reduced mRNA levels of them expressed in the IPF group (p<0.01 and p<0.0001, respectively) compared to the control (Figures 4A and 4B).

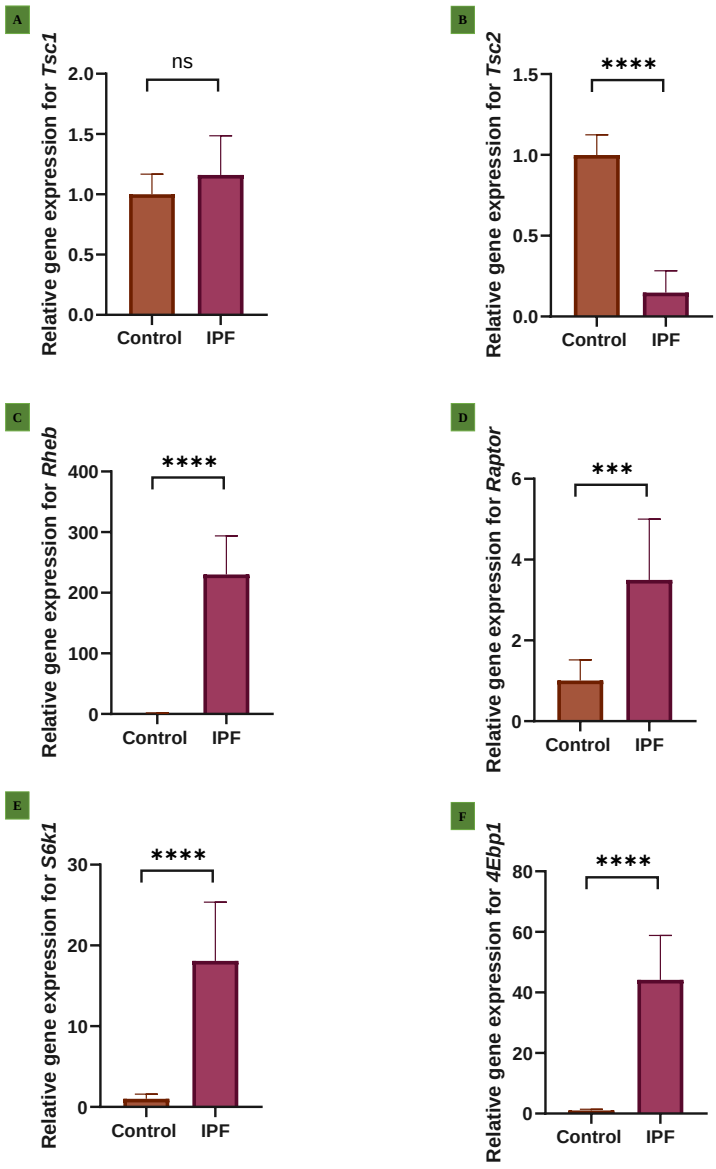

Figure 3. The gene expression alterations within the lung tissues from the experimental groups. The data were normalized to *Gapdh* mRNA levels and represented as relative fold changes. A) Relative expression of *Tsc1* was not significantly altered in the IPF group compared with the control. B) Relative expression of *Tsc2* was significantly decreased in the IPF group compared with the control. C) Relative expression of *RHEB* was significantly increased in the IPF group compared with the control. D) Relative expression of RAPTOR was significantly increased in the IPF group compared with the control. E) Relative expression of *S6k1* was significantly increased in the IPF group compared with the control. F) Relative expression of *4ebp1* was significantly increased in the IPF group compared with the control. n=7, ***p<0.001, ****p<0.0001. Tuberous sclerosis complex1,2 (TSC1, TSC2), Ras homolog enriched in brain (RHEB), Eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1), and S6 kinase (S6K).

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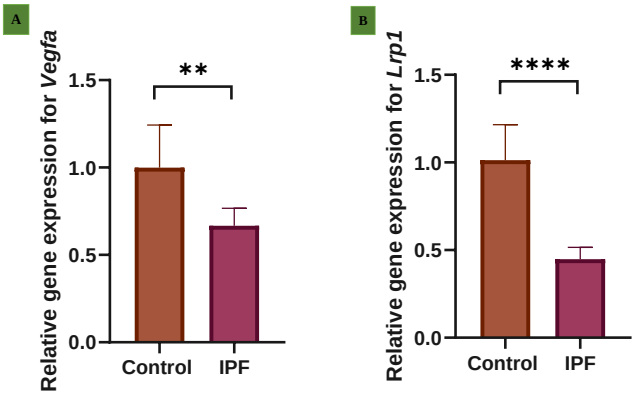

Figure 4. Gene expression of *Vegf* and *Lrp1* within the lung tissues from the experimental groups. Data were normalized to *Gapdh* mRNA levels and are represented as fold changes. A) The relative expression of *Vegfa* was significantly decreased in the IPF group compared with the control. B) Relative expression of *LRP1* was significantly decreased in the IPF group compared with the control. n=7, **p<0.01, ****p<0.0001. Vascular Endothelial Growth Factor Alpha (VEGFa), low-density lipoprotein receptor related protein 1(Lrp1).

DISCUSSION

Considering the pathogenic pathways shared by IPF and cancer, we examined the expression of genes that contribute to these common pathways in BLM-induced lung fibrosis.

The induction of the mouse model was performed by direct intratracheal injection of bleomycin, considered the most commonly used animal model to study the pathogenesis of IPF, resembling human disease.[20,21] Histologic findings showed increased cell infiltration and ECM deposition, decreased alveolar spaces, and remodeled lung architecture. Along with these fibrotic changes, we found that *Tsc2* mRNA was downregulated in lung tissue; however, the gene expression of *Tsc1* did not change. Besides, the gene expression of both *Rheb* and *Raptor* (regulatory-associated protein of mTOR), a specific subunit related to the activation of the *mTORC1* complex, was observed to be overexpressed.

TSC1 and *TSC2* are tumor suppressors and form a complex that acts as a key inhibitor of cell growth and protein synthesis. The TSC1–TSC2 complex negatively regulates the activity of *mTORC1* through inhibition of RHEB. Loss of *TSC1* or *TSC2* function is reported to be involved in a broad range of disorders that underlie abnormalities in cell growth, proliferation, differentiation, and migration processes, such as proliferative diseases and malignancies.[22] In the context of fibrosis, we have shown that the expression of *Tsc2* is significantly reduced in comparison with controls; in line with our data, Wang et al. have also demonstrated the reduced protein expression of *TSC2* in fibrotic lungs.[23]

We further observed the upregulation of *Rheb* and the increased expression of the activation-related subunits of *mTORC1*, *Rptor*, in fibrotic lung tissues. *Rheb* is a major activator of *mTORC1*, which promotes downstream fibrotic pathways. Consistent with our findings, Jiang et al. showed a profibrotic role for *Rheb*/*mTORC1* signaling in fibroblast activation and interstitial kidney fibrosis.[24]

The S6 kinase 1 (p70S6K1 or S6k1) and *4EBP1* are two effector molecules that function downstream of the mTORC1 and are directly involved in the regulation of protein synthesis, cell proliferation, and survival. Our data demonstrated the mRNA overexpression of both *p70S6k1* and *4Ebp1* in fibrotic lungs. The activation of *p70S6K1* leads to an increase in protein synthesis and cell proliferation; thereby, overexpression of this kinase contributes to tumorigenesis.[25,26] Moreover, increased expression of *p70S6K1* in breast cancer was associated with aggressive disease and a poor prognosis.[27] Hence, *p70S6K1* is suggested as a putative target for preventing or inhibiting tumor growth.[28] Although it needs more validation in further studies, our findings suggest that *S6k1* could also be evaluated as a therapeutic target for lung fibrosis. Moreover, the association of aberrant expression of *S6k1* with disease severity and prognosis in patients with IPF needs further evaluation.

4EBP1 is a translational repressor that interacts with eukaryotic initiation factor 4E (*eIF4E*) and inhibits the cap-dependent translation. Defects in the function of 4EBP1 have been implicated in various human cancers upon its downregulation or increased phosphorylation.[29] As 4EBP1 represses translation, it is expected that reduced expression of *4Ebp1* would be associated with the fibrotic phenotype of the lungs. Instead, we found overexpression of the *4EBP1* gene in the IPF model, which may indicate that *4Ebp1* acts as a part of a compensatory mechanism to halt the overexpression of ECM proteins in IPF. Our data is the first report of *4Ebp1* expression levels in lung fibrosis; in this regard, overexpression of *4Ebp1* has also been reported in breast cancer, resulting from increased phosphorylation of 4EBP1, which leads to cancer progression.[30,31,32,33] *4Ebp1* overexpression may contribute to IPF development in the same manner. In addition, *S6k1* and *4EBP1* mRNA expression levels were reported to be correlated and were associated with tumorigenesis and poor prognosis.[31] However, the prognostic value of *S6k1* and *4Ebp1* mRNA expression in IPF remains to be further evaluated.

While VEGF is a well-established component of the mechanisms involved in cancer cell proliferation and metastasis, data about the role and expression profile of VEGF in IPF are mixed and not conclusive. In one study, elevated *Vegf* mRNA was observed in endothelial progenitor cells of IPF patients. Others have found no changes in plasma levels, and some studies reported reduced VEGF in the lungs.[34,35,36,37]. Since we have observed the downregulation of *Vegf* mRNA along with fibrotic changes in the lung, we suggest that this molecule may not participate in IPF development or progression. Controversially, two new FDA-approved drugs for IPF, originally designed as antiangiogenic agents for the treatment of cancer, target VEGF as well as FGF and PDGF. These drugs retarded IPF progression; however, they were not successful in improving survival and quality of life.[38] Whether this failure is due to the inhibition of VEGF or has other reasons remains to be further explored.

LRP1 was found to control cell growth and ECM turnover in cancer; however, it has rarely been studied in IPF. Despite extensive investigation in patients with cancer, the role of LRP1 fibrosis is still controversial.[39,40,41,42] Our results showed reduced expression of *Lrp1* in fibrotic lungs. It seems that lower *Lrp1* expression would be associated with IPF pathogenesis, suggesting a possible protective role for LRP1 in lung fibrosis. Figure 5 illustrates a schematic representation of the sequence of events that may occur in the course of fibrogenesis in terms of the gene alterations in the TSC2/RHEB/mTORC1 axis, also known to be dysregulated in cancer.

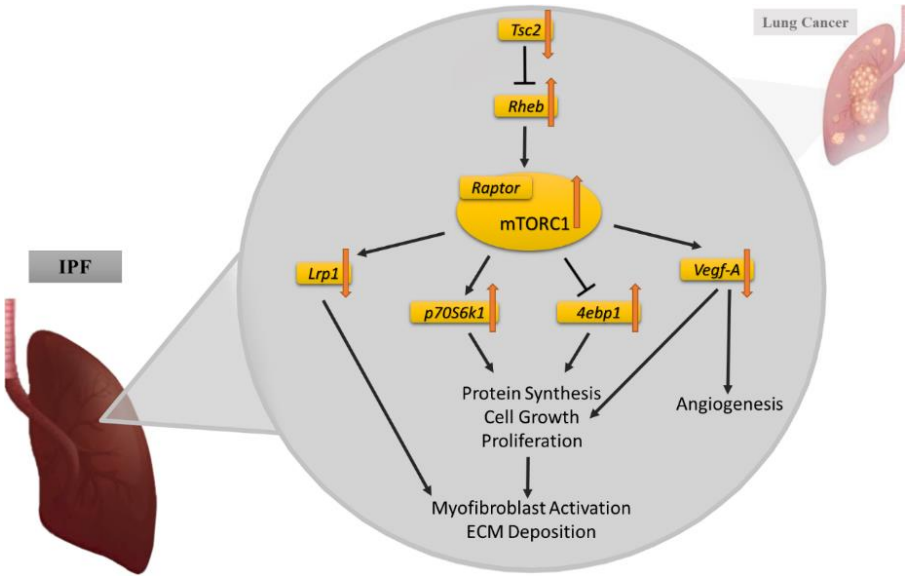

Figure 5. schematic representation of the alterations of genes in the IPF mouse model previously known to be involved in cancer. The proposed sequence of events that may occur in the context of fibrogenesis is depicted here. The same events are known to be involved in the pathogenesis of malignancies.

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We showed the contribution of an aberrant TSC2/RHEB/mTORC1 axis in association with a fibrotic phenotype, which could promote collagen synthesis, ECM deposition, and fibrogenesis in IPF through *S6k1* and *4Ebp1* expression. Moreover, VEGF-A and LRP1, known for promoting cancer progression, might also play a preventive role in lung fibrosis. Although preliminary, our data may pave the way for the characterization and development of more effective strategies for drug repositioning from cancer to IPF or other related disorders.

STATEMENT OF ETHICS

All animal experiments were carried out with the approval of the Animal Ethics Committee of the Iran University of Medical Sciences (ethics code: IR.IUMS.FMD.REC.1396.9321126001).

FUNDING

This study was funded by a grant from the Iran University of Medical Sciences (Grant No. 96-04-30-32399).

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGEMENTS

Not applicable.

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