

# Effects of Akt Inhibition on The Regulatory and Proteomic Profiles of Alveolar Macrophages Enriched from Lung Lavages of Pulmonary Fibrosis Patients

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## ABSTRACT

**Objective:** pulmonary fibrosis caused by pneumoconiosis is an irreversible disease, and there is a lack of treatment at present. The alveolar macrophages of patients were treated with Akt inhibitor MK-2206 to explore the changes of transcriptome expression and enrichment pathway, so as to provide reference targets for clinical medication. **Materials:** Purification and lab assays of alveolar macrophages from whole lung lavages of pulmonary fibrosis patients. **Methods:** RNA-seq was used to analyze alveolar macrophages before and after MK-2206 treatment to obtain subgroups of differentially expressed genes, which were further subjected to the analysis of the function and pathway enrichment. Key targets of main pathways were obtained through protein-protein interaction network analysis, providing information for the underlying connected regulatory mechanisms. **Results:** 143 up-regulated and 110 down-regulated genes were obtained by differential gene analysis. The up-regulated genes were mainly enriched in longevity regulation pathway, autophagy pathway and mTOR pathway, and the down-regulated genes were mainly enriched in inflammatory response and cytokine- cytokine interaction pathway. MK-2206 regulated alveolar macrophages in pulmonary fibrosis through the above two aspects. **Conclusion:** MK-2206, as a specific inhibitor of the Akt, could potentially affect its downstream pathway to regulate the process of autophagy and apoptosis. In addition, it also reduces the release and interaction of cytokines, and jointly regulate the process of pulmonary fibrosis. Clinically, MK-2206 can combine and cooperate with other targets to provide reference for drug treatment.

**Keywords:** *Pneumoconiosis, Pulmonary Fibrosis, Akt Inhibitor, Alveolar Macrophages, Autophagy*

## Introduction

Pneumoconiosis is an irreversible occupational chronic disease caused by long-term exposure to dust particles. The number of cases increased by 66% from 1990 to 2017 all over the world (Shi *et al.*, 2017). Unfortunately, so far there is no effective treatment to get rid of it. The production of a large number of cytokines, excessive proliferation of fibroblasts and deposition of extracellular matrix (ECM) are the main manifestations of pulmonary fibrosis (Hewlett *et al.*, 2018; Gharaee-Kermani *et al.*, 2009).

The process of epithelial-mesenchymal transition (EMT) plays an important role, and it is mainly caused by the activation of TGF- $\beta$ /Smads pathway, Wnt pathway and MAPK pathway by TGF- $\beta$ 1 (Willis and Borok, 2007). In recent years, it has also been found that there is closely related among endoplasmic reticulum stress (ERs), autophagy and EMT (Tanjore *et al.*, 2011; Ghavami *et al.*, 2018).

In clinical treatment, pulmonary lavage is mainly used to remove inflammatory factors and alveolar macrophages to improve the condition of pneumoconiosis patients. Alveolar macrophages (AM) is the first line of defense in the process of pulmonary fibrosis and can produce immune factors such as tumor necrosis factor (TNF) and transforming growth factor  $\beta$  (TGF- $\beta$ ) (Zhu *et al.*, 2017). After AMs are stimulated, mitochondria would not only produce a large of reactive oxygen species (ROS) to induce autophagy, but also increase the expression of TGF- $\beta$ 1, which leads to the EMT (Larson-Casey *et al.*, 2016). Autophagy is a conserved cellular program used to transform organelles and proteins through lysosome dependent degradation pathways. It captures and degrades damaged mitochondria to prevent cell death, which is a means of cell survival. However, excessive autophagy can also lead to programmed cell death (Galluzzi *et al.*, 2008). In various cancers and pulmonary fibrosis, the regulation of autophagy and apoptosis plays a key role in the development of diseases (Zhao *et al.*, 2020).

Protein kinase B (PKB/Akt) is a serine/threonine kinase downstream of PI3K, including three isomers of Akt1, Akt2 and Akt3 (Revathidevi and Munirajan, 2019). Akt can bind 3-phosphatidylinositied-dependent protein kinase 1 (PDK1) to promote terminal phosphorylation, what activate hypoxia inducible factor (HIF-1 $\alpha$ ), mammalian target of rapamycin (mTOR) and other factors in the downstream participating in cell proliferation and differentiation (Song *et al.*, 2019). At present, it is an important inhibition target of anti-cancer research. The principle is that Akt inhibitor can significantly increase apoptosis and inhibit tumor development. However, it was found that the autophagy program was activated simultaneously in this process. PI3K/Akt pathway control the autophagy process by regulating the activity of downstream mTOR (Xu *et al.*, 2020). Nevertheless, MK-2206, an Akt inhibitor, has been widely used in the treatment of cancer (Hirai *et al.*, 2010). It played a key role in several recent clinical trials of treatments for breast, pancreatic and gastric cancers (Chien *et al.*, 2020; Murphy *et al.*, 2020; Mehnert *et al.*, 2019). Similarly, MK-2206 can induce the production of low-density lipoprotein receptor (LDLR) and stimulate the uptake of LDL (Bjune *et al.*, 2018).

However, so far, there is no study about the effect of MK-2206 on pulmonary fibrosis in pneumoconiosis patients. This study treated alveolar macrophages purified from lung lavages of pulmonary fibrosis patients with MK-2206 to specifically inhibit Akt, aiming to assay and explore the associated intracellular changes in protein expression and pathway enrichment, thus providing supporting evidence for the underlying mechanistic regulatory networks for pulmonary fibrosis.

## Materials and Methods

### *Procedures of Whole Lung Lavage (WLL)*

The study design has been reviewed and approved by the institution's ethics committee. Patients diagnosed of pulmonary fibrosis (pneumoconiosis) received WLL at West China Fourth Hospital as the recommended medical treatment. During WLL, 0.9% normal saline solution was heated to 37°C, subjected to lung lavage 20 minutes after unilateral pure oxygen ventilation, and terminated when the drainage fluids turn from turbid to fully transparent. The total volume of lung lavage fluids was generally 4000-6000 ml, influenced by the patient's lung volume and pneumoconiosis stages, which were routinely discarded as medical wastes. In this study, the patient, before WLL, was informed of the proposed research and gave permission in the Informed Consent Form to authorize the usage of his lung lavage fluids for biomedical research purposes. Accordingly, after WLL, lung lavage fluids collected into the aseptic containers were not discarded but immediately transferred to the laboratory for further biomedical experiments.

### *Cell Isolation from Lung Lavage Fluids*

The lung lavage fluid was filtered through double-layer gauze to remove mucus and other clumped stuffs, aliquoted to 50 ml tubes, and then centrifuged at 1800 rpm for 5 min. Supernatants were discarded and the pellets were washed 2-3 times with PBS. The pellets were aggregated and resuspended in culture medium, made up of DMEM medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1X penicillin-streptomycin mixture (HyClone). Cells were imaged under a light microscope to evaluate quality, measured for density, and cultured in 25 cm<sup>2</sup> plastic flasks (Corning) in an incubator (5% CO<sub>2</sub>, 37°C). For cryopreservation, cells were resuspended in culture medium supplemented with 10% dimethyl sulfoxide (Sigma-Aldrich) and frozen in stock tubes at a -80°C freezer.

### *Inhibition Treatment and Design*

Cells were incubated at 37°C in a humidified 5% CO<sub>2</sub> incubator and divided into two groups: Group A (alveolar macrophages in pneumoconiosis patients), Group B (Akt inhibited with MK-2206). Cells in the control group were grown in the culture medium, and cells in the experiment group were grown in the culture medium supplemented with 10 nM MK-2206. Both control and experiment groups were repeated with independent triplicates.

### *RNA-seq Experiment and Bioinformatic Analysis*

Total RNA was extracted from cells, and mRNA was purified from total RNA using poly-T oligo-

attached magnetic beads. Fragmentation of mRNA was carried out using divalent cations under elevated temperature, followed by cDNA synthesis using random hexamer primers. After adenylation of 3'-end DNA fragments, adaptors with hairpin loop structure were ligated to prepare for hybridization. The library fragments were purified with AMPure XP system (Beckman Coulter) to select cDNA fragments of about 400 bp in length. After PCR reactions, products were purified and the library quality was assessed on a Bioanalyzer 2100 system (Agilent). All RNA-seq experiments were repeated in independent triplicates.

Differential expression was based on the DESeq2 analysis pipeline, and the thresholds of absolute  $\log_2$ (fold change) over 1 and adjusted P value less than 0.05 were used to identify differentially expressed genes (DEGs). Overrepresentation analysis (ORA) and gene set enrichment analysis (GSEA) were conducted with reference to the gene ontologies (GOs) and KEGG pathways (Wu *et al.*, 2021).

### ***Construction of Protein-Protein Interaction (PPI) Network***

The Search Tool for the Retrieval of Interacting Genes (STRING) database (<http://string-db.org/>) (Szkarczyk *et al.*, 2021), is an online tool designed to explore and analyze PPI information. In order to evaluate the interaction between DEGs after inhibition experiments, STRING is used to map DEGs, and the interaction with combination score > 0.4 is selected. The top 10 most connected genes in the network are defined as hub genes to identify key elements in biological processes (BP).

### ***Statistical Analysis***

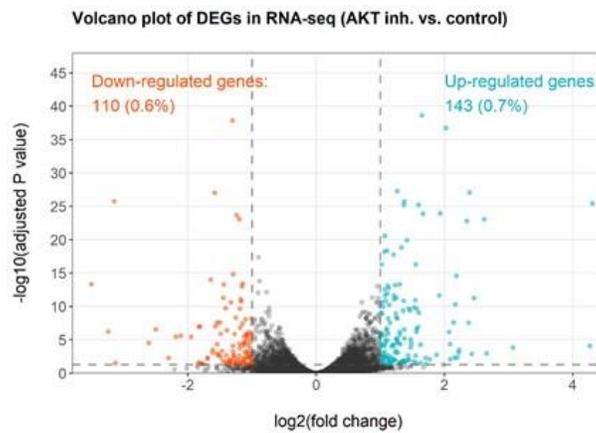
Data analysis and visualization were performed in the R statistical software. For single null-hypothesis tests, a significance level of 0.05 was used. For multiple testing where the control of false discovery rate (FDR) was necessary, raw P values were adjusted to obtain adjusted P values.

## **Results**

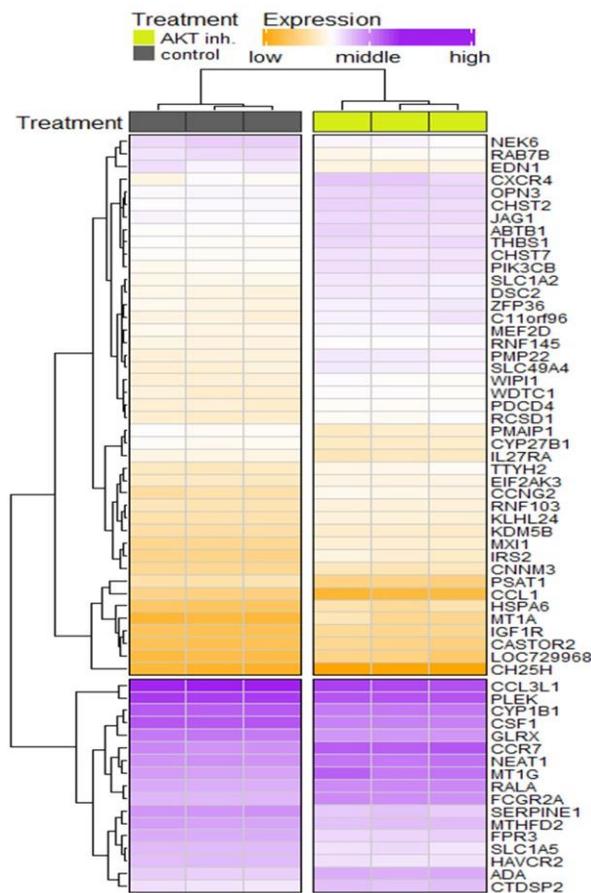
### ***Differentially Expressed Genes Amongst Various Treatment Groups***

Using RNA-seq as the next-generation high-throughput sequencing technology, such gene expression changes could be revealed by the profiles of differentially expressed genes (DEGs). For the control treatment vs. the experimental treatment, three replicates were prepared and assayed in parallel. Set the absolute  $\log_2$  (multiple change) threshold to 1 and the adjusted p-value threshold to 0.05 to control the error discovery rate (FDR). In lung lavage cells that inhibited Akt targets with MK-2206, a total of 253 genes were identified as DEGs (143 up-regulated genes and 110 down-regulated genes) compared with the control, as shown in volcanic diagram (Fig. 1). The heatmap of the first 60 DEGs sorted

by adjusted  $P$  value, in which three control samples and three experimental samples were stably clustered into different groups again, revealing the significant transcriptional patterns of relative up-regulation and down-regulation after Akt inhibition experiment with MK-2206 (Fig. 2).



**Figure 1:** RNA-seq analysis of inhibition of Akt in alveolar macrophages. Volcanic plot obtained after inhibiting Akt target with MK-2206. The up- and down-regulated genes before and after inhibition were annotated. Thresholds of absolute  $\log_2$  (fold change) of 1 and adjusted  $P$  value of 0.05 were applied to identify DEGs.



**Figure 2:** Heatmap of the most significant DEGs in lung lavage cells after inhibition treatment. Akt inhibited with MK-2206 versus control.

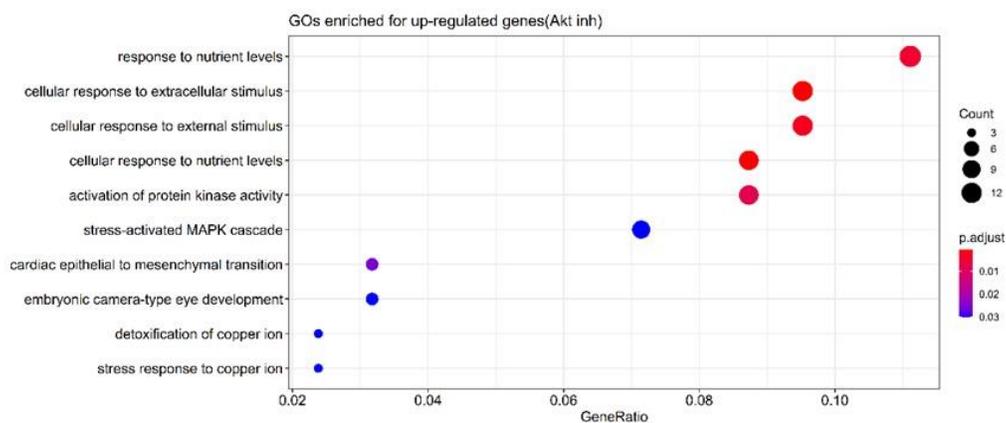
## Enrichment Analysis of DEGs

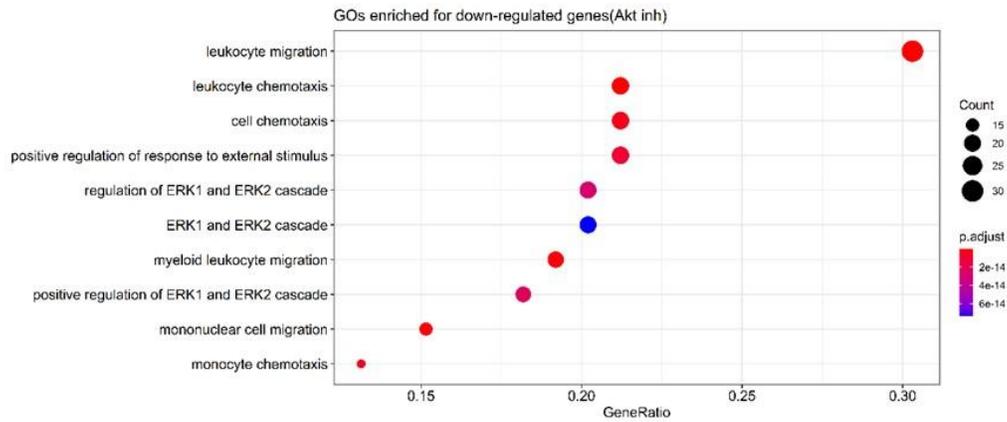
Differential expression analysis of RNA-seq determined the list of DEGs in lung lavage cells after different inhibition experiments. In order to clarify the biological function of DEGs, we used GO enrichment analysis for functional annotation, and KEGG pathway analysis to determine the important pathways changed in the study. Overrepresentation analysis (ORA) only considers the identity of DEGs.

GO enrichment analysis revealed that the up-regulated genes were mainly enriched in 'response to nutrient levels', 'cellular response to extracellular stimulus', 'cellular response to external stimulus', 'cellular response to nutrient levels', 'activation of protein kinase activity' and 'cardiac epithelial to mesenchymal transition'. Down-regulated DEGs are enriched in cytokine-related functions and pathways, including 'leukocyte migration, leukocyte chemotaxis', 'cell chemotaxis', 'positive regulation of response to external stimulus', 'regulation of ERK1 and ERK2 cascade', 'ERK1 and ERK2 cascade', 'myeloid leukocyte migration', 'positive regulation of ERK1 and ERK2 cascade', 'mononuclear cell migration' and 'monocyte chemotaxis' (Fig. 3).

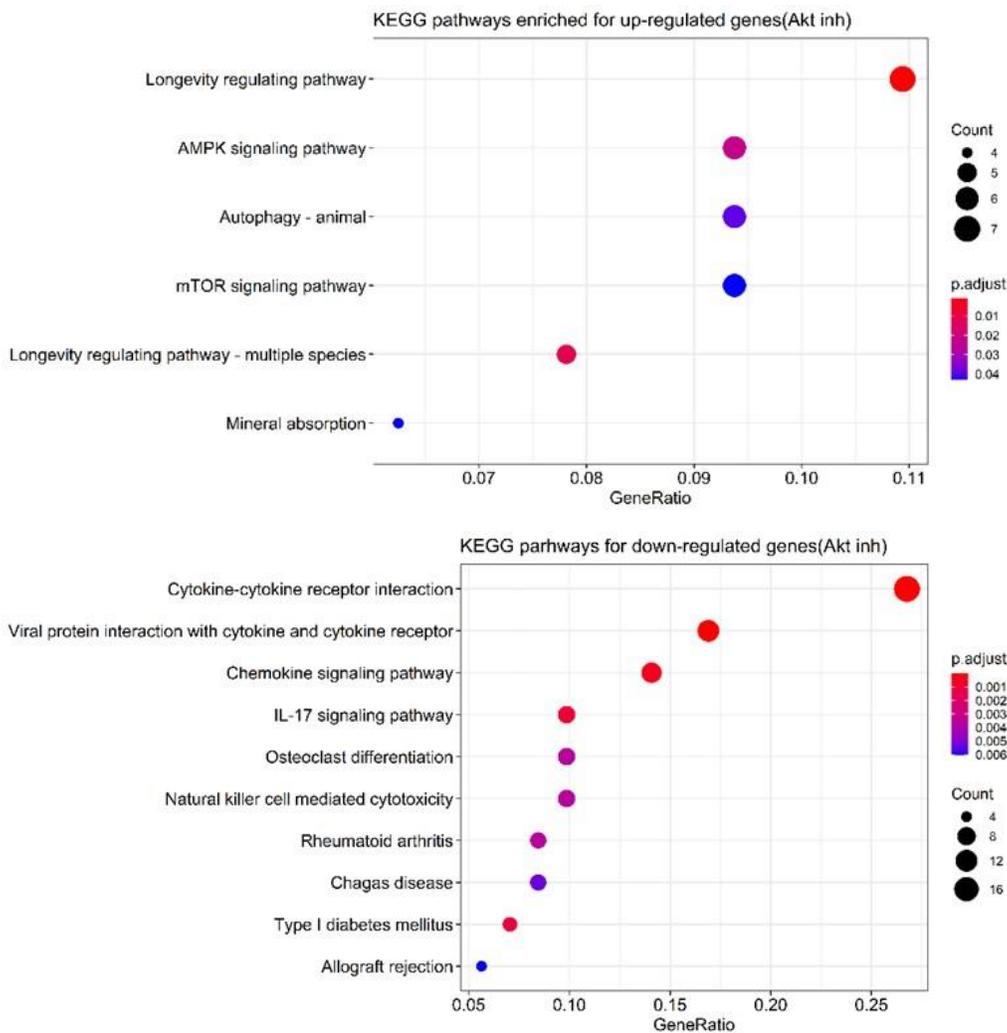
In addition, the ORA of KEGG pathway analysis of up-regulated genes revealed the key member 'Autophagy-animal' and identified the first three significantly enriched pathways, including 'Longevity regulating pathway', 'AMPK signaling pathway' and 'mTOR signaling pathway'. (Fig. 4). The down-regulated genes revealed key members such as 'Cytokine-cytokine receptor interaction', 'Osteoclast differentiation' and 'Natural killer cell mediated cytotoxicity' and significantly enriched signal pathways, including 'Chemokine signaling pathway' and 'IL-17 signaling pathway', which are related to the role of cytokines (Fig. 4).

Together, these significantly enriched GO terms and KEGG pathways could aid further understanding of the roles of these DEGs involved in the occurrence and development of pulmonary fibrosis.





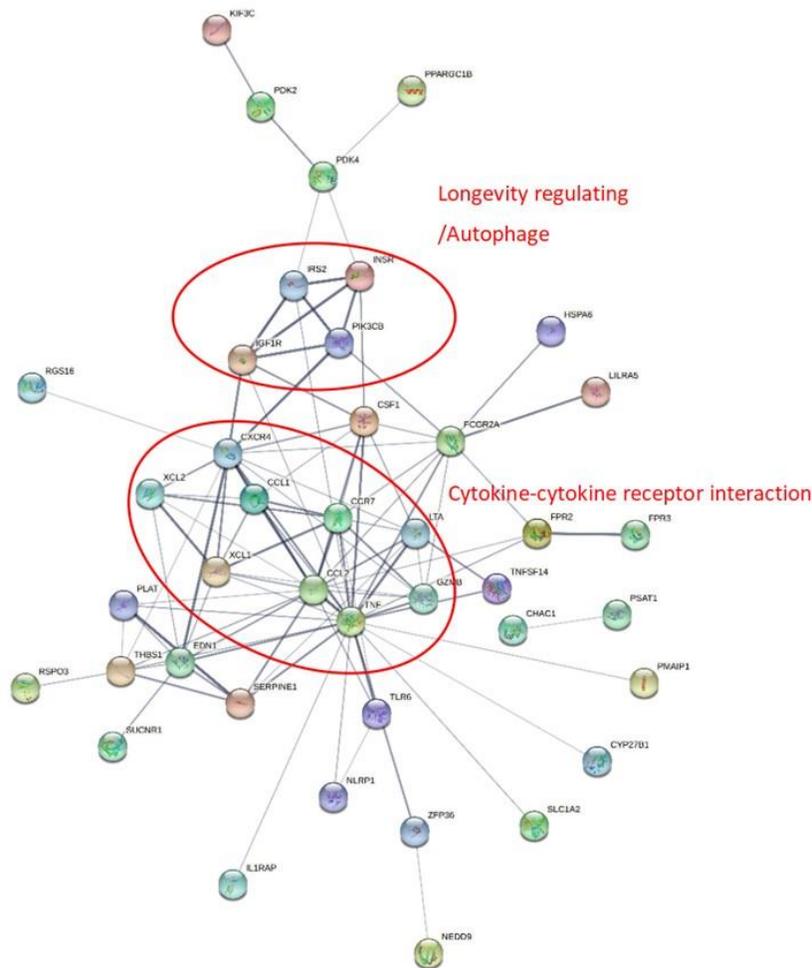
**Figure 3:** Go enrichment analysis of DEGs obtained by the inhibition tests for Akt. Go enrichment analysis of up-regulated genes (top) and down-regulated genes (bottom) after inhibition of Akt with MK-2206.



**Figure 4:** KEGG pathway analysis of DEGs obtained by the inhibition tests for Akt. KEGG pathway analysis of up-regulated genes (top) and down-regulated genes (bottom) after inhibition of Akt with MK-2206.

## PPI Network Analysis

After inhibiting Akt target, a total of 253 genes were identified as DEGs, and 96 of them ( $\log_2\text{FoldChange} > 1.2$ ,  $P \text{ adj} < 0.001$ ) were included in the STRING online database. A total of 35 genes were filtered into the PPI network complex, including 90 nodes and 106 edges, and 61 genes were located outside the DEGs PPI network (Fig. 5). Among the network, the functional module composed of WIPI1, IGF1R, PIK3CB, ULK1, IRS2, ATG2A and RAB7B is related to autophagy.

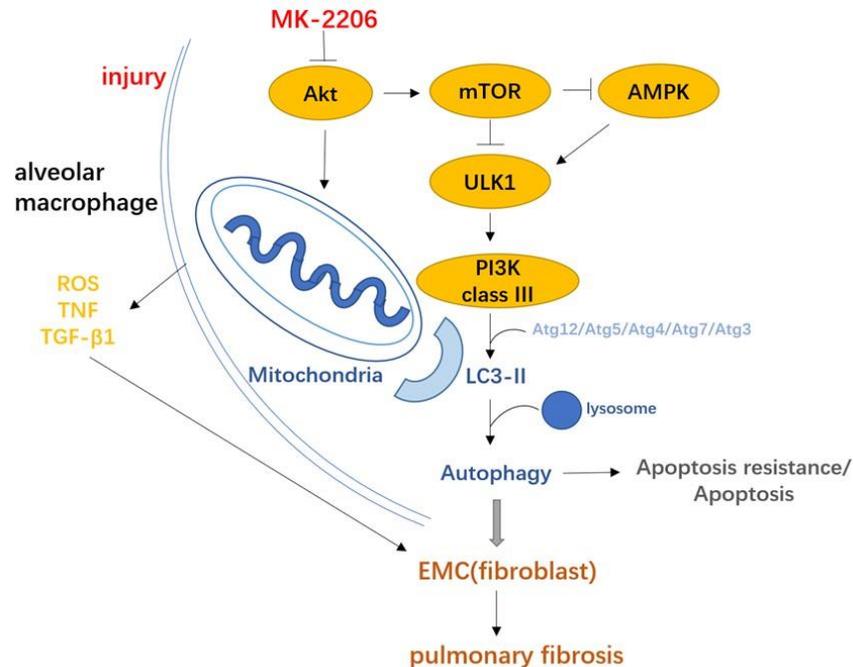


**Figure 5:** PPI analysis of DEGs obtained by the inhibition tests for Akt. PPI obtain the key genes and their interaction network after inhibition of Akt with MK-2206. The main two parts correspond to the enrichment pathway, including up-regulated autophagy/longevity regulation pathway(top) and down-regulated cytokine- cytokine interaction pathway(bottom).

## A Potential Scheme of Cascade Pathways

In this study, the inhibitor MK-2206 was used to inhibit the Akt on cells isolated from the lung lavage fluids of pulmonary fibrosis patients. The DEGs in the inhibited and uninhibited groups were analyzed by GO and KEGG enrichment to explore the gene expression and related biological functions of

the Akt pathway in the alveolar macrophages of patients with pneumoconiosis. As a reference for drug targets of pulmonary fibrosis, the potential regulatory mechanism is as assumed and depicted in the context of cellular cascade pathways (Fig. 6).



**Figure 6:** Regulatory mechanism after the inhibition tests for Akt in alveolar macrophages.

## Discussion

Firstly, the imbalance of wound repair in the lung leads to excessive tissue proliferation and finally fibrosis. In this process, a large number of inflammatory immune factor are produced, such as TNF, TGF and IL. The expression of TNF and IL-6 in patients with coal worker's pneumoconiosis was significantly increased (Boitelle *et al.*, 1997). This study confirmed the role of MK-2206 in inhibiting the expression of inflammatory factors. GO analysis revealed that the most significantly enriched processes were cell response to the outside world, cell migration, cell chemotaxis, and regulation of the ERK1 and ERK2 cascade. ERK1/2 is an extracellular signal-regulated kinase of the mitogen-associated protein kinase (MAPK) cascade, related to the proliferation of alveolar epithelial cells in vitro after exposure to asbestos. It have been shown that crocidolite fibers cause ERK1/2 activation through phosphorylation and epidermal growth factor receptor (EGFR) aggregation (Cummins *et al.*, 2003). On the other hand, the study showed that up-regulated genes were mainly enriched in autophagy, AMPK and mTOR signaling pathway. Previous studies have shown that autophagy is related to pulmonary fibrosis (Xie *et al.*, 2019). By inhibiting the PI3K/Akt/mTOR signaling pathway with different inhibitors, it can increase the stability and expression of a key regulator of cystic fibrosis. Among them, the most effective inhibitor

showed a rescue effect by regulating autophagy (Reilly *et al.*, 2017).

MK-2206 affects the activity of mTOR by inhibiting Akt. mTOR can regulate cell longevity and growth by accepting the stimulation signals of external nutrition, amino acids and oxygen. It is a key target affecting autophagy pathway (Bjedov and Rallis, 2020). mTOR includes two protein complexes: TORC1 and TORC2. After activated by phosphorylation, mTORC1 can bind to downstream ULK1, resulting in its inactivation and finally terminate autophagy. On the contrary, if mTOR is inactivated under starvation, AMPK can mediate phosphorylation of ULK1 to promote autophagy (Hosokawa *et al.*, 2009). AMPK is a kinase that regulates energy homeostasis output and input of cells. It is activated under the condition of low ATP in cells (Gwinn *et al.*, 2008). ULK is the only core protein with serine/threonine kinase activity in autophagy signaling pathway. ULK1 complex, composed of ULK1, FIP200 and mATG13, is a link between upstream nutrient or energy receptors (mTOR and AMPK) and downstream autophagosomes (Lin and Hurley, 2016).

It is an essential prerequisite for the formation of autophagosomes to activate downstream PI3KC3 which consisting of hVps34, Beclin-1, p150 and Atg14 by ULK1 (Russell *et al.*, 2013). The abnormal expression of Beclin-1 in a variety of tumor tissues is associated with poor prognosis (Liang *et al.*, 1999). After that, autophagosomes are gradually formed under the action of Atg. LC3-II is a part of the membrane of autophagosome and is often used as a biomolecular marker of autophagy, and accordingly western-blot experiments could evaluate the status of autophagy (Kabeya *et al.*, 2000). Complete autophagosomes can combine with lysosomes to form autophagic lysosomes, as a way of self-protection (D'Arcy, 2019).

MK-2206 is often used in combination with other inhibitors. Given the heterogeneity between individuals, the effect of MK-2206 alone in the treatment of breast cancer was limited because the treatment would be compromised under the limitation of insufficient target inhibition (Xing *et al.*, 2019). It is more effective to determine the treatment scheme with joint regulation of multiple targets. Rapamycin and MK-2206 could induce autophagy and necrotic apoptosis of neuroblastoma cells, and the expression of markers such as ATG5, LC3 and PIPK3 were significantly increased. By inhibiting any multiple sites on PI3K/Akt/mTOR pathway, the effect of controlling cell growth was better than that of any single inhibitor. It had vertical synergy on this pathway (Woo *et al.*, 2017).

## Conclusion

For the specific inhibition of Akt, Mk-2206 not only reduces inflammatory factors secreted from alveolar macrophages in pneumoconiosis patients, but also regulates cell growth and apoptosis by

affecting downstream mTOR pathway, AMPK pathway and autophagy pathway. The above changes can potentially alleviate the process of pulmonary fibrosis and provide candidate targets for the drug screening and testing purposes in translational research.

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