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Molecular Mechanisms of Pulmonary Fibrosis: The Interaction of Epithelial-mesenchymal Transition and AMPK Pathways in a Bleomycin-induced Model

Omid Sadatpour[1,2], Hoda kavosi[2,3], Mahdi Mahmoudi[2,3], Mehdi Mohammadi[1], Hiva Saffar[4], Elham Farhadi[2,3], and Mohammad Vodjgani[1]

[1] *Department of Immunology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran*
[2] *Rheumatology Research Center, Tehran University of Medical Sciences, Tehran, Iran*
[3] *Research Center for Chronic Inflammatory Diseases, Tehran University of Medical Sciences, Tehran, Iran*
[4] *Department of Pathology, Shariati Hospital, Tehran University of Medical Sciences, Tehran, Iran*

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ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is a progressive lung disease marked by excessive extracellular matrix (ECM) deposition, largely mediated by activated fibroblasts. Epithelial-mesenchymal transition (EMT), regulated by transcription factors such as *TGF-β*, *Twist1*, and *Snail*, is a critical mechanism in fibrosis progression. AMP-activated protein kinase (AMPK) has been implicated in modulating fibrotic pathways, but its role in EMT remains unclear. This study aimed to explore the interaction between EMT and AMPK signaling in pulmonary fibrosis.

A bleomycin-induced pulmonary fibrosis mouse model was used. Histological analysis assessed fibrosis and inflammation, while gene expression (*TGF-β*, *Twist1*, *Snail*) was measured by qPCR. Protein levels of E-cadherin, α-SMA, and phosphorylated AMPK were analyzed using Western blotting to evaluate EMT and AMPK activity.

Bleomycin-treated mice showed significant lung inflammation and fibrosis, particularly in the lower region of the left lung. Gene expression analysis revealed elevated *TGF-β*, *Twist1*, and *Snail* in fibrotic areas. Protein analysis demonstrated increased α-SMA and decreased E-cadherin, confirming EMT induction. Notably, AMPK phosphorylation was significantly reduced in fibrotic regions, occurring concurrently with EMT activation.

These findings indicate an inverse relationship between AMPK signaling and EMT in pulmonary fibrosis. EMT may serve as a direct therapeutic target, either by inhibiting transcription factors such as *Snail* and *Twist1* or by modulating upstream metabolic regulators including AMPK.

Keywords: AMP-activated protein kinases; Bleomycin; Epithelial-mesenchymal transition; Fibrosis; Idiopathic pulmonary fibrosis

Corresponding Authors: Mohammad Vodjgani, PhD;
Department of Immunology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran. Tel: (+98 21) 2387 2545, Fax: (+98 860) 2243 9970, Email: vojganim@sina.tums.ac.ir

Elham Farhadi, PhD;
Rheumatology Research Center, Tehran University of Medical Sciences, Tehran, Iran. Tel: (+98 918) 385 8107, Fax: (+98 21) 2243 9970, Email: Farhadie@tums.ac.ir

INTRODUCTION

The classification of idiopathic pulmonary fibrosis (IPF) underwent a transformation in the 21st century, prompted by the ineffectiveness of anti-inflammatory therapies that were associated with increased mortality rates.[1,2] Current understanding emphasizes the role of genetic and environmental risk factors, with recurrent micro-injuries to the aging alveolar epithelium leading to abnormal interactions between epithelial cells and fibroblasts. It is currently classified as a pulmonary and inflammatory disorder characterized by the excessive synthesis of extracellular matrix (ECM) components by activated fibroblasts within lung connective tissue. This pathological process contributes to the exacerbation of respiratory complications and the development of pneumonitis.[3–5] The disease has a higher prevalence in males and is rarely diagnosed in individuals under 50, with a median diagnosis age of approximately 65 years.[6] Genetic factors, such as single-nucleotide polymorphisms (SNPs) that increase the risk of lung fibrosis, combined with chronic turnover of epithelial cells throughout life, lead to telomere shortening that, alongside exposure to environmental factors, increases the risk for developing IPF.[1,7–10] The initial stimuli activate fibroblasts and vascular endothelial cells, causing vascular injury. This results in endothelial dysfunction, reduced blood flow, and hypoxia in affected tissues, setting the stage for fibrosis. Inflammatory cells, platelets, and surrounding components release mediators, such as interleukins (IL-1, IL-6), interferon-γ (IFN-γ), tumor necrosis factor-alpha (TNF-α), and transforming growth factor-beta (TGF-β), a key factor in fibrosis.[11–13] The non-canonical *TGF-β* pathway promotes the differentiation of myofibroblasts from fibroblasts and facilitates endothelial-mesenchymal (EndMT) and epithelial-mesenchymal (EMT) transitions via SMAD signaling. *TGF-β* also stimulates endothelial cells to produce endothelin-1 (ET-1), a vasoconstrictor involved in fibrotic responses and smooth muscle cell proliferation. Elevated ET-1 levels, combined with reduced caveolin-a protein that modulates *TGF-β* signaling, link vasculopathy to fibrosis. Additionally, pericytes differentiate into myofibroblasts when exposed to *TGF-β* and growth factors, such as PDGF and CTGF.[14,15]

EMT is a complex process involving molecular signaling pathways and transcription factors. This phenomenon is distinguished by the downregulation of epithelial markers, including E-cadherin and tight junction proteins, alongside the upregulation of mesenchymal markers, such as vimentin, N-cadherin, fibronectin, and alpha-smooth muscle actin (α-SMA). This transition promotes cellular migration and invasion. EMT plays key roles in tissue development, repair, and in diseases like fibrosis and cancer metastasis.[16,17] EMT is regulated by various signaling pathways, including *TGF-β*, Wnt/β-catenin, *Notch*, *HIF*, *PI3K/Akt/mTOR*, Rho GTPases, and *MAPK/ERK*. These pathways control key transcription factors, such as *Snail*, *Twist*, and *ZEB1*, which in turn regulate the expression of epithelial and mesenchymal markers.[18–20]

Snail (*SNAI1*) is a key transcription factor in EMT, primarily by downregulating E-cadherin, reducing cell adhesion, and enhancing cell motility. It also promotes mesenchymal traits by upregulating vimentin and N-cadherin.[21,22]

Twist1, a basic helix-loop-helix (bHLH) transcription factor, regulates EMT by downregulating E-cadherin. It is crucial for developmental processes and tissue remodeling and contributes to cancer cell migration and fibrosis by promoting a mesenchymal phenotype.[23–25]

Bleomycin, a chemotherapeutic drug, is frequently employed to induce lung fibrosis in experimental models owing to its capacity to provoke oxidative stress, DNA damage, and inflammatory responses, ultimately leading to the activation of fibroblasts and the accumulation of ECM. *TGF-β* isoforms and their receptors play a central role, as they are upregulated in bleomycin-induced pulmonary fibrosis, contributing to fibrotic progression by activating fibroblasts and altering cellular localization patterns. Both *Twist1* and *Snail* are induced by *TGF-β*, and additionally, *TGF-β* signaling is amplified by these transcription factors, promoting EMT and collagen production-key features of tissue fibrosis. A significant mechanism through which bleomycin induces fibrosis is the activation of EMT.[23,26,27]

AMP-activated protein kinase (AMPK) serves as a critical energy sensor that plays a vital role in the regulation of cellular energy homeostasis. It is activated in response to low ATP levels, increasing AMP concentrations. AMPK has a complex relationship with EMT, influencing metabolic processes and cellular responses to stress, exercise, and nutrient scarcity.[28–32] AMPK plays a protective role in lung fibrosis by inhibiting key fibrotic pathways. Its activation reduces ECM deposition and myofibroblast activation, both

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crucial in fibrosis. AMPK achieves this by suppressing *TGF-β* signaling and promoting autophagy to clear damaged cells, preventing fibrosis development. However, AMPK activity is often reduced in fibrotic conditions, leading to metabolic dysregulation, inflammation, and worsened fibrosis.[33,34] AMPK is predominantly known as a negative regulator of fibrotic signaling, primarily through its inhibition of the *TGF-β/Smad3* pathways and its facilitation of autophagy. However, its involvement in EMT remains a subject of debate and is influenced by contextual factors. Numerous studies have indicated that AMPK activation can suppress EMT by downregulating key transcription factors, such as *Snail* and *Twist1*, thereby maintaining epithelial integrity and diminishing mesenchymal transition, particularly in models of lung fibrosis. In contrast, other research—particularly within oncological frameworks—suggested that AMPK activation may facilitate EMT, potentially via metabolic reprogramming or the indirect activation of EMT-related transcription factors like *Twist1*. These conflicting results imply that AMPK may have either pro- or anti-EMT effects contingent upon the specific cell type, signaling environment, or pathological condition. This inconsistency highlights the necessity for a more comprehensive understanding of AMPK's role in the regulation of EMT, especially in the context of fibrotic lung disease, which is the primary focus of the current study.[28,35,36] Therefore, we aimed to assess EMT-involved genes *Twist1*, *Snail*, and *TGF-β* and their relation with AMPK status in fibrotic lung areas in the bleomycin model of pulmonary fibrosis (PF) and decipher the relationship between EMT-related genes and AMPK.

MATERIALS AND METHODS

Animal Model

C57BL/6 male mice, aged 8 to 10 weeks, were procured from the center for experimental studies at the Pasteur Institute of Iran, located in Tehran. All methodologies and experimental protocols were approved by the Tehran University of Medical Sciences (TUMS) ethics committee with the corresponding ethics code provided (IR.TUMS.AEC.1403.047). Eight mice were randomly assigned to 2 groups: a control group (n=4) and a bleomycin (BLM) group (n=4). The BLM group received an administration of 8 mg/kg of bleomycin (Chemex Company, Argentina), which was dissolved in phosphate-buffered saline (PBS) and surgically instilled into the trachea. Conversely, the control group was administered PBS via the same route. Both groups were euthanized on day 28 posttreatment, at which point the lungs were excised, and the lobes were subsequently sectioned for further analysis. Only male mice were used, consistent with previous studies showing that male C57BL/6 mice exhibit a more robust fibrotic response to bleomycin than females, thereby improving model consistency and reproducibility.[37] However, future studies should consider potential sex-based differences in fibrotic signaling pathways, including AMPK and EMT.

Micro-CT Scanning

In this study, at day 28 post-bleomycin treatment, we utilized an in vivo X-ray micro-computed tomography (micro-CT) scanner (LOTUS-inVivo, Behin Negareh Co., Tehran, Iran)[38] at the preclinical core facility (TPCF) of Tehran University of Medical Sciences. The LOTUS-inVivo system is equipped with a cone beam micro-focus X-ray source and a flat panel detector. To optimize image quality, the X-ray tube voltage and current were calibrated to 50 kV and 150 μA, respectively, with a frame exposure time of 0.25 seconds at 2 magnifications. The total duration of the scan was 18 minutes, and the slice thickness of the reconstructed images was set to 25 μm. All protocol parameters were managed using the LOTUS-inVivo-ACQ software. The acquired three-dimensional data was reconstructed employing the LOTUS inVivo-REC software utilizing a standard Feldkamp, Davis, Kress (FDK) algorithm.

Hematoxylin & Eosin (H&E) and Masson-trichrome Staining

Tissue specimens were obtained and subsequently prepared for histological analysis following established protocols. For standard examination, the tissues were fixed in 10% neutral-buffered formalin for a duration of 24 hours, after which they were embedded in paraffin. Sections were then sliced to a thickness of 5 μm and affixed to glass slides for the purpose of staining. Hematoxylin and eosin (H&E) and Masson trichrome staining were applied to evaluate the inflammation and collagen depositions, respectively.

RNA Extraction, cDNA Synthesis, and SYBR Green Real-time PCR

Lung specimens preserved in Trizol (Yektatajhiz, Iran) solution were initially minced and subsequently lysed using a homogenizer, following the manufacturer's protocol. RNA isolation was performed utilizing YTzol pure RNA reagent (Yektatajhiz, Iran) following the manufacturer's instructions. The quality and quantity of the isolated RNA were evaluated through a Nano Drop spectrophotometer (Thermo Fisher, USA). Following this, the RNA samples underwent reverse transcription using the cDNA synthesis kit (Yektatajhiz, Iran), according to the manufacturer's guidelines. The expression levels of the genes *Twist1*, *Snail*, and *TGF-β* were subsequently analyzed using specific primers and the Applied Biosystems Step One Plus Real-Time PCR System (Foster City, CA, USA). Mouse *β-actin*-specific primers served as the reference gene for data normalization. The gene expression data were analyzed employing the $2^{-\Delta\Delta C(T)}$ method.[39] All of the experiments were carried out in triplicate.

Total Protein Isolation and Western Blot

Western blot analyses were performed as previously described, with some modifications.[40] Lung tissues were first mechanically homogenized using a tissue homogenizer to physically disrupt the tissue structure. The homogenized samples were then lysed in RIPA buffer supplemented with protease and phosphatase inhibitors to extract total proteins. The lysates were removed by centrifugation at 14 000 rpm for 20 minutes at 4°C. Protein concentration was determined by the Bradford protein quantification kit (DB0017, DNAbioTech, Iran) according to the manufacturer's instructions. The tissue lysates were mixed with an equal volume of 2×Laemmli sample buffer. Lysates (20 µg) were then subjected to SDS-PAGE after a 5-minute boiling and subsequently transferred to a 0.2 µm immune-Blot™ polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, CA, USA). The membranes were then blocked with 5% BSA (Sigma Aldrich, MO, USA) in 0.1% Tween 20 for 1 hour. Then, the membranes were incubated with Anti-E-cadherin (ab308347, Abcam), Anti-Alpha SMA (ab7817, Abcam), Anti-p-AMPK (2531S, Cell Signaling), Anti-AMPK (ab32047, Abcam), and anti-GAPDH control antibodies (1:2500, ab8245, Abcam, UK) as a loading control overnight at 4°C. Subsequently, membranes were washed 3 times with TBST and incubated with goat anti-rabbit IgG H&L (HRP) (1:10 000, ab6721; Abcam) secondary antibody for 1 hour. The membranes were then incubated with enhanced chemiluminescence (ECL) reagent (Clarity Western ECL Substrate, Bio-Rad Laboratories, CA, USA). After detection of the target proteins, membranes were stripped using Western Blot Stripping Buffer (ab270550, Abcam, UK) following the manufacturer's instructions. Following stripping, the membranes were re-blocked and incubated with the GAPDH antibody for normalization purposes. Densitometry of protein bands was performed using the gel analyzer Version 2010a software (NIH, USA), such that the percentage area under the curve of each band was divided by the percentage area under the curve of its corresponding GAPDH band, and then the calculated values were compared between groups as we described previously.[41]

Statistical Analyses

All statistical analyses were conducted utilizing GraphPad Prism ver 10.4.1 software. A Mann-Whitney test was applied to compare the data. A p value of ≤ 0.05 was considered statistically significant. All experiments were performed with sample sizes indicated in the figure legends (n=4 mice per group unless otherwise specified).

RESULTS

Micro-CT Scanning Images Show Acute Fibrosis in the Bleomycin Group

The results of micro-CT at day 28 present visible progressive changes in the lung architecture consistent with acute fibrosis. As shown in Figure 1, the lower part of the left lung was most affected by fibrosis compared to other parts of the lung. Based on intensity thresholds and Hounsfield unit (HU) analysis, the fibrotic region in the lower lobe of the left lung displayed an approximately 43% increase in density compared to non-fibrotic regions. This semi-quantitative analysis supports the rationale for selecting this area for downstream molecular and histological assessments. Therefore, the aforementioned part of the lung was used for the rest of the experiments to see changes in fibrosis areas.

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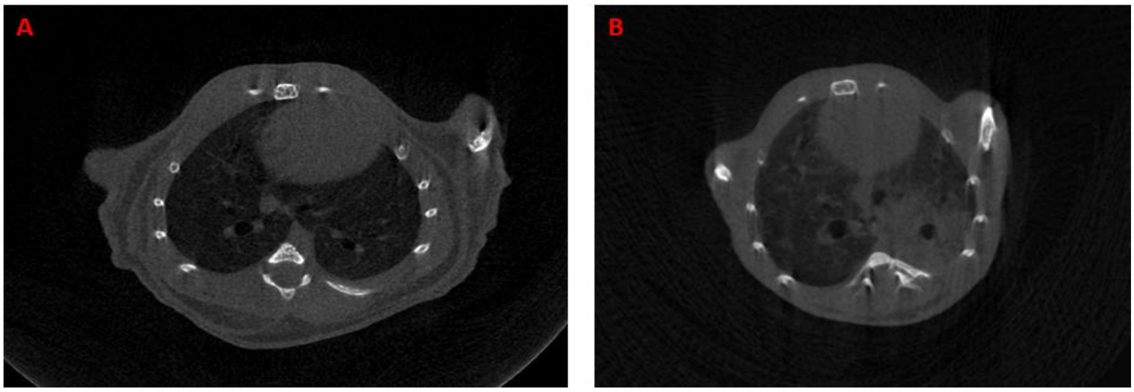

Figure 1. Representative micro-CT images of control lung (A) and bleomycin-treated lung (B). The findings demonstrate distinctly observable progressive alterations in lung architecture that align with fibrosis.

Increased Fibrosis and Inflammation in the Lung Tissues of the Bleomycin Group

H&E-stained lung sections showed infiltration of mononuclear inflammatory cells (lymphocytes and macrophages) in more than 70% of the lung alveoli, with a severe degree of inflammation in the BLM group, which is significantly higher than the control (Figure 2A–B). Emphysema-like alterations were identified by the presence of enlarged alveolar spaces and the degradation of alveolar walls, resulting in a reduction in alveolar density and a general increase in the size of airspaces. These structural modifications indicate alveolar damage that aligns with emphysematous remodeling. This observation is consistent with the fibrosis and inflammation noted, suggesting that bleomycin not only induces fibrotic changes but also contributes to emphysematous injury within the lung parenchyma. Damage and degeneration are also present in the mucous layers of the bronchioles. Fibrinous deposits in the vascular wall are also positive, indicating endothelial reactions in response to the damaging agent. A moderate to high degree of fibrosis is seen in Masson trichrome staining, with remarkably increased collagen depositions identified by blue color and dense alveolar spaces in the BLM group compared to controls (Figure 3A–B).

Increased Expression of Key Genes Involved in the EMT Process

The present investigation aimed to assess the expression levels of key genes associated with EMT. Specifically, *Twist1*, *Snail*, and *TGF-β* are recognized as critical contributors to the EMT process, the primary mechanism underlying the pathogenesis of fibrotic disease, IPF. Upon analyzing the gene expression data, it was found that the expression levels of *TGF-β*, *Twist1*, and *Snail* were significantly elevated in the bleomycin (BLM) group compared to the control group (*TGF-β* (p=0.02), *Twist1* (p=0.02), *Snail* (p=0.02)) (Figure 4).

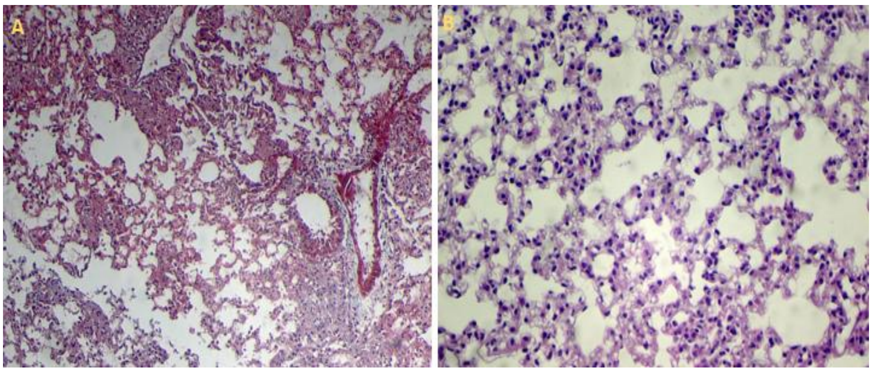

Figure 2. Hematoxylin and eosin staining of lung specimens. A. In the bleomycin (BLM) group, notable airway remodeling and heightened inflammation were observed, characterized by reduced alveolar spaces and increased immune cell infiltration. B. The control group shows normal lung architecture.

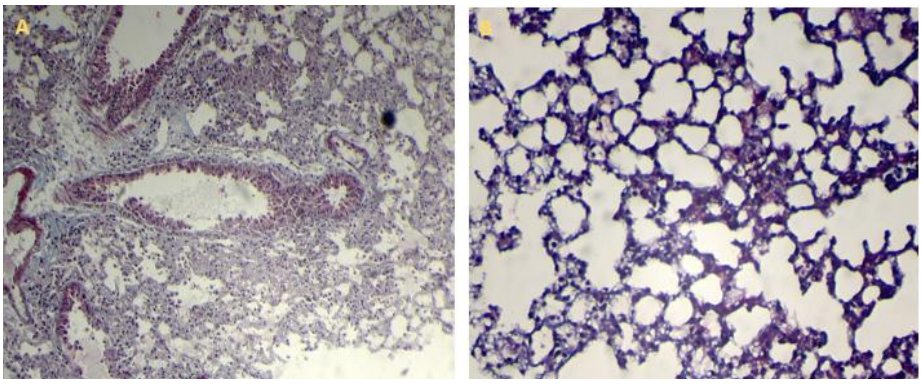

Figure 3. Masson trichrome staining of lung specimens. A. In the bleomycin-treated group, notable airway remodeling and fibrotic alterations are observed, indicated by reduced alveolar spaces and increased collagen deposition (blue). B. The control group shows minimal collagen.

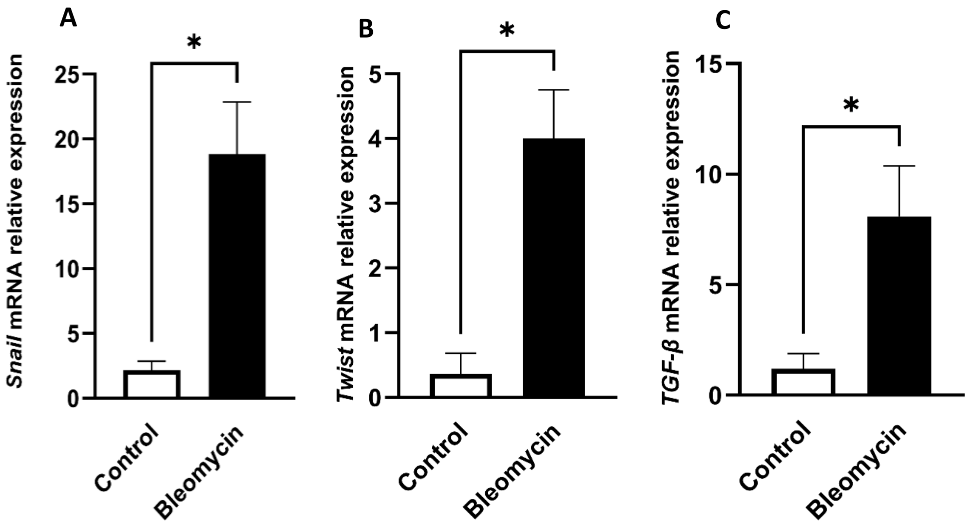

Figure 4. Relative gene expression of EMT-related genes. A. Relative expression of *Twist1* was significantly increased in the bleomycin (BLM) group compared with the control group. B. Relative expression of *Snail* was significantly increased in the BLM group compared to the control group. C. Relative expression of *TGF-β* was significantly increased in the BLM group. Relative expression of the *β-actin* gene was used as the reference gene for data normalization. *$p \leq 0.05$.

Decreased E-cadherin and Increased α-SMA Proteins Show Induction of the EMT Process in the Bleomycin Group

To assess the EMT process more deeply, we investigated two important epithelial and mesenchymal markers. As expected, we saw higher protein levels of α-SMA and lower protein levels of E-cadherin (α-SMA (p=0.002), E-cadherin (p=0.002)) (Figure 5), which is demonstrative of the EMT process happening inside the affected areas of the lung.

Decreased Phosphorylation of AMPK in the Bleomycin Group

Mice receiving bleomycin and undergoing lung fibrosis showed a diminished ratio of phosphorylated AMPK to total AMPK compared to the control group (p=0.003) (Figure 6).

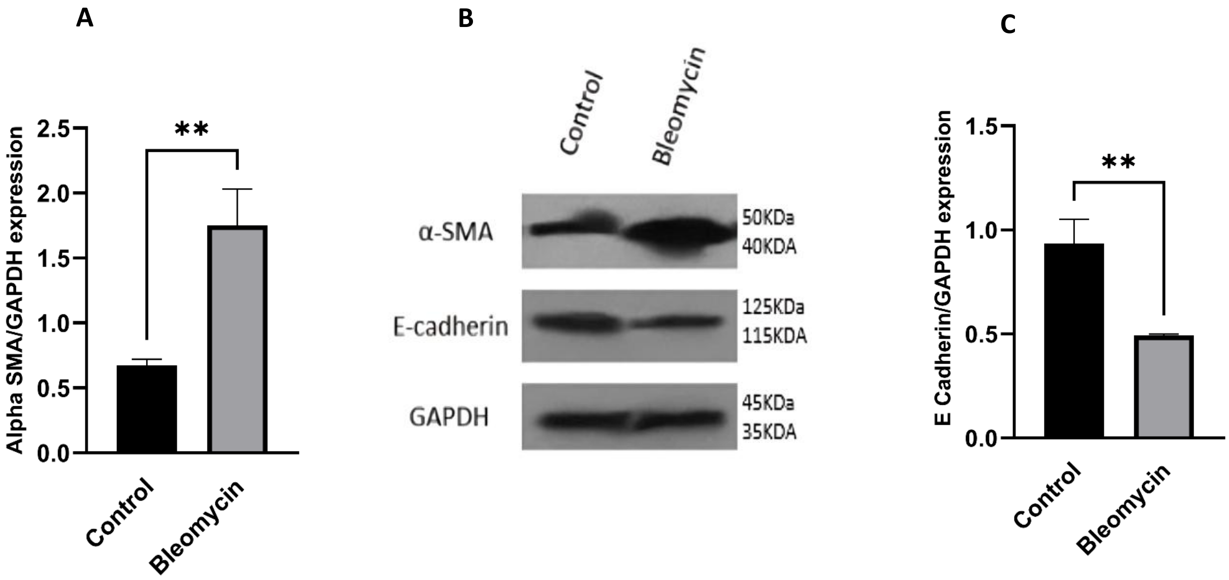

Figure 5. Protein levels of E-cadherin and α-SMA in fibrosed areas of the lung. A–C. Bleomycin-affected lung shows higher α-SMA and lower E-cadherin protein levels. ****p*<0.05, ***p*<0.01 (n=3 in each group).

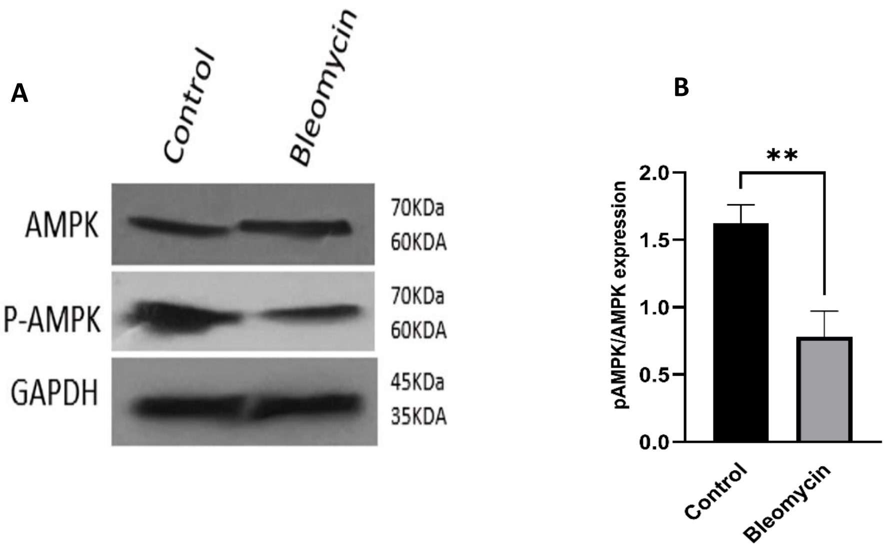

Figure 6. Protein levels of AMPK and p-AMPK in fibrosed areas of the lung. A–B. Bleomycin-affected lung shows a lower phosphorylation level of AMPK. ****p*<0.05, ***p*<0.01 (n=3 in each group).

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DISCUSSION

Pulmonary fibrosis (PF) is a progressive lung disorder characterized by excessive ECM, EMT, and the deterioration of normal lung architecture. A critical component in the pathogenesis of PF is the conversion of epithelial cells into myofibroblasts, a process referred to as EMT. This transformation is essential for the fibrotic response and significantly contributes to the development of PF. Various molecular factors, such as *Twist*, *Snail*, and *TGF-β*, regulate EMT. Furthermore, AMP-activated protein kinase (AMPK) signaling has been identified as a vital pathway in fibrosis, functioning as a metabolic sensor and influencing multiple fibrotic processes.[5,42,43] Bleomycin, which is used in this study to induce pulmonary fibrosis, also causes EMT, which plays a critical role in the pathogenesis of pulmonary fibrosis, as it triggers the transformation of epithelial cells into mesenchymal-like cells. This transition is associated with increased collagen deposition and tissue scarring, which contributes to the fibrotic response in the lungs. Research indicates that bleomycin, a chemotherapeutic agent, induces EMT through signaling pathways involving *TGF-β*, Smad proteins, and other regulatory factors, leading to altered cell-cell junctions and the acquisition of migratory properties in epithelial cells.[44–46] In this study, we examined the interactions among *Twist*, *Snail*, *TGF-β*, EMT, and AMPK signaling, thereby elucidating their collective roles in the progression of PF.

Micro-CT imaging conducted on day 28 demonstrated notable alterations in lung architecture, indicative of acute fibrosis, with the most significant changes observed in the lower region of the left lung. Histological examination corroborated these observations, revealing considerable inflammation characterized by mononuclear cell infiltration in over 70% of alveoli, which aligns with findings from previous studies on bleomycin-induced fibrosis. Furthermore, damage to the bronchiolar mucosa and the presence of fibrin deposits within vascular walls suggested acute injury, consistent with earlier research. Masson trichrome staining revealed substantial collagen deposition, a critical marker of fibrosis, which was more pronounced in the bleomycin (BLM) group compared to control subjects.[46-48]

Twist1, a basic helix-loop-helix transcription factor, is integral to the pathogenesis of PF, particularly through its role in EMT. Increased expression of *Twist1* has been documented in various models of lung fibrosis, including those induced by viral infections and bleomycin, as well as in tissues from patients with IPF.[24,49] Overexpression of *Twist1* in lung epithelial cells is correlated with the expression of mesenchymal markers, such as N-cadherin and fibronectin, which are indicative of EMT.[24] Conversely, the knockdown of *Twist1* was found to maintain the epithelial phenotype, thereby underscoring its essential role in EMT during the progression of fibrosis. Similarly, in models of bleomycin-induced fibrosis, the overexpression of *Twist1* in collagen-producing fibroblasts led to an increase in collagen synthesis and the upregulation of genes associated with fibrotic processes, further emphasizing its involvement in fibrosis.[50] Regarding the relationship between *TGF-β* and *Twist1*, it should be noted that *TGF-β* activates *Twist1* expression through a *Smad3*-dependent mechanism, and once *Twist1* is upregulated by *TGF-β*, it does not just act as a passive transcription factor. Instead, *Twist1* amplifies the *TGF-β* signaling pathway and binds to the promoter regions of collagen genes, thus enhancing collagen production. This feedback loop leads to the activation of fibroblasts and further fibrosis in tissues.[23,26,40] Collectively, these findings highlight the significant role of *Twist1* in the initiation and progression of PF through its regulation of EMT. We have also shown increased expression of *Twist1* in the fibrotic part of the lung in the bleomycin model of lung fibrosis.

Snail is a zinc-finger transcription factor that is integral to the process of EMT, which is associated with the pathogenesis of PF. In the context of PF, *Snail* facilitates the transformation of epithelial cells into mesenchymal-like fibroblasts, thereby contributing to the excessive deposition of extracellular matrix and subsequent tissue scarring. Recent investigations have shed light on the regulatory mechanisms that control *Snail* expression and its functional consequences in PF. In models of bleomycin-induced PF, *Snail* expression is notably upregulated in lung tissues, particularly within epithelial cells undergoing EMT. This finding has been corroborated in regions of the lung affected by fibrosis in our study. *Snail* promotes EMT by repressing the expression of E-cadherin, a crucial epithelial marker, which was confirmed in our study, while simultaneously enhancing the expression of mesenchymal markers, such as α-SMA and vimentin.[51] We have analyzed the α-SMA

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protein level in affected areas and indicated enhanced levels of α-SMA. *TGF-β*, which is also increased in fibrotic regions of the lung, can induce *Snail* expression via the *MAPK* signaling pathway, a non-Smad pathway, in epithelial cells. *Snail* then facilitates fibrosis and EMT.[52] In models of radiation-induced PF, the knockdown of *Snail* restores E-cadherin expression and suppresses mesenchymal markers, indicating that *Snail* serves as a central mediator of EMT in PF. Therefore, targeting *Snail* expression may represent a promising therapeutic approach for PF.[51,53] In summary, *Snail* is a vital transcription factor in the pathogenesis of PF, primarily through its role in facilitating EMT.

The activation of AMPK has been associated with the inhibition of *TGF-β* signaling, indicating that AMPK may act as a protective mechanism against excessive fibrotic responses. In fact, AMPK has the capacity to directly and indirectly influence *TGF-β* signaling pathways. Under normal physiological conditions, *TGF-β* activation results in the phosphorylation of Smad proteins, which promotes the expression of fibrotic genes. However, in the context of AMPK activation, there is a reduction in *Smad3* phosphorylation, leading to a diminished fibrotic response. This observation suggests that AMPK functions as a negative regulator of the *TGF-β*/*Smad* signaling pathway.[54,55] Furthermore, bleomycin-induced fibrosis has been associated with heightened oxidative stress, which in turn activates the *TGF-β* signaling pathway. The activation of AMPK in response to oxidative stress plays a crucial role in preserving cellular energy homeostasis and may diminish the generation of reactive oxygen species (ROS). This reduction in ROS production can indirectly inhibit *TGF-β* activation and the ensuing fibrotic process. Consequently, the interplay between AMPK, *TGF-β*, and bleomycin-induced fibrosis highlights a complex relationship between metabolic regulation and fibrotic signaling that ultimately favors the more powerful stimuli.[56,57] Our findings demonstrate a decrease in AMPK phosphorylation in fibrotic regions of the lung, which aligns with previous research linking reduced AMPK activity to fibrotic progression. However, the relationship between AMPK and EMT-related genes remains controversial. While some studies have reported concurrent upregulation of AMPK and *Twist1*, others have observed an inverse association.[28,58] In the present study, we observed that the expression of *Twist1* and *Snail* was elevated in fibrotic lung areas, whereas AMPK phosphorylation was downregulated. This inverse pattern suggests a possible inhibitory mechanism, where *Snail* transcription factors may suppress AMPK phosphorylation by binding to regulatory gene promoters, thereby limiting AMPK activation.[59] *Snail* and *Twist1* may exert inhibitory effects on AMPK activity through both transcriptional repression and post-translational mechanisms. For instance, *Snail* has been shown to repress *LKB1*, which is a critical upstream kinase responsible for the activation of AMPK, resulting in diminished phosphorylation of AMPK. Furthermore, AMPK phosphorylates *Snail*, thereby modulating its stability, which suggests the existence of a feedback loop that regulates the progression of EMT. Additionally, *Twist1* engages with metabolic pathways that affect AMPK activity, thereby further establishing a connection between EMT factors and the regulation of AMPK. These pathways offer a plausible molecular framework for the observed inverse relationship between the expression levels of *Snail*/*Twist1* and AMPK activation in the context of fibrosis.[59–61] In such a scenario, AMPK's potential promotive effect on *Twist1*, as suggested in some cancer models, would be mitigated. The divergent roles of AMPK and *Twist1* in different pathological contexts could also explain these findings. In cancer, AMPK activation has been shown to promote EMT and metastasis via *Twist1* upregulation. In contrast, in pulmonary fibrosis, AMPK activation appears to suppress *TGF-β*-induced EMT, which includes inhibition of both *Twist1* and *Snail* expression. Under energy stress conditions, AMPK can regulate *Snail* by phosphorylating it and, as a result, decreasing its stability and inhibiting its oncogenic functions.[62] Notably, once *Snail* is activated and downstream EMT processes are initiated, it may further inhibit AMPK activation, reinforcing this regulatory loop. It was shown previously, too, that *Snail* is a factor that can be activated independently of AMPK. Like in the case of tumor metastasis progression via EMT occurrence, which is enhanced through *Snail* upregulation that is AMPK independent.[63] While our results indicate a reduction in AMPK phosphorylation within fibrotic lung tissue, we did not directly assess downstream targets, including *SMAD3* phosphorylation, reactive oxygen species (ROS) production, or autophagy-related pathways, all of which are recognized to be influenced by AMPK. Subsequent research will be necessary to clarify the impact of AMPK activation or inhibition on these interrelated signaling pathways in the context of pulmonary fibrosis.[64] Despite the complexity of the process, EMT continues to represent a more straightforward and actionable therapeutic target in the context of fibrotic diseases. Numerous pharmacological agents have been examined for their potential to activate AMP-activated protein kinase (AMPK) and produce anti-fibrotic effects in the context of pulmonary fibrosis. Metformin, a widely recognized AMPK activator, has been demonstrated to inhibit transforming growth factor-beta (TGF-β)-induced EMT and collagen deposition in both *in vitro* and *in vivo* models of lung fibrosis.[65] Likewise, 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), another AMPK agonist, has shown efficacy in reducing fibrotic responses by mitigating fibroblast activation and ECM production.[66] More recently, salicylate derivatives and direct AMPK activators, such as PF-06409577, have been investigated for their ability to modulate AMPK signaling with enhanced specificity and potency. These agents not only inhibit *TGF-β*/*Smad3* signaling but may also influence oxidative stress and autophagy, thereby addressing multiple pathways implicated in fibrosis.[67] Although these findings are encouraging, additional preclinical and clinical studies are necessary to evaluate the safety, dosage, and long-term efficacy of AMPK-targeted therapies in patients with pulmonary fibrosis. Therapeutic interventions designed to inhibit EMT transcription factors, such as *Snail* and *Twist1*, or their upstream regulators, including *TGF-β*, may effectively mitigate fibrosis by preventing the transformation of epithelial cells into mesenchymal cells that produce ECM. By concentrating on the inhibition of EMT-either directly or through the application of metabolic modulators like AMPK-therapeutic strategies may slow the progression of fibrosis in the lungs and potentially in other organs affected by fibrotic conditions.

Limitations

One of the limitations of this study is the small sample size (n=4 per group), which may reduce statistical power and limit the applicability of the results. However, we observed persistent molecular, histological, and imaging outcomes across replicates, providing support for the validity of our conclusions. Nonetheless, future studies with larger animal numbers are necessary to confirm these findings and to account for potential biological variability in the fibrotic response. Another limitation of this study is the absence of dual-marker immunofluorescence imaging to directly demonstrate co-localization of epithelial and mesenchymal markers (e.g., E-cadherin and α-SMA). Although our molecular and histological data support the occurrence of EMT, future studies using co-staining, confocal microscopy, or spatial transcriptomics will be valuable to confirm EMT transitions at the cellular level within fibrotic lung regions. Another notable limitation of this study is the use of the bleomycin-induced fibrosis model, which primarily represents an acute and partially reversible lung injury, unlike the chronic and progressive nature of human idiopathic pulmonary fibrosis (IPF). Consequently, molecular mechanisms identified here, including *Snail*/*Twist1* regulation of AMPK signaling, may differ in timing or magnitude in human disease. Additionally, species-specific differences and the artificial nature of bleomycin injury may limit direct translation of findings. Future studies employing chronic fibrosis models and human tissue validation are warranted to better understand the clinical relevance.

This study investigates the roles of *Twist*, *Snail*, *TGF-β*, and AMPK signaling in the progression of PF, with particular emphasis on their interactions during EMT. *Twist* and *Snail* are identified as pivotal transcription factors in EMT, facilitating the transformation of epithelial cells into myofibroblasts and thereby advancing the fibrotic process. Furthermore, AMPK, recognized as a crucial metabolic regulator, appears to function as a protective mechanism against fibrosis by inhibiting *TGF-β* signaling pathways. Our findings showed that bleomycin can induce fibrosis and EMT by inducing *TGF*, *Twist1*, and *Snail* expression. In the BLM group, AMPK activation is inhibited, which indicates that induction of *Twist* and *Snail* is AMPK-independent inside fibrotic areas. These results imply that EMT represents a viable and direct therapeutic target for mitigating fibrosis, either through the inhibition of core transcription factors (*Snail*, *Twist1*) or via metabolic modulation of pathways such as AMPK that intersect with EMT regulation.

STATEMENT OF ETHICS

This study was conducted in accordance with the ethical standards of the Tehran University of Medical Sciences Ethics Committee. Ethical approval was obtained under the code IR.TUMS.AEC.1403.047.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

AI ASSISTANCE DISCLOSURE

No generative AI tools were used in the preparation, writing, or editing of this article. All content was developed solely by the authors.

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