Long non-coding RNA *H19* deficiency ameliorates bleomycin-induced pulmonary inflammation and fibrosis

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Research

**Keywords:** Pulmonary Fibrosis, IncRNA H19, Smad, S1pr2

**Posted Date:** October 1st, 2020

**DOI:** https://doi.org/10.21203/rs.3.rs-41983/v4

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**Version of Record:** A version of this preprint was published at Respiratory Research on November 2nd, 2020. See the published version at https://doi.org/10.1186/s12931-020-01534-6.
Abstract

Background: The poor understanding of pathogenesis in idiopathic pulmonary fibrosis (IPF) impaired development of effective therapeutic strategies. The aim of the current study is to investigate the roles of long non-coding RNA H19 (IncRNA H19) in the pulmonary inflammation and fibrosis of IPF.

Methods: Bleomycin was used to induce pulmonary inflammation and fibrosis in mice. The mRNAs and proteins expression in lung tissues was determined by quantitative real-time polymerase chain reaction (qRT-PCR) and western blot. H19 knockout (H19−/−) mice were generated by CRISPR/Cas9.

Results: The expression of H19 mRNA was up-regulated in fibrotic lungs patients with IPF as well as in lungs tissues that obtained from bleomycin-treated mice. H19−/− mice suppressed bleomycin-mediated pulmonary inflammation and inhibited the Il6/Stat3 signaling. H19 deficiency ameliorated bleomycin-induced pulmonary fibrosis and repressed the activation of TGF-β/Smad and S1pr2/Sphk2 in the lungs of bleomycin-treated mice.

Conclusions: Our data suggests that H19 is a profibrotic IncRNA and a potential therapeutic target for pulmonary fibrosis.

Background

Idiopathic Pulmonary Fibrosis (IPF) is a progressive and highly lethal pulmonary fibrotic lung disease with poor treatment and unknown etiology, which rises significantly with age and higher in men [1-4]. Patients with IPF present similar characteristics to the usual interstitial pneumonia (UIP), including extracellular matrix deposition, alveolar architectural disruption, and subpleural honeycombing [5]. The patients with IPF usually have clinical experiences from cough to respiratory insufficiency and have a median survival time of 3 to 5 years after diagnosis [1, 4]. Unfortunately, there are currently no effective therapies capable of stabilizing or improving lung function for patients with IPF.

Long non-coding RNAs (IncRNAs) are defined as non-protein encoding RNA molecules that are more than 200 bp long in length [6]. IncRNAs have been shown to play important roles in different physiological activities, such as gene imprinting, cell proliferation, differentiation, apoptosis, migration, and immune responses [7, 8]. Recent studies have shown that aberrant expression of IncRNAs are associated with a number of human diseases, including cardiovascular, neurodegenerative, lung diseases, tumors and infections [9-15]. The IncRNA H19 is an imprinted and maternally expressed gene that plays a vital role in the controlling the cell proliferation and differentiation [16-18]. The others and our recent studies both indicate that hepatic H19 level is correlated with the severity of cholestatic injury and liver fibrosis in mice [19, 20]. Furthermore, H19 was also related to progression of lung cancer and lung fibrosis [21-24]. Although these studies suggest a causal link among H19 and pulmonary injury, it remains unknown whether and to what extent H19 is involved in the regulation of pulmonary fibrosis in vivo. In present study, we identified H19 as an up-regulated IncRNA in the lungs of pulmonary fibrosis. We further
determined the functional roles and underlying mechanisms of *H19* in pulmonary fibrosis, which suggested *H19* acts as a profibrotic lncRNA in the lungs.

**Materials And Methods**

**Materials**

Hydroxyproline Assay Kit (Cat.No.MAK008-1KT, Sigma-Aldrich, St. Louis, MO), PowerUp SYBR-Green Master Mix kit (Cat. No. A25742) and a High Capacity cDNA Reverse Transcription kit (Cat. No. 4368814) were obtained from Applied Biosystems (Foster City, CA). NuPAGE 10% Bis-Tris gel (Cat.No.NP0301BOX, Invitrogen, Carlsbad, CA). Bleomycin (Cat.No. HY-17565, MedChemExpress, LLC, NJ), The antibodies used in this study were showed in Table S1.

**A mouse model of bleomycin-induced pulmonary fibrosis**

*H19*Δ (H19 ΔExon1-5) mice were generated by CRISPR/Cas9-mediated genome engineering in C57BL/6J mice as our previously described [20]. The animal procedures were approved by the Shanghai Jiao tong University School of Medicine affiliated Xin Hua hospital Animal Care and Use Committee (XHEC-F-2020-008). The mice (about 8 weeks old) were divided into four groups: wild type (Wt) sham (n=8 -12), *H19*Δ sham (n=6 -10), wild type (Wt) treated with bleomycin (BLM) (n=8 -12) and *H19*Δ treated with bleomycin (BLM) (n=8 -12). For bleomycin administration, mice were anaesthetized with 2% isoflurane, and then instilled intratracheally with bleomycin (3.5 mg/kg body weight) in 100 ml PBS, as previously described [25, 26]. After 4 weeks, the lung tissues were collected for RNA, protein, collagen content analyses and histological analysis. The degree of fibrosis was quantitated using an Ashcroft score in a blinded manner according to the described method [27].

**Fluorescence in situ hybridization (FISH)**

H19 FISH in mouse lung tissue was performed using a commercially available RNAscope Multiplex Fluorescent Reagent Kit v2 (Advanced Cell Diagnostics, Newark, CA) and RNAscope® Probe-Mm-H19 (#423751, Advanced Cell Diagnostics, Newark, CA) according to the manufacturer’s instruction. Fluorescent staining targeting Sftpc protein was performed followed H19 FISH.

**Quantitative real-time polymerase chain reaction (qRT-PCR)**

Total RNA was extracted from left lung of mice using the RNeasy kit (Qiagen, Hilden, Germany) according to the protocol of the manufacture and 2 mg of total RNA was used to synthesize the 1st cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). The real-time PCR reactions were performed on the ViiA 7 Real-Time PCR System (Applied Biosystems, Foster City, CA) using PowerUp SYBR-Green Master Mix kit (Applied Biosystems, Foster City, CA). All samples were assayed in triplicate, and data were normalized to endogenous control *Hprt1*. Relative RNA expression levels were calculated using the ΔΔCt method. The primers are listed in Table S2.
**Western blotting**

A total 30 mg left lung tissues was homogenized in 300 μl RIPA buffer (Invitrogen, Carlsbad, CA) supplemented with a protease inhibitor cocktail (Servicebio, Wuhan, China). After determining the protein concentration, the equal amounts of protein were separated on NuPAGE 10% Bis-Tris gels (Invitrogen, Carlsbad, CA) and transferred onto polyvinylidene difluoride (PVDF) membranes. After blocking in 5% nonfat milk at room temperature for 60 min, membranes were incubated with the primary antibodies overnight at 4°C. The membranes were washed three times for 30 min with TBST (containing 0.1% Tween-20), and then incubated with secondary antibodies. After final washes with TBST, the signals were detected using ECL chemiluminescence reagent Kit (Pierce, Rockford, IL, USA). The primary antibodies performed in this study as showed in Table S1.

**Histology and immunofluorescence (IF)**

The right lung tissues were immediately fixed in 10% neutral buffered formalin for 24 h and go through dehydration, clearing and paraffin embedding. Sections were mounted on positively charged slides after cutting at 4 mm thick, baked at 65 °C for 1 h and then stored at room temperature (RT) for later use. Fibrosis was performed using mason's trichrome (Genmed Scientifics, Wilmington, DE, USA) and Sirius red stain (Servicebio, Wuhan, China) following the protocols of manufactures. For immunofluorescence (IF) assay, the slides were incubated with xylol and descending concentrations of ethanol. Endogenous peroxidases were blocked by using 0.3% H₂O₂ for 10 min at RT. After antigen retrieval, blocking was performed using 5% bovine serum albumin for 30 min at RT. The antibodies used here were listed on the Table S1.

**Hydroxyproline assay**

The amount of collagen in the lung tissues was determined by a Hydroxyproline Assay Kit according to the manufacturer's protocol (Cat.No.MAK008-1KT, Sigma-Aldrich, St. Louis, MO). Briefly, about 10 mg lung tissues were homogenized in 100 μl water with 100 μl concentrated hydrochloric acid (HCl, 12 M) and hydrolyzed at 120°C for 3 hours. Transfer 20 μl of supernatant at 60°C until completely desiccated. Chloramine T/Oxidation Buffer Mixture was added at room temperature for 5 minutes, followed by the addition of Diluted DMAB Reagent and incubation at 60°C for 90 minutes. Measure the absorbance of samples and standards at 560 nm, and hydroxyproline content was expressed as μg per mg lung tissue.

**Statistical analysis**

All data were expressed as mean ± SD (standard deviation). For comparisons of different groups, statistical significance was determined by Student's t-test or ANOVA analysis. P-value less than 0.05 was considered statistically significant.

**Results**
**H19 is up-regulated in fibrotic lungs**

Analysis of publicly available datasets showed that IncRNA \( H19 \) expression was more highly expressed in lung tissue from patients with IPF compared to normal lung tissue, but there was no significant difference between IPF patients and other interstitial lung diseases (ILD) (Figure 1A) [28, 29]. Fibrotic genes, including \( ACTA2 \), \( COL1A2 \) and \( MMP7 \), increasingly expressed in lung tissue from patients with IPF compared to lung tissue from patients with other interstitial lung diseases and to normal lung tissue (Figure 1A) [28, 29]. In addition to, \( H19 \) expression increased upon bleomycin-induced lung fibrosis in rats. \( H19 \) expression peaked after about 2 weeks of bleomycin-treatment, and then \( H19 \) levels returned to an amount comparable to controls (Figure 1B) [30]. In current study, Fluorescent in situ hybridization (FISH) assay showed that \( H19 \) expression increased in lungs of bleomycin-treated mice (2 weeks) and located at alveolar epithelium and capillaries (Figure 1C). Furthermore, immunofluorescent staining of surfactant protein C (Sftpc), a marker for type 2 epithelial cells (AEC2s), indicated that \( H19 \) was also expressed and up-regulated in AEC2s following bleomycin-treatment (Figure 1C). The qRT-PCR assay confirmed that \( H19 \) increasingly expressed in the lungs of mice with bleomycin (BLM) treatment (Figure 1D and 1E).

**\( H19 \) deficiency represses bleomycin-induced lung inflammatory response**

\( H19 \) knock out (\( H19^{-/-} \)) mice here were used to elucidate the roles of \( H19 \) in bleomycin -induced pulmonary inflammation and fibrosis. After 4-week BLM-treatment, immunofluorescence (IF) staining showed the \( H19^{-/-} \) BLM mice had less CD45\(^+\) cells accumulated in the lungs than that in lungs of \( Wt \) BLM mice (Figure 2A). RT-PCR analysis showed that CD11b and Ccr2 genes expression was reduced significantly in lungs of \( H19^{-/-} \) mice when compared to that of \( Wt \) mice (Figure 2B). The inflammatory markers, including \( F4/80 \), \( CD11b \), \( Ccl20 \), \( Il1b \) and \( Ccr2 \) increased in \( Wt \) BLM mice compared to \( WT \) sham mice, but decreased in \( H19^{-/-} \) BLM mice (Figure 2B). Western blot results indicated that protein expression levels of \( Il6 \) and \( p-Stat3 \) were decreased in lungs of \( H19^{-/-} \) BLM mice relative to the \( Wt \) BLM (Figure 2C and 2D).

**\( H19 \) knockout ameliorates bleomycin-induced pulmonary fibrosis**

As shown in Figure 3, histopathological analysis firstly showed reduced fibrosis in the \( H19^{-/-} \) BLM mice (Figure 3A and 3B). The haematoxylin-eosin (H&E) staining and Collagen I immunofluorescence (IF) staining showed the fibrosis increased in \( Wt \) BLM mice compared to the \( Wt \) sham, but not in lungs of \( H19^{-/-} \) BLM mice (Figure 3A). The Masson's Trichrome staining and Sirius red staining further indicated that 4-week BLM significantly induced lung fibrosis in \( WT \) mice, but had much less impact in \( H19^{-/-} \) mice (Figure 3A). Consistently, quantitation of lung fibrosis in a blinded manner revealed the Ashcroft score decreased significantly in \( H19^{-/-} \) BLM mice when compared to \( Wt \) BLM mice (Figure 3B). Furthermore, the pulmonary hydroxyproline levels were significantly increased in \( Wt \) BLM mice, but not in \( H19^{-/-} \) BLM mice (Figure 3C). At molecular level, \( H19^{-/-} \) mice had decreased expression of \( Tgfb1 \) and \( Acta2 \) mRNA in the lungs in relation to \( Wt \) animals (Figure 3D). The mRNA levels of \( Tgfb1 \), \( Acta2 \) and \( Col1a1 \) (Figure 3D) and
protein expression of Col1a1 (Figure 3E and 3F) reduced in $H19^{-/-}$ BLM mice compared to the $Wt$ BLM mice.

**H19 depletion attenuated the pathways of TGF-β/Smad and S1pr1/Sphk2 in fibrotic lungs**

TGF-β/Smad signaling is the key regulating pathway in fibrogenesis [31]. In this study, we showed that the TGF-β mRNA level and protein levels of p-Smad2 and p-Smad3 were increased in the $Wt$ BLM mice, compared with $Wt$ mice, while these proteins reduced in $H19^{-/-}$ BLM mice (Figure 4A and 4B). Our previous study had reported that S1pr2 and SphK2 played an important role in promoting liver fibrosis [20]. As shown in Figure 4, Western blot results indicated that protein expression levels of S1pr2 and SphK2 were increased in lungs of $Wt$ BLM mice compared to $Wt$ sham mice, but decreased in lungs of $H19^{-/-}$ BLM (Figure 4C and 4D).

**H19 deficiency decreased AEC2s proliferation in lungs of bleomycin-treated mice**

In the lung sections from the $H19^{-/-}$ BLM mice, immunofluorescence results showed that expression of Sftpc was decreased relative to that of the $Wt$ BLM mice (Figure 5A). Additionally, the number of Ki67-positive cells was also reduced in the $H19^{-/-}$ BLM mice compared to the $Wt$ BLM mice (Figure 5A). Consistently, western blot results indicated that protein expression levels of Sftpc reduced in the $H19^{-/-}$ BLM mice compared to the $Wt$ BLM mice (Figure 5B and 5C). In addition to, the proteins of p-Egfr decreased in lungs of $H19^{-/-}$ BLM mice compared to the $Wt$ BLM mice (Figure 5B and 5C).

**Discussion**

Presently, the poor understanding in the pathogenesis of IPF has resulted in a lack of effective therapies. In current study, we found that $H19$ was up-regulated in the fibrotic lungs of IPF patients and bleomycin-treated mice. Functionally, $H19$ deficiency reduced pulmonary inflammation and fibrosis may via inhibiting Il6/Stat3, TGF-β/Smad or S1pr2/Sphk2. Moreover, $H19$ deficiency reduced the proliferation of alveolar type II cells in vivo.

$H19$ is an imprinted and maternally expressed transcript, which is one of the few well-characterized IncRNA [32, 33]. Aberrant expression of $H19$ has been related to a variety of human diseases [34-38]. The current study showed that IPF patients had higher levels of $H19$ mRNA in lungs when compared to the control subjects using a public datasets, which indicates that $H19$ may play an important role in the pathogenesis of IPF. Similarly, $H19$ mRNA also increased in a mice and rat models of bleomycin-induced pulmonary fibrosis. To elucidate the roles of $H19$ in the pathogenesis of IPF, we firstly generated a $H19$ deficiency mouse ($H19^{-/-}$). In vivo studies showed that bleomycin-induced pulmonary inflammation was attenuated in $H19^{-/-}$ mice, demonstrating $H19$ could promote the inflammatory response after the pulmonary injuries. In this response, CD11b and Ccr2 were reduced in the lung tissues of $H19^{-/-}$ mice. Li and other colleague recently reported that $H19$ significantly induced the expression and secretion of chemokine (C-C motif) ligand 2 (CCL-2) that could accumulate the monocytes from circulation into livers.
It thus suggests that \textit{H19} may promote pulmonary inflammation via attracting the CD11b monocytes into the lung after injuries. STAT3 has been reported to play a critical role in the pulmonary inflammation. In the injured lung, STAT3 rapidly activated and increased the production of the proinflammatory molecules \textit{IL1\beta}, \textit{IL6}, \textit{TNF-a}, \textit{iNOS} and \textit{CCL2} [40-43]. We here showed bleomycin-induced the expression of \textit{Il6} and activated \textit{Stat3} in the lungs, and these effects were attenuated by \textit{H19} depletion. It is indicated \textit{H19} deficiency inhibited bleomycin-induced pulmonary inflammation may through attenuating the \textit{Il6}/\textit{Stat3} signaling.

\textit{In vivo} studies, we further revealed \textit{H19} deficiency significantly blocked bleomycin-induced pulmonary fibrosis and reduced the cell proliferation. TGF-\beta/smad signaling is one of the key pathways responsible for pulmonary fibrosis [31, 44-46]. We here showed that bleomycin induced \textit{Tgfb1} mRNA expression and activated the Smad2 and Smad3, but not in the \textit{H19}\textsuperscript{-/-} mice. \textit{In vitro}, a recent study reported that \textit{H19} can target miR-140 and regulate the TGF-\beta/Smad3 pathway [24]. Zhu et. al., reported that \textit{H19} could enhance TGF-\beta signaling in both hepatic stellate cells and hepatocytes and facilitate liver fibrosis [47]. \textit{H19} was also showed to accelerate TGF-\beta1-induced tenogenic differentiation \textit{in vitro} and promoted tendon healing in a mouse tendon defect model [48]. It thus suggests that \textit{H19} enhances the pulmonary fibrosis maybe partly via activation of TGF-\beta/smad signaling. Sphingosine-1-phosphate and its receptor S1pr2 have been shown to promote lung fibrosis [49-53]. Our previous study showed that \textit{H19} could activate the S1pr2/SphK2 signalling pathway in the cholestatic livers. The current study indicated that S1pr2/SphK2 signalling was activated in the bleomycin-treated lungs, but these effects were attenuated by \textit{H19} depletion. We suggested that \textit{H19} increased lung fibrosis during the bleomycin-treatment partly via downregulation of S1pr2/SphK2 signalling. Epidermal growth factor receptor (EGFR) activating is a major driver of lung adenocarcinoma, which is essential to lung cancer cell proliferation [54]. The present study suggested \textit{H19} deficiency represses EGFR activating, which indicating \textit{H19} enhances EGFR signaling and lead to promote the cell proliferation in the injured lung.

**Conclusion**

In summary, we demonstrated that the \textit{H19} is a profibrotic lncRNA in the lung and that regulating TGF\beta/Smad and S1pr2/Sphk2, two novel mechanisms, underlying \textit{H19} activity in pulmonary fibrosis.

**Abbreviations**

IPF, idiopathic pulmonary fibrosis; lncRNA, long non-coding RNA \textit{H19}; UIP, usual interstitial pneumonia; RT-PCR, real-time polymerase chain reaction; IF, immunofluorescence; H&E, hematoxylin-eosin; EGFR, Epidermal growth factor receptor; TGF-\beta, receptor; transforming growth factor-\beta; S1PR2, sphingosine-1-phosphate receptor 2; SphK2, sphingosine kinase 2; ACTA2, alpha 2 smooth muscle actin; COL1A2, collagen type I alpha 2; MMP7, Matrix metalloproteinase 7

**Declarations**
Acknowledgments

The authors extend their gratitude to Shanshan Chen and Yang Liu for their technical support and assistance in performing the experiments.

Author's contributions

X. Wan and Y. Xiao developed study concept and design, acquisition of data, analysis and interpretation of data. X. Wan, X. Tian, J. Du and Y. Lu performed and analyzed most of the experiments. X. Wan and Y. Xiao wrote the manuscript. All of the authors approved this version of the manuscript to be published.

Funding

This study was supported by the National Natural Science Foundation of China (81770517)

Availability of data and materials

Original data can be requested from corresponding author.

Ethics approval and consent to participate

The animal experiments were approved by the Shanghai Jiao tong University School of Medicine affiliated Xin Hua hospital Animal Care and Use Committee(XHEC-F-2020-008).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing financial interest.

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**Figures**
Figure 1

H19 increased in lungs of pulmonary fibrosis. (A) Data for H19 mRNA expression extracted from the GEO database, comparing whole extracts of lung tissue from human patients diagnosed with IPF (n = 50) and other interstitial lung diseases (ILD) (n = 35) and from control subjects (n = 55) (GSE47460). (B) Data for H19 mRNA expression extracted from the GEO microarray database, comparing whole extracts of lung tissue from bleomycin-induced lung fibrosis in rat (n = 5 animals per bleomycin-treated group and n = 22
(C) Representative images of FISH targeting H19 (red) and immunofluorescence (IF) staining of Sftpc in lungs of Sham and bleomycin-treated mice. (D) The relative H19 mRNA levels in the lungs of Sham wild type (Wt, n=8), Sham H19 knockout (H19-/-, n=6) Wt bleomycin (BLM, n=8) and H19-/- BLM (n=10) mice were determined by real-time RT-PCR. The Hprt1 was used as an internal control. (E) Representative images of the DNA agarose gels of H19 and Hprt1.

Figure 2
H19 knockout inhibited bleomycin-induced pulmonary inflammation in mice. (A) Representative immunofluorescence (IF) images of CD45 positive cells in lungs of Sham Wt (n=10), Sham H19-/- (n=7), Wt BLM (n=9) and H19-/- BLM (n=9) mice. (B) The relative mRNA levels of the inflammatory marker genes including F4/80, CD11b, Ccl20, IL1b and Ym1, in the lungs of Sham Wt (n=10), Sham H19-/- (n=7), Wt BLM (n=9) and H19-/- BLM (n=9) mice. The Hprt1 was used as an internal control. (C) Representative images of the immune blots of Il6, p-Stat3, Stat3 and β-Actin. (D) Relative protein expression levels of Il6 and p-Stat3 were normalized using β-actin or Stat3. Statistical significance: *p<0.05; **p<0.01; ns, not significant.
H19 deficiency ameliorated bleomycin-induced pulmonary fibrosis in mice. (A) Representative images of hematoxylin-eosin (H&E) staining, Masson's trichrome staining, Sirius red staining and collagen I immunofluorescence staining of Sham Wt, Sham H19^{-/-}, Wt BLM and H19^{-/-} BLM mice. (B) Ashcroft score for Sham Wt (n=10), Sham H19^{-/-} (n=7), Wt BLM (n=9) and H19^{-/-} BLM (n=9) mice. (C) Quantifying collagen content with hydroxyproline assay in the lungs of Sham Wt, Sham H19^{-/-}, Wt BLM and H19^{-/-} BLM mice.
BLM mice. Each group, n=6-8 (D) Real-time PCR analysis for Tgfb1, Col1a1 and Acta2 in the lungs of Sham Wt, Sham H19-/-, Wt BLM and H19-/- BLM mice. Each group, n=8-10 (E) Western blot analysis for collagen type I the lungs of Sham Wt, Sham H19-/-, Wt BLM and H19-/- BLM mice. (F) Quantification of the panel (E).
H19 depletion altered the TGF-β/Smads and S1pr2/Sphk2 pathways in lungs of bleomycin-induced mice. (A) Western blot analysis for Smad1/5, Smad2, Smad3, Samd4 and their phosphorylated forms the lungs of Sham Wt, Sham H19-/-, Wt BLM and H19-/- BLM mice. (B) Quantification of the panel (A). (C) Western blot analysis for S1pr2 and Sphk2 in the lungs of Sham Wt, Sham H19-/-, Wt BLM and H19-/- BLM mice. (D) Quantification of the panel (C).

Figure 5
H19 depletion reduced cell proliferation in lungs of bleomycin-induced mice. (A) Representative images of co-staining for Sftpc and Ki67 in the lungs of Sham Wt, Sham H19-/-, Wt BLM and H19-/- BLM mice. (B) Western blot analysis for Sftpc, p-Egfr, Egfr, Lin28 and β-actin in the lungs of Sham Wt, Sham H19-/-, Wt BLM and H19-/- BLM mice.

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