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ALKBH5 Modulates Asthma Progression by Downregulating N6-methyladenosine Methylation

Xiang Fan[1], Chen Wei[2], and Yongguang Han[1]

[1] Henan University of Chinese Medicine, Zhengzhou, China
[2] Department of Pediatrics, The Third Affiliated Hospital of Zhengzhou University, Zhengzhou, China

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ABSTRACT

Asthma is a chronic respiratory disease that is characterized by airway inflammation, excessive mucus production, and airway remodeling. Prevention and treatment for asthma is an urgent issue in clinical studies. In recent years, N6-methyladenosine methylation (m6A) has emerged as a promising regulatory approach involved in multiple diseases. ALKBH5 (alkB homolog 5) is a demethylase widely studied in disease pathologies. This work aimed to explore the regulatory mechanisms underlying the ALKBH5-regulated asthma.

We established an interleukin-13 (IL-13)-stimulated cell model to mimic the in vitro inflammatory environment of asthma. ALKBH5 knockdown in bronchial epithelial cells was performed using siRNAs, and the knockdown efficacy was analyzed by quantitative PCR (qPCR). Cell viability and proliferation were measured by cell counting kit 8 (CCK-8) and colony formation assay. The ferroptosis was assessed by measuring the total iron, Fe2+, lipid reactive oxygen species (ROS), malondialdehyde (MDA), and superoxide dismutase (SOD) levels. The enrichment of N6-methyladenosine methylation (m6A) modification was detected by the MeRIP assay.

Knockdown of ALKBH5 significantly elevated the survival and colony formation ability of bronchial epithelial cells in the IL-13 induction model. The levels of total iron, Fe2+, lipid ROS, and MDA were remarkedly elevated, and the SOD level was reduced in IL-13-induced bronchial epithelial cells, and depletion of ALKBH5 reversed these effects. Knockdown of ALKBH5 elevated the enrichment of m6A modification and expression of glutathione peroxidase 4 (GPX4). Knockdown of GPX4 abolished the pro-proliferation and anti-ferroptosis effects of siALKBH5.

Knockdown of ALKBH5 improved the proliferation of bronchial epithelial cells and alleviated cell ferroptosis.

Keywords: Asthma; ALKBH5; Ferroptosis; N6-methyladenosine methylation

INTRODUCTION

Asthma is a chronic respiratory disease that affects over 300 million people worldwide, and its global incidence is on the rise and has become a significant economic burden.[1,2] Asthma is a complex condition influenced by both genetic factors and environmental exposures, which are considered the primary causative elements.[1] It is characterized by airway inflammation, excessive mucus production, and airway remodeling.[3] Even though great advancements have been made in

Corresponding Author: Yongguang Han, PhD;
Henan University of Chinese Medicine, Zhengzhou, China.
Tel: (+86 181) 3782 0150, E-mail: hyg1207@hactcm.edu.cn

basic and medical research, asthma still pose challenges for individuals of all ages, which results in great morbidity. Conventional medications show effective for patients with mild and moderate asthma, but less effective for those with severe disease.[4,5] Identification of new and effective regulatory mechanisms could provide insights into more precise treatments.

Ferroptosis is a form of cell death that is dependent on the presence of iron.[6] It primarily occurs due to the excessive accumulation of iron-related reactive oxygen species (ROS) within the cell, coupled with a reduced capacity of glutathione peroxidase 4 (GPX-4) to eliminate ROS effectively.[7] These factors lead to the production of lipid peroxides and an imbalance between their generation and degradation.[8] As a result, the cell becomes unable to efficiently eliminate the excessive lipid peroxides, ultimately resulting in cell death.[9] Research findings suggest that individuals with asthma demonstrate increased levels of airway inflammation, lipid peroxidation, and ROS. Moreover, they exhibit markers associated with ferroptosis, indicating that ferroptosis may play a role in the pathogenesis of allergic asthma.[10] Studies indicated that ferroptosis inhibitor Liproxstatin-1 markedly attenuated lipid ROS accumulation and ameliorated cell viability, as well as reduced the expression of inflammatory factors IL-33, TSLP, IL-8, and IL-6.[11] A traditional Chinese medicine component Quercetin is reported to ameliorate ferroptosis-associated neutrophilic airway inflammation accompanied by inhibiting M1 macrophage polarization.[12]

RNA modification is a form of epigenetic regulation that has been identified to exist widely at the transcriptome level.[13] Among the RNA modifications, N6-methyladenosine (m6A) is demonstrated as the most frequent modification type of the eukaryotic mRNA and is involved in various biological processes.[14] The m6A modification is a reversible process regulated by m6A "writers", "erasers" and "readers". ALKBH5 (alkB homolog 5) is a demethylase that is reported to be a critical regulator for cancers.[15] In this work, we aimed to investigate the potential effects and regulatory role of ALKBH5 during asthma progression and determine the involvement of ferroptosis-related molecular mechanisms.

MATERIALS AND METHODS

Cell Model

Bronchial epithelial cell line BEAS-2B and HBE cells were purchased from Procell (Wuhan, China). All cells were cultured in 1640 medium (Thermo, USA) which was supplemen with 10% fetal bovine serum (FBS; Thermo) and incubated in a humidified incubator 37°C with5% CO_2. To establish the in vitro model, the cells were incubated in a culture medium containing 10 ng/ml IL-13 (Sigma, MA, USA) for 24 h.

Cell Counting Kit 8 (CCK-8) Assay

Cell viability was detected using CCK-8 assay. In brief, cells were seeded into a 96-well plate with 5,000 cells in each well. After 24 hours incubation, the CCK-8 reagent was added into each well and incubated for an additional 2 hours. The optical density at 450 nm was measured using a microplate reader (Thermo, USA).

Colony Formation

BEAS-2B and HBE cells were suspended in culture medium as single cells. Cells were then plated into 6-well plate at a density of 1,000 cells per well. Cells were incubated at 37°C for two weeks. After that, colonies were stained with crystal violet (Beyotime, China). The images were captured with a digital camera.

Detection of Ferroptosis

The level of lipid reactive oxygen species (ROS) was measured by C11-BODIPY probe as per manufacturer's protocol. The intracellular total iron and Fe2+ level was detected using an Iron assay kit (Abcam, USA). The malondialdehyde (MDA) and SOD activity was detected with the MDA assay kit (Sigma, USA) and SOD activity detection assay kit (Beyotime, China), respectively.

Quantitative PCR (qPCR)

The cells were homogenized with TRIzol reagent (Invitrogen, USA) to collect total RNA. A total of 1 µg RNA was reverse transcribed to cDNA using the First strand cDNA synthesis kit (Takara, Japan) as per manufacturer's protocol. Relative RNA levels of GPX4 and ALKBH5 were assessed using a SYBR green system (Takara, Japan). RNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western Blotting Assay

Total proteins were obtained using the radioimmunoprecipitation assay (RIPA) buffer (SolarBio, China). A total of 30 µg proteins were separated with SDS-PAGE gels and were blotted onto the PVDF membrane (Millipore, MA, USA). After blocking in 5% skimmed milk, the membranes were

incubated with primary anti-ALKBH5 and anti-GPX4 antibodies (Abcam, USA) at 4°C overnight. The next day, the protein bands were reacted with a secondary anti-mouse or anti-rabbit antibody at room temperature for 1 h. Protein bands were then hatched with the enhanced chemiluminescence (ECL; Millipore, USA) and visualized with an imaging system.

MeRIP Assay

The enrichment of m6A modification on GPX4 RNA was evaluated by MeRIP assay. In brief, cells were lysed to collect total RNA. The enrichment of m6A was assessed by the MeRIP assay kit (RiboBio, China) according to the manufacturer's instruction. The enriched RNA level of GPX4 was then detected by qPCR.

Statistics

Data in this study were shown as mean ± standard deviation (SD) of three independent experiments. The statistical difference between two or more groups was analyzed with Student's t-test or one-way analysis of variance (ANOVA), respectively. All statistical analyses were performed using GraphPad Prism 7.0 software (GraphPad Software, San Diego, CA, USA). Differences with a $p<0.05$ were considered significant.

RESULTS

Knockdown of ALKBH5 Facilitates the Proliferation of Bronchial Epithelial Cells

We analyzed the effects of ALKBH5 on bronchial epithelial cells during asthma by using an IL-13-induced cell model and knockdown of ALKBH5. The transfection with siALKBH5 could effectively downregulate the RNA level of ALKBH5 in BEAS-2B and HBE cells (Figure 1A), and siALKBH5-1 was selected for subsequent experiments. The results from CCK-8 revealed that siALKBH5 could recover the growth of BEAS-2B and HBE cells that were reduced by IL-13 induction (Figure 1B). Besides, IL-13 induction reduced the colony number formed by BEAS-2B and HBE cells, whereas the knockdown of ALKBH5 reversed this effect (Figure 1C).

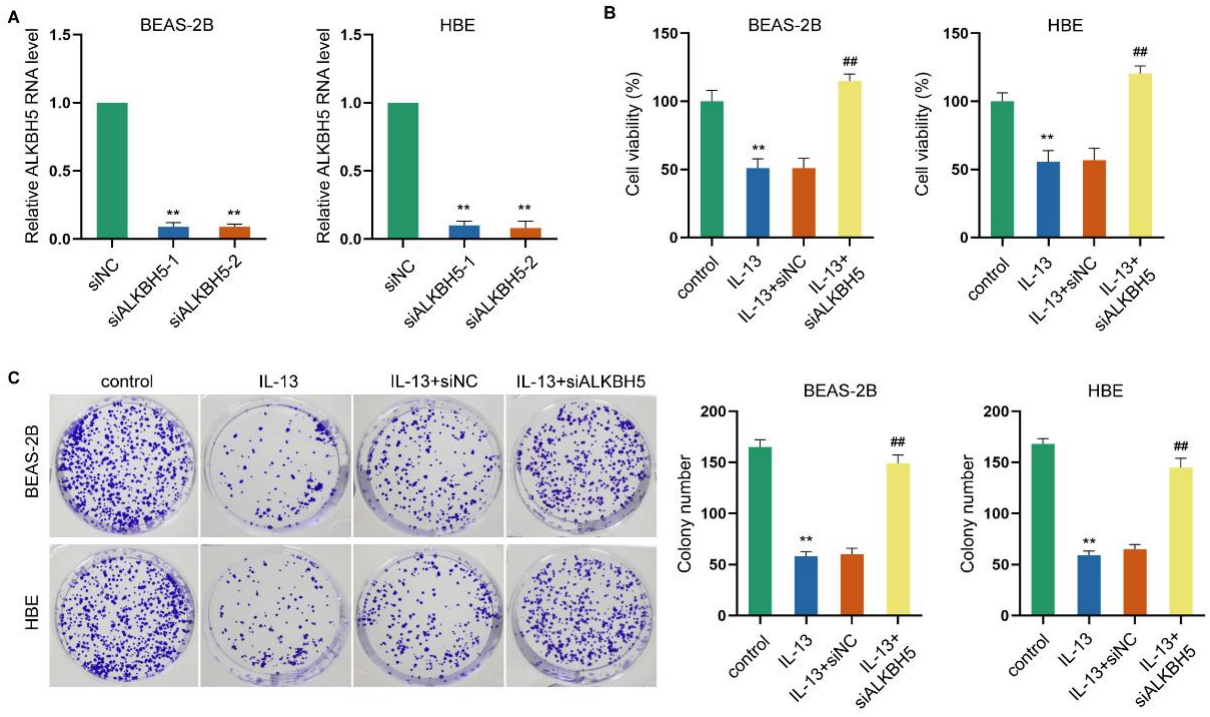

Figure 1. Knockdown of ALKBH5 facilitates the proliferation of bronchial epithelial cells. (A) BEAS-2B and HBE cells were depleted of ALKBH5, and RNA level of ALKBH5 was detected by quantitative PCR (qPCR) assay. (B) Cell viability was detected by cell counting kit 8 (CCK-8) assay. (C) Colony formation ability of cells. **$p<0.01$ vs control; ##$p<0.01$ vs IL-13 group.

Knockdown of ALKBH5 Alleviates Ferroptosis of Bronchial Epithelial Cells

Ferroptosis is characterized by the accumulation of Fe2+, total iron, lipid ROS, and MDA, and a reduced level of SOD. Here, we observed that IL-13 induction induced significant elevation of Fe2+, total iron, lipid ROS, and MDA, and reduction of SOD, whereas knockdown of ALKBH5 alleviated IL-13-induced ferroptosis in BEAS-2B (Figure 2A) and HBE cells (Figure 2B).

ALKBH5 Epigenetically Regulates GPX4 Expression

We next analyzed the epigenetic modification of GPX4 mRNA by using MeRIP assay. As shown in Figure 3A, the m6A modification level of GPX4 was notably elevated under depletion of ALKBH5 (Fig. 3A). Moreover, knockdown of ALKBH5 caused elevation of GPX4 mRNA and protein level (Figure 3B and C).

ALKBH5 Modulates Bronchial Epithelial Cells Proliferation and Ferroptosis via Targeting GPX4

To determine the role of GPX4 in ALKBH5-regulated bronchial epithelial cell proliferation and ferroptosis, we performed GPX4 knockdown in ALKBH5-depleted cells. Under IL-13 induction, the bronchial epithelial cells exhibited notable proliferation suppression and ferroptosis, which was significantly recovered by ALKBH5 depletion. However, the knockdown of GPX4 suppressed cell viability (Figure 4A) and elevated accumulation of Fe2+, total iron, and lipid ROS (Figures 4B and C) under the transfection of siALKBH5.

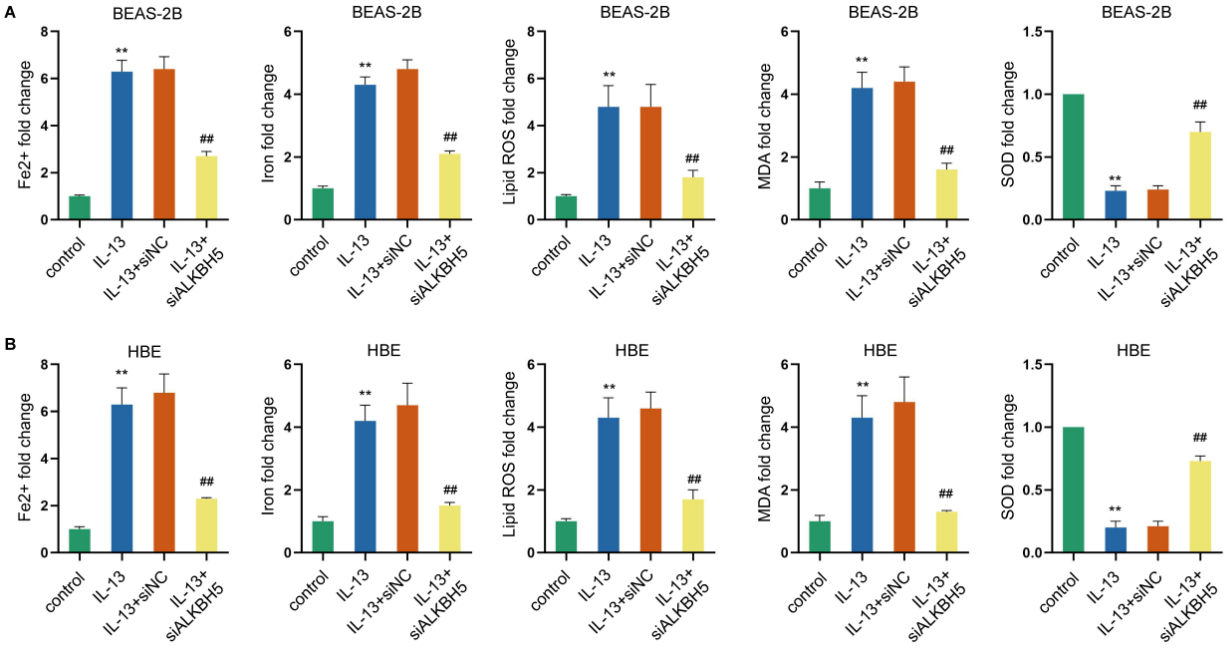

Figure 2. Knockdown of ALKBH5 alleviates ferroptosis of bronchial epithelial cells. BEAS-2B and HBE cells were stimulated with IL-13 and depleted of ALKBH5. (A) The levels of Fe2+, total iron, lipid reactive oxygen species (ROS), malondialdehyde (MDA), and superoxide dismutase (SOD) in (A) BEAS-2B and (B) HBE cells were measured. **p<0.01 vs control; ##p<0.01 vs IL-13 group.

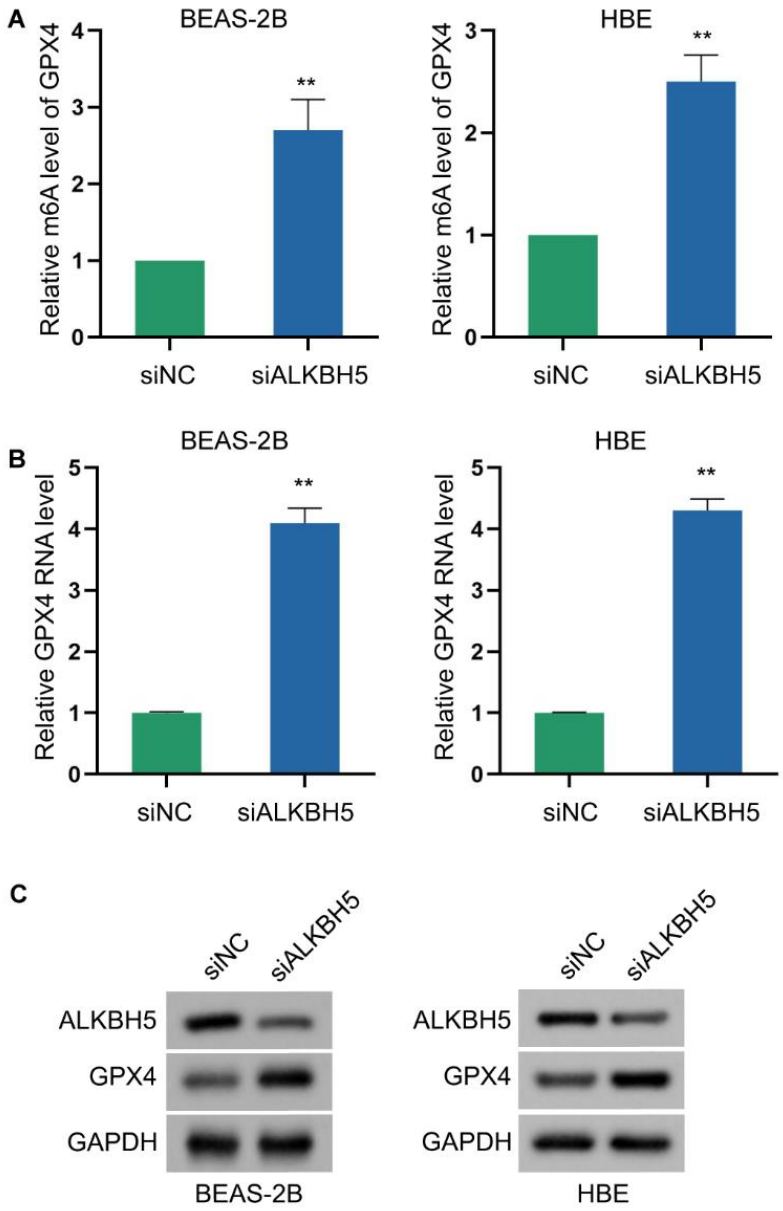

Figure 3. ALKBH5 epigenetically regulates glutathione peroxidase 4 (GPX4) expression. BEAS-2B and HBE cells were depleted of ALKBH5. (A) The m6A enrichment onGPX4 mRNA was measured by MeRIP assay. (B and C) The RNA and protein level of GPX4 was measured by quantitative PCR (qPCR) and western blotting assay. **p<0.01 vs siNC group.

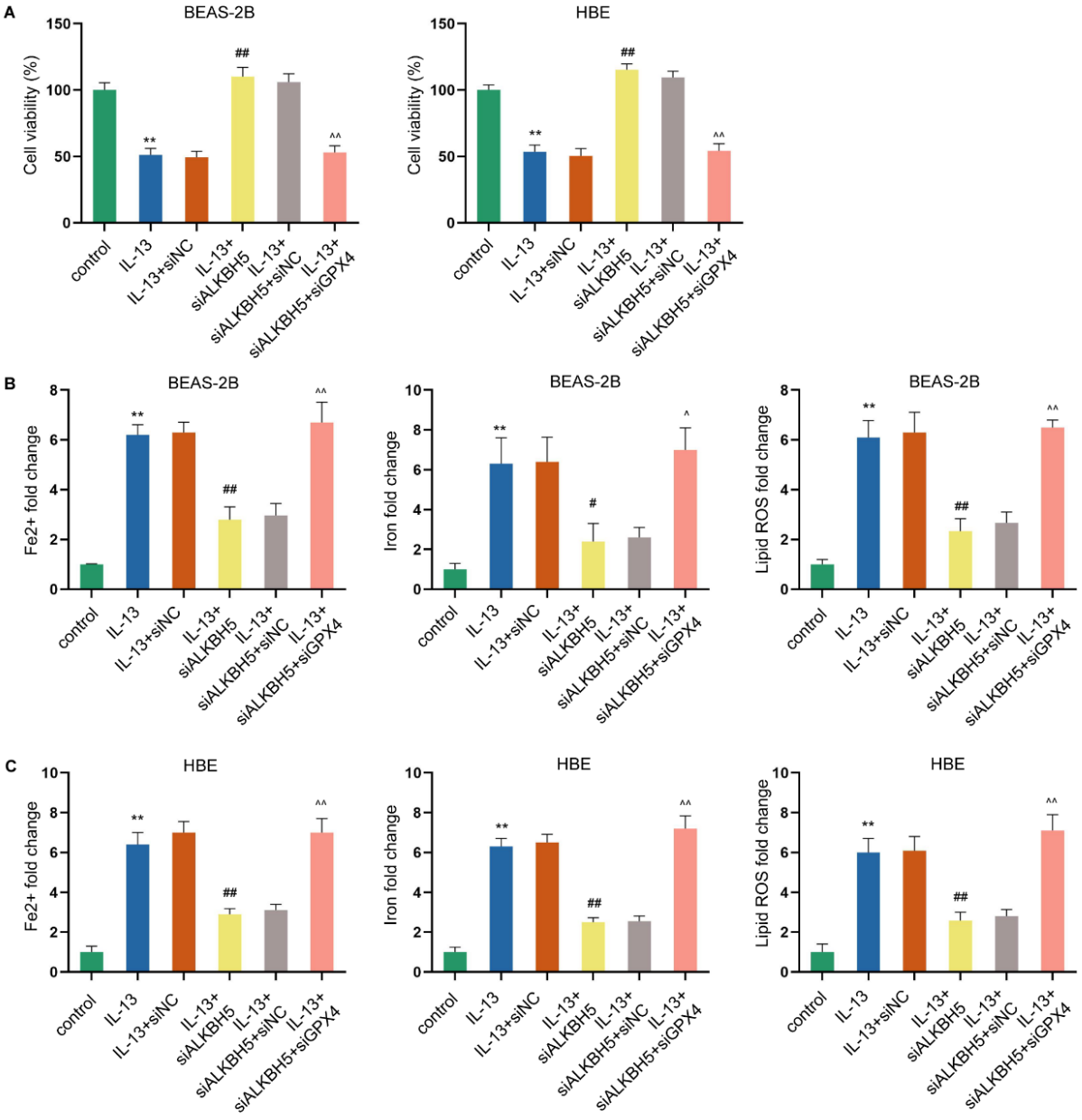

Figure 4. ALKBH5 modulates bronchial epithelial cells proliferation and ferroptosis via targeting glutathione peroxidase 4 (GPX4). BEAS-2B and HBE cells were stimulated with IL-13 and depleted of ALKBH5 and GPX4. (A) Cell viability was detected by superoxide dismutase (SOD) assay. The levels of Fe2+, total iron, lipid reactive oxygen species (ROS), malondialdehyde (MDA), and SOD in (C) BEAS-2B and (D) HBE cells. **p<0.01 vs control; #p<0.05, ##p<0.01 vs IL-13 group; ^p<0.05, ^^p<0.01 vs IL-13+siALKBH5 group.

DISCUSSION

In this study, we explored the potential role of ALKBH5 in asthma and identified that knockdown of ALKBH5 could improve the viability and proliferation of bronchial epithelial cells, simultaneously suppressing the ferroptosis. ALKBH5 is discovered to exclusively catalyze the removal of m6A on ssRNAs.[16] It has been observed that ALKBH5 is primarily located in nuclear speckles, which play a role in the formation of mRNA-processing factors. This suggests that ALKBH5 primarily targets nuclear nascent RNAs as its main

substrates.[16] Additionally, ALKBH5 demonstrates a preference for interacting with the distal 5′ region of coding sequences.

ALKBH5 exhibits high expression in the testis and lung under normal conditions, followed by the spleen, kidney, and liver, and low expression in the heart and brain.[16] The abnormal level of ALKBH5 is closely correlated with human pathologies. ALKBH5 deficiency in mice leads to impaired spermatogenesis due to abnormal levels of key genes that regulate spermatogenic maturation.[15] ALKBH5 has been shown to play a significant role in various biological processes, including immune response,[17-19] heart regeneration,[20] osteogenic differentiation,[21,22] brain development,[23] DNA damage caused by ROS,[24] and angiogenesis after ischemia.[25] Recent research has suggested that ALKBH5 is commonly dysregulated in multiple types of cancer and plays an important role as an m6A demethylase.[26] The expression of ALKBH5 can be altered in a way that promotes or suppresses carcinogenesis, depending on the specific type of cancer.[15,27-29] In leukemia-initiating cells, the promoter regions of ALKBH5 exhibit significantly increased levels of active histone markers, such as H3K4me3, H3K9ac, H3K79me2, and H3K4me2. At the same time, there is a decrease in the levels of repressive histone markers like H3K27me3 and H3K9me3. These alterations in histone modifications lead to a more open chromatin structure.[30] The increased levels of ALKBH5 can enhance the expression of HBx mRNA, which in turn creates a positive feedback loop and consequently contributes to the development of liver cancer associated with hepatitis B virus (HBV) infection.[31] Overexpression of ALKBH5 reduced the infarct size, restored cardiac function, and promoted cardiomyocyte proliferation after myocardial infarction.[20]

ALKBH5 repressed mitochondrial fission, suppresses the mitochondrial membrane potential, and oxygen consumption rate, and the proliferation and migration of hepatic stellate cells and ameliorates liver fibrosis.[32] These previous findings established the potential application of ALKBH5 as a therapeutic target in medical use. However, the specific role of ALKBH5 in asthma is unclear. Here, we identified that ALKBH5 knockdown could reduce the IL-13-induced accumulation of lipid ROS and Fe2+ and alleviate ferroptosis of bronchial epithelial cells, which is dependent on the m6A modification of GPX4. Consistent with our findings, a previous study on thyroid cancer suggested that overexpression of ALKBH5 could reduce the protein expression of SLC7A11 and GPX4 and induce ferroptosis.[33] Certain limitations still exist in current work, such as further exploration of the ALKBH5-regulated GPX4 expression during asthma and other mechanisms underlying ALKBH5-mediated epigenetic regulation. The verification of the ALKBH5-GPX4 axis in an *in vivo* model is needed in further study.

To summarize, we determined that the knockdown of ALKBH5 improved the proliferation of bronchial epithelial cells and alleviated cell ferroptosis, simultaneously elevating the expression of GPX4 via elevating its m6A modification level. Our findings presented ALKBH5 as a promising target for the treatment of asthma.

STATEMENT OF ETHICS

The protocol was approved by the ethics committee of Henan University of Chinese Medicine, No.H358911. Informed consent was obtained from all study participants. All the methods were carried out in accordance with the Declaration of Helsinki.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Not applicable

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