

ORIGINAL ARTICLE

TRIM11 promotes cell proliferation of non-small cell lung cancer through the inhibition of ferroptosis by AMPK

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Abstract

Lung cancer is one of the leading causes of cancer-related deaths worldwide, with non-small cell lung cancer (NSCLC) being the most prevalent type. This study investigates the role of TRIM11 gene in NSCLC and its underlying mechanism. NSCLC patients were recruited from our hospital and showed upregulated TRIM11 mRNA and protein expressions. Patients with high TRIM11 expression had lower survival rates. TRIM11 gene was found to promote cell proliferation and reduce ROS-induced ferroptosis in NSCLC. Additionally, TRIM11 gene induced AMPK expression and its regulation affected TRIM11's effects on cell proliferation and ferroptosis in NSCLC. IP analysis revealed that TRIM11 protein interacted with AMPK protein in NSCLC. These data confirmed that TRIM11 promotes cell proliferation and reduces ROS-induced ferroptosis in NSCLC through AMPK. Hence, TRIM11 is a potential target for the treatment of NSCLC and other cancers.

KEYWORDS

AMPK, ferroptosis, non-small cell lung cancer, TRIM11

1 | INTRODUCTION

Lung cancer is the second most common cancer in terms of incidence and the leading cause of cancer-related deaths worldwide.¹ According to the 2020 GLOBOCAN report on global cancer statistics, there were 2.207 million new cases of lung cancer worldwide, ranking second only to breast cancer.² The number of deaths due to lung cancer was 1.796 million, making it the most deadly cancer type.³ In China, lung cancer is not only the most common cancer in terms of incidence but also mortality.³ In 2020, the estimated number of new cases of lung cancer in China was 816 000, accounting for 17.9% of all new cancer cases, and the number of deaths was 715 000, accounting for 23.8% of all cancer deaths.⁴ Based on age-standardized rates (ASR), the incidence rate of lung

cancer in Chinese men and women was 47.8 per 100 000 and 22.8 per 100 000, respectively, and the mortality rate was 41.8 per 100 000 and 19.7 per 100 000, respectively.⁵

The occurrence of cancer is related to many factors, such as smoking and passive smoking, silica, asbestos, tin, coal tar, diet, oil fume, air pollution, etc.⁶ Both smoking and passive smoking exposure are important risk factors for lung cancer, and smoking can increase the risk of lung cancer by more than three times.⁷ In our country, 72.4% of non-smokers are exposed to passive smoking, which is extensive, and the impact on non-smokers' lung cancer cannot be ignored.⁶ Lung cancer ranks first in the death and incidence of malignant tumors in China.⁸ Lung cancer is closely related to smoking and passive smoking exposure.⁶ In recent years, China has taken a

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series of measures to control smoking and prevent lung cancer, but the burden of lung cancer is still serious, the smoking rate of men is still high, and non-smokers are very widely exposed to passive smoking.⁹

Ferroptosis is a recently discovered programmed cell death mode that is distinct from apoptosis, necrosis, and autophagy.¹⁰ Its primary mechanism involves the catalysis of lipid peroxidation of highly expressed unsaturated fatty acids in cells under the influence of divalent iron or lipoxygenase, leading to cell death.¹¹ Ferroptosis is implicated in the development of many diseases, including tumors, neurodegenerative diseases, rheumatoid arthritis, ischemia–reperfusion, and cardiovascular-related diseases.¹¹ Lung cancer is the most deadly malignant tumor globally, and its treatment strategy is continuously evolving.¹¹ In recent years, an increasing number of research findings indicate a close connection between ferroptosis and non-small cell lung cancer (NSCLC).¹² Some molecules that play a significant regulatory role in the development, treatment, and onset of NSCLC, such as KRAS, TP53, and EGFR, are also implicated in ferroptosis.¹² Additionally, ferroptosis in NSCLC is closely tied to chemotherapy, radiotherapy, and immunotherapy. Some preclinical studies have shown that ferroptosis can serve as a “catalyst,” and combining ferroptosis with the aforementioned treatment methods can significantly enhance treatment effectiveness, opening up a new research avenue for NSCLC treatment.¹³

AMPK is an energy sensor in the body that enhances energy utilization, regulates autophagy and mitochondrial fusion and fission, and participates in various pathophysiological activities.¹⁴ Recent studies have demonstrated that AMPK activation can alleviate brain I/R injury by regulating energy metabolism, oxidative stress, and mitochondrial function.^{15,16} Furthermore, the use of AMPK activators in tumor cells has been reported to regulate lipid synthesis and metabolism, thus reducing the incidence of cell ferroptosis.¹⁷ Additionally, it has been reported that upregulating the expression of p-AMPK can reduce lipid peroxide levels after I/R and subsequent nerve injury in rats.¹⁸

The TRIM11 protein, a member of the TRIM family, is involved in regulating cell apoptosis, proliferation, and cell cycle.¹⁹ As a tumor promoting factor, TRIM11 can enhance tumor cell proliferation and invasion through various mechanisms.²⁰ High expression of TRIM11 was observed in advanced stage gliomas, while low expression was associated with poor differentiation and favorable prognosis.²¹ Additionally, TRIM11 can activate MAPK or PI3K/AKT signaling pathways to promote tumor cell proliferation, invasion, and migration.²² Shang et al. identified that TRIM11 suppresses ferritinophagy in pancreatic ductal adenocarcinoma.²³ So, this study elucidates the

role and potential mechanism of TRIM11 in ferroptosis of NSCLC.

2 | MATERIALS AND METHODS

2.1 | Patients with NSCLC

This study was approved by the ethics committee of our hospital. All the serum samples (number = 40) were immediately snap frozen in liquid nitrogen and stored at -80°C . Pathological evaluation was performed according to the WHO classification by two experienced clinical pathologists. All methods were carried out in accordance with relevant guidelines and regulations. Informed consent was obtained from all participants.

2.2 | Cell culture and transfection

BEAS-2B, NCI-H1437, NCI-H1299, NCI-H322, and A549 cells were performed in compliance with ATCC protocols and incubated in a 5% CO_2 atmosphere at 37°C . Plasmids were transfected into NSCLC cell lines using Lipofectamine 2000.

2.3 | Microarray experiments

Microarray experiments were performed at the Genminix Informatics (China). Gene expression profiles were analyzed with the Human Exon 1.0 ST GeneChip (Affymetrix).

2.4 | Quantitative polymerase chain reaction (qPCR)

Total RNAs were isolated with RNA isolator total RNA extraction reagent (Takara), and cDNA was synthesized using PrimeScript RT Master Mix (Takara). qPCR was performed with the ABI Prism 7500 sequence detection system according to the Prime-Script™ RT detection kit. Relative levels of the sample mRNA expression were calculated and expressed as $2^{-\Delta\Delta\text{CT}}$. Primer sequence: TRIM11: forward: 5'-GTGCCTATGGAGCTGAGGAC-3'; reverse: 5'-CAGGATCAGCTCAGGGTTG-3'; β -actin: forward: 5'-TACCTCATGAAGATCCTCACC-3'; reverse: 5'-TTTCGTGGATGCCACAGGAC-3'. High TRIM11 expression of NSCLC patients ≥ 3 fold of TRIM11 expression of normal patients, and low TRIM11 expression of NSCLC patients < 3 -fold of TRIM11 expression of normal patients.

2.5 | Immunofluorescent staining

Cells were fixed with 4% paraformaldehyde for 15 min and incubated with using 0.15% Triton X-100 for 15 min at room temperature. Cells were incubated with TRIM11 (ab191217, 1:500, abcam) and p-AMPK (3934, 1:500, Cell Signaling Technology, Inc.) at 4°C overnight after blocking with 5% BSA for 1 h. Cells was incubated with goat anti-rabbit IgG-cFL 488 or anti-rabbit IgG-cFL 555 antibody (1:100) for 2 h at room temperature and stained with DAPI for 15 min and washed with PBS for 15 min. The images of cells were obtained using a Zeiss Axioplan 2 fluorescent microscope (Carl Zeiss AG, Oberkochen, Germany).

2.6 | Proliferation assay and EDU staining

For cell counting kit-8 (CCK-8), after 48 h of transfection, a total of approximately 2×10^3 cells/well was seeded in 96-well plate. After culturing at indicated time (0, 1, 2, 3, and 4 day), the cellular proliferation was detected using CellTiter-GloR Luminescent Cell Viability Assay (Promega, Madison, WI, USA) according to manufacturer's instructions.

For ethynyl deoxyuridine (EdU) incorporation assay, EdU (10 mM) was added to each well, and cells were fixed with 4% formaldehyde for 30 min. After washing, EdU was detected with Click-iTR EdU Kit, and images were visualized using fluorescent microscope (Olympus).

2.7 | Western blot

Tissue or cells samples were lysed with ice-cold RIPA buffer with complete protease and phosphatase inhibitors. The protein concentrations were measured using BCA protein assay kit. Total proteins were separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with primary antibodies: AMPK (1:1000, ab169197, abcam) and p-AMPK (1:1000, 3934, Cell Signaling Technology, Inc.), TRIM11 (1:1000, ab191217, abcam), GPX4 (1:1000, ab267373, abcam), and β -Actin (1:5000, Santa Cruz Biotechnology) after blocking with 5% BSA in TBS, followed by incubation with peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). The signals were detected with the ECL system and exposed by the ChemiDoc XRS system with Image Lab software (Bio-rad).

2.8 | Statistical analyses

Survival analysis was performed using the Kaplan–Meier method and log-rank test for survival curves. GraphPad Prism 6 was used for the statistical analysis. $p < 0.05$ was considered statistically significant. Comparisons of data between groups were followed using Student's *t* test or one-way analysis of variance (ANOVA), followed by Tukey's post hoc test.

3 | RESULTS

3.1 | TRIM11 expression level in patients with NSCLC

We first evaluated the expression of TRIM11 in patients with NSCLC. The results showed that TRIM11 mRNA and protein expressions were upregulated in patients with NSCLC, compared with normal (Figure 1A,B). Furthermore, TRIM11 mRNA expressions in NSCLC cell lines were higher compared to normal lung cells (Figure 1C). Kaplan–Meier survival analysis revealed that NSCLC patients with high TRIM11 expression had a lower survival rate (Figure 1E,F). These findings suggest that TRIM11 plays an activating role in NSCLC.

3.2 | TRIM11 gene promoted cell proliferation of NSCLC

We conducted experiments to determine the effect of TRIM11 on cell proliferation in NSCLC. The results showed that TRIM11 gene facilitated cell proliferation, as evidenced by increased EDU cells and cell metastasis in NSCLC, compared with negative (Figure 2A–C). On the other hand, si-TRIM11 inhibited cell proliferation, as demonstrated by reduced EDU cells and cell metastasis in NSCLC, compared with si-nc (Figure 2A–C). Furthermore, TRIM11 gene upregulation decreased the activity levels of caspase-3/9 and Bax protein expression and induced Bcl-2 protein expression in NSCLC, compared with negative (Figure 2D–F), while si-TRIM11 promoted caspase-3/9 activity levels and Bax protein expression and suppressed Bcl-2 protein expression in NSCLC, compared with si-nc (Figure 2D–F).

3.3 | TRIM11 gene reduced ROS-induced ferroptosis of NSCLC

We then investigated the impact of TRIM11 on the ferroptosis induced by ROS in NSCLC. We found that

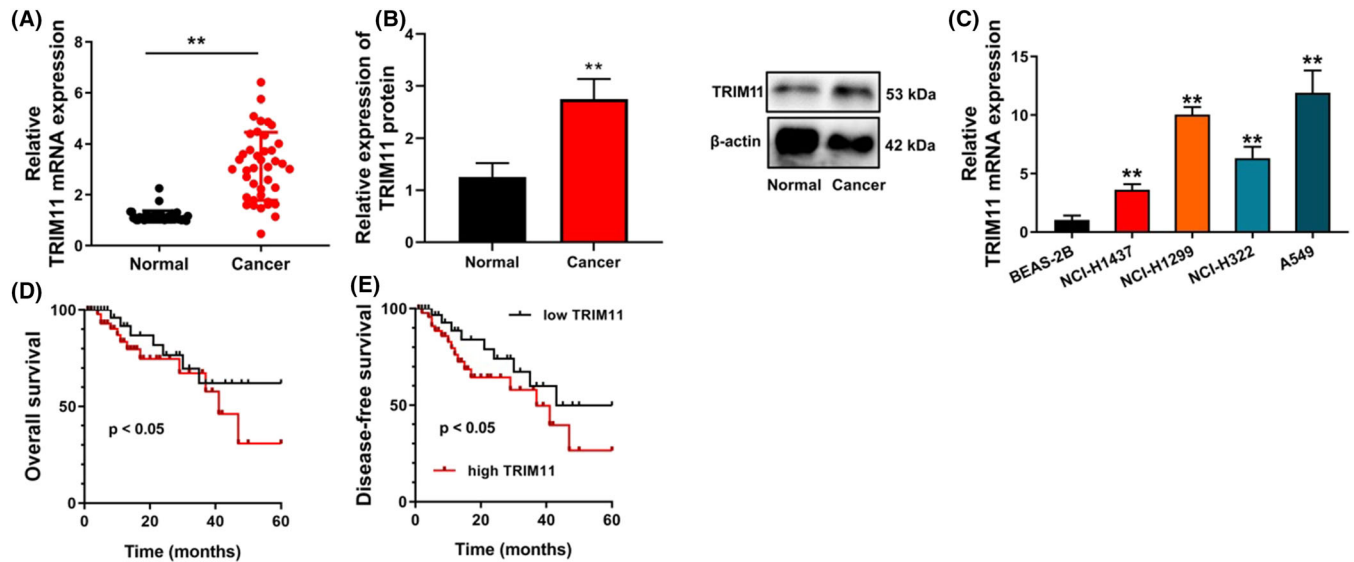


FIGURE 1 TRIM11 expression level in patients with NSCLC. TRIM11 mRNA and protein expressions in patients with NSCLC (A and B), TRIM11 mRNA expressions in NSCLC cell line (C), and OS and DFS (D and E). ** $p < 0.01$ compared with normal volunteers group or BEAS-2B group.

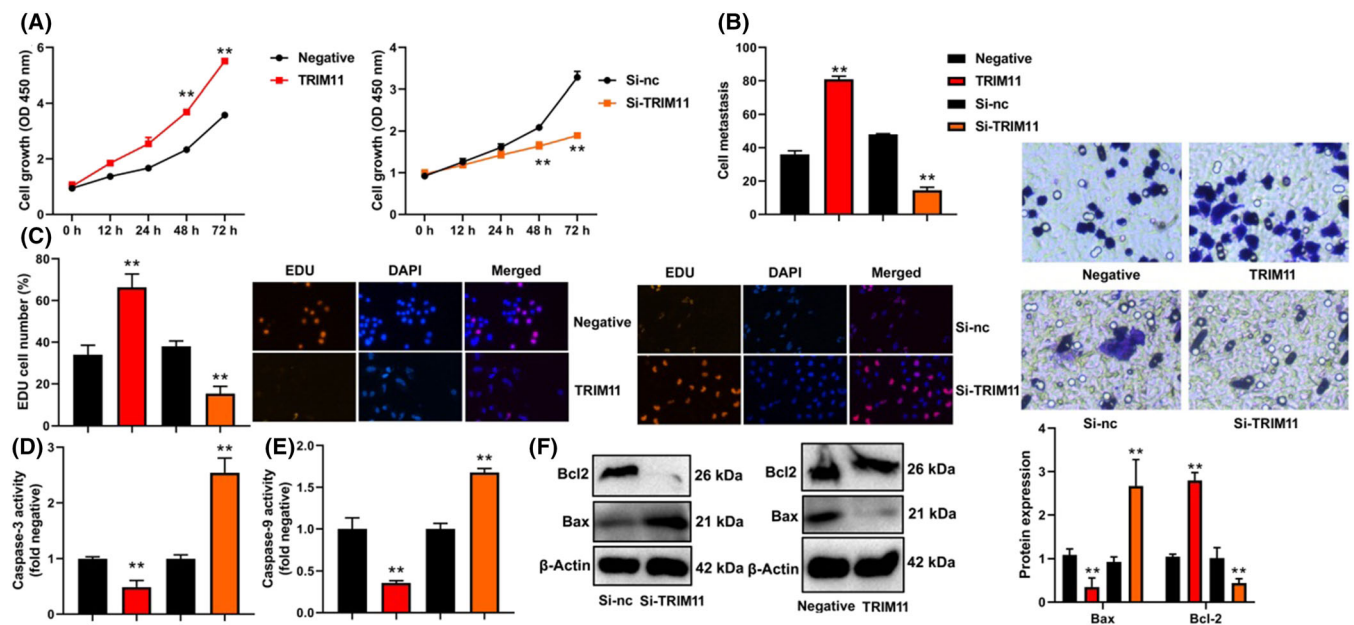


FIGURE 2 TRIM11 gene promoted cell proliferation of NSCLC. Cell growth (A), migration rate (B), EDU assay (C), and caspase-3/9 activity level (D and E). ** $p < 0.01$ compared with negative group or si-nc group.

upregulation of TRIM11 gene activity increased mitochondrial damage as evidenced by JC-1 disaggregation and increased MPT as observed in the calcein AM/CoCl₂ assay. Additionally, we observed a reduction in LDH activity levels and a decrease in the proportion of PI-positive cells. Furthermore, TRIM11 gene upregulation inhibited iron concentration levels and expanded GSH and GPX4 protein expressions in NSCLC, compared with negative

(Figure 3). On the other hand, silencing of the TRIM11 gene reduced mitochondrial damage and MPT, increased LDH activity levels and the proportion of PI-positive cells, promoted iron concentration levels, and suppressed GSH and GPX4 protein expressions in NSCLC, compared with negative (Figure 3). Overall, our findings suggest that TRIM11 gene activity reduces ROS-induced ferroptosis in NSCLC by inhibiting mitochondrial damage.

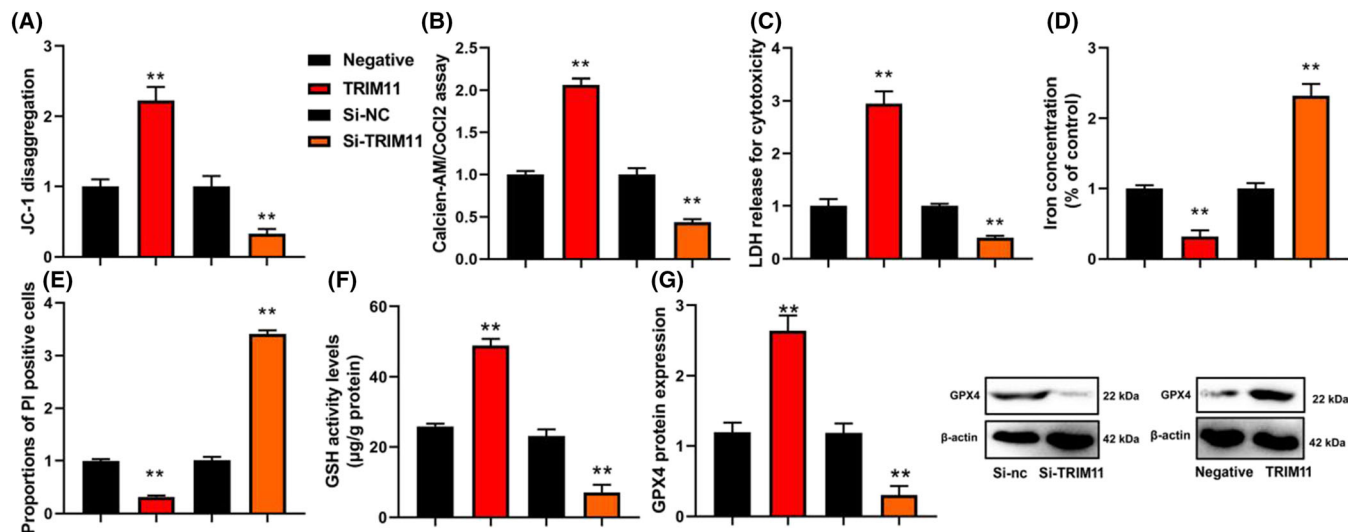


FIGURE 3 TRIM11 gene reduced ROS-induced ferroptosis of NSCLC. JC-1 disaggregation (A), calcein-AM/CoCl₂ levels (B), LDH activity (C), iron concentration levels (D), calcein/PI (E), GSH (F), and GPX4 protein expression (G). ***p* < 0.01 compared with negative group or si-nc group.

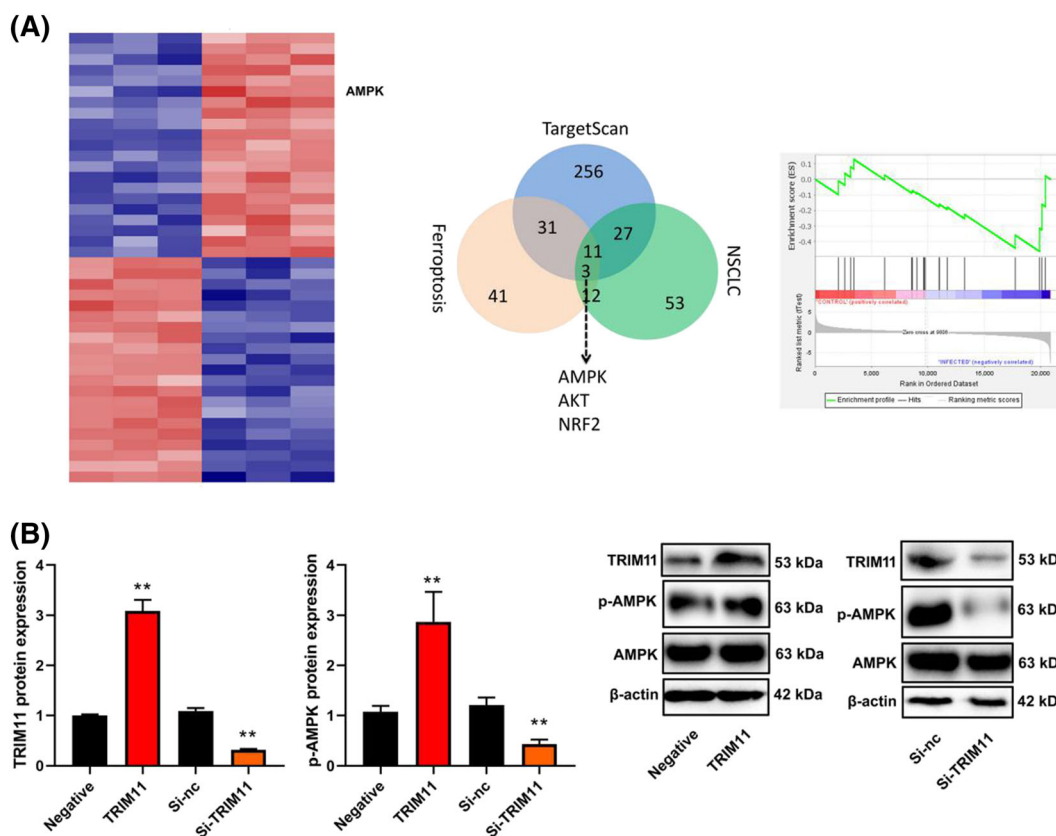


FIGURE 4 TRIM11 gene induced AMPK expression in NSCLC. Results of microarray analysis and KEGG terms (A) and TRIM11 and p-AMPK protein expression (B). ***p* < 0.01 compared with negative group or si-nc group.

3.4 | TRIM11 gene induced AMPK expression in NSCLC

The study delved into the mechanism behind TRIM11's role in ferroptosis in NSCLC through microarray experiments. The results indicated that AMPK could potentially be a target of TRIM11 in ferroptosis of NSCLC (as depicted in Figure 4A). Upregulation of TRIM11 led to an increase in p-AMPK protein expression, whereas si-TRIM11 decreased p-AMPK protein expression in NSCLC (as seen in Figure 4B). These findings shed light on the potential involvement of AMPK in TRIM11-mediated ferroptosis in NSCLC.

3.5 | The regulation of AMPK affected the effects of TRIM11 on cell proliferation of NSCLC

The study investigated the potential mechanism by which TRIM11 affects the progression of gastric NSCLC through AMPK expression. The use of an AMPK inhibitor (100 nM of AMPK-IN-3) resulted in reduced cell proliferation and EDU cells, inhibited cell metastasis, and increased caspase-3/9 activity levels in NSCLC with

upregulated TRIM11 gene expression (as shown in Figure 5A–E). On the other hand, an AMPK agonist (20 nM of Ampkinone) reduced cell proliferation and EDU cells, increased cell metastasis, and decreased caspase-3/9 activity levels in NSCLC with downregulated TRIM11 gene expression (as depicted in Figure 5F–J). These findings suggest a potential role of AMPK in the regulation of TRIM11-mediated cell progression in gastric NSCLC.

3.6 | The regulation of AMPK affected the effects of TRIM11 on ferroptosis of NSCLC

In addition, we observed that the AMPK inhibitor reduced mitochondrial damage and mitochondrial permeability transition (MPT), increased lactate dehydrogenase (LDH) activity levels and the proportion of propidium iodide (PI)-positive cells, promoted iron concentration levels, and suppressed glutathione (GSH) and glutathione peroxidase 4 (GPX4) protein expressions in NSCLC with upregulated TRIM11 gene expression (as shown in Figure 6A–G). Conversely, the AMPK agonist increased mitochondrial damage and MPT, reduced

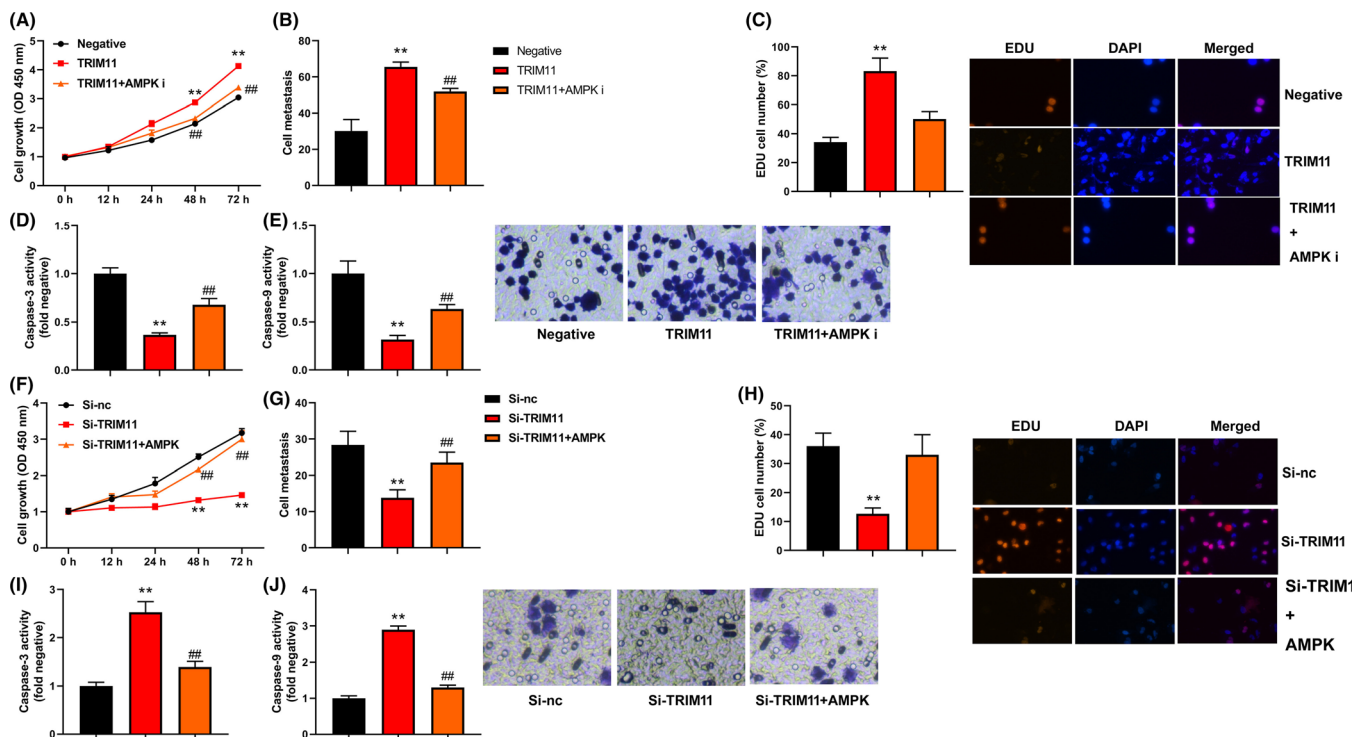


FIGURE 5 The regulation of AMPK affected the effects of TRIM11 on cell proliferation of NSCLC. Cell growth (A), migration rate (B), EDU assay (C), and caspase-3/9 activity level (D and E) in vitro model by TRIM11 + AMPK inhibitor; cell growth (F), migration rate (G), EDU assay (H), and caspase-3/9 activity level (I and J) in vitro model by si-TRIM11 + AMPK agonist. ** $p < 0.01$ compared with negative group or si-nc group; ### $p < 0.01$ compared with TRIM11 or si-TRIM11 group.

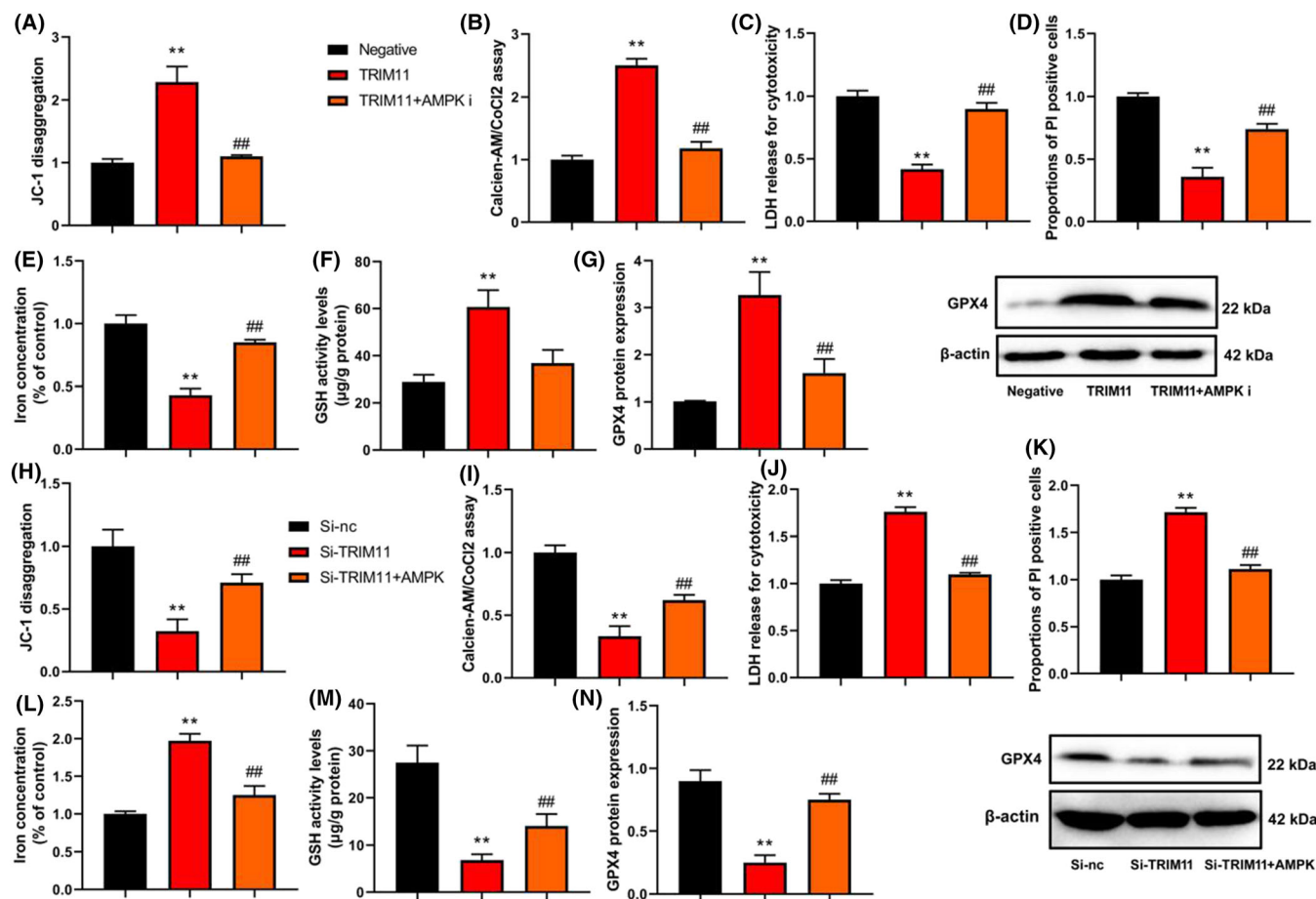


FIGURE 6 The regulation of AMPK affected the effects of TRIM11 on ferroptosis of NSCLC. JC-1 disaggregation (A), calcein-AM/CoCl₂ levels (B), LDH activity (C), and iron concentration levels (D), calcein/PI (E), GSH (F), and GPX4 protein expression (G) in vitro model by TRIM11 + AMPK inhibitor; JC-1 disaggregation (H), calcein-AM/CoCl₂ levels (I), LDH activity (J), and iron concentration levels (K), calcein/PI (L), GSH (M), and GPX4 protein expression (N) in vitro model by si-TRIM11 + AMPK agonist. ** $p < 0.01$ compared with negative group or si-nc group; ## $p < 0.01$ compared with TRIM11 or si-TRIM11 group.

LDH activity levels and the proportion of PI-positive cells, inhibited iron concentration levels, and expanded GSH and GPX4 protein expressions in NSCLC with downregulated TRIM11 gene expression (as depicted in Figure 6H–N). These results suggest that AMPK may play a crucial role in the regulation of TRIM11-mediated cell progression in gastric NSCLC by modulating cellular metabolism and redox homeostasis.

3.7 | TRIM11 protein interlinked AMPK protein

We investigated whether ferroptosis is functionally involved in TRIM11/AMPK-mediated treatment for NSCLC. Confocal microscopy revealed that TRIM11 upregulation increased p-AMPK expression in NSCLC (as shown in Figure 7A). Immunoprecipitation (IP) analysis demonstrated that TRIM11 protein

interacted with AMPK protein in NSCLC (as depicted in Figure 7B). Furthermore, the AMPK inhibitor suppressed p-AMPK expression in NSCLC (as demonstrated in Figure 7C), while the AMPK agonist induced p-AMPK expression in NSCLC (as illustrated in Figure 7D). These findings suggest that TRIM11 promotes cell proliferation in NSCLC by inhibiting ferroptosis through AMPK expression.

4 | DISCUSSION

Lung cancer is a prevalent and deadly malignancy worldwide, with non-small cell lung cancer (NSCLC) is the most common histological type of lung cancer, accounting for 85%.^{24,25} In China, lung cancer is the most common cancer and the leading cause of cancer-related deaths.^{24,25} Unfortunately, NSCLC is often diagnosed in advanced stages, leading to poor prognosis.²⁶ TRIM11,

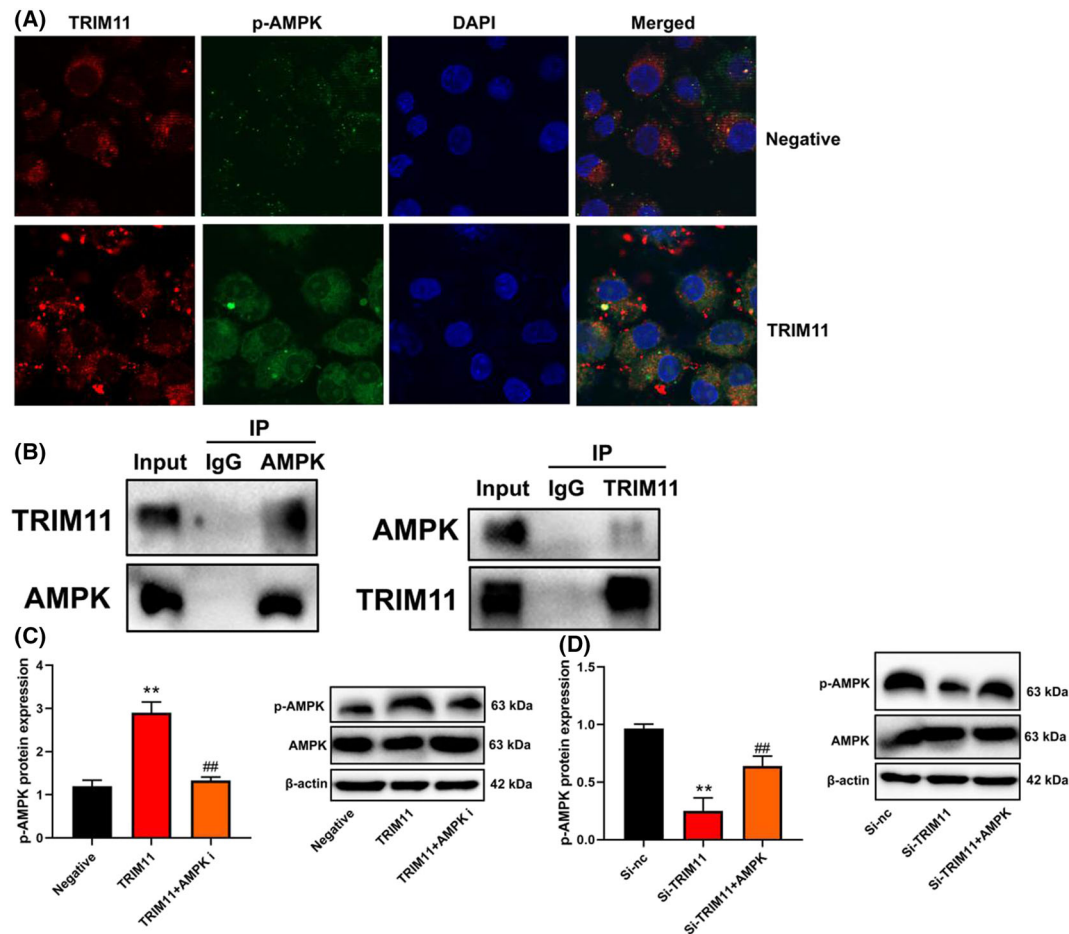


FIGURE 7 TRIM11 protein interlinks AMPK protein. TRIM11 and p-AMPK expression (confocal microscope, A), TRIM11 protein interlinks AMPK protein (IP, B), p-AMPK expression (C and D). ** $p < 0.01$ compared with negative group or si-nc group; ## $p < 0.01$ compared with TRIM11 or si-TRIM11 group.

which has been linked to the migration and invasion of nasopharyngeal carcinoma, was found to be upregulated in NSCLC patients at both the mRNA and protein levels. Patients with high TRIM11 expression had lower survival rates.²⁷ Therefore, inhibiting TRIM11 may offer protection against NSCLC. Our study demonstrated that TRIM11 mRNA and protein expressions were upregulated in NSCLC patients. Furthermore, patients with high TRIM11 expression had lower survival rates. Previous research by Zhao et al. also showed that TRIM11 facilitated the migration and invasion of nasopharyngeal carcinoma cells.²⁸ These results suggest that TRIM11 is upregulated in NSCLC and that inhibiting TRIM11 may offer protection against this disease. In this study, we only analyzed the serum expression of TRIM11, which was one insufficient for this experiment. We will analyze TRIM11 expression in tissue of patients with NSCLC.

Lung cancer is a prevalent and deadly malignancy worldwide, with non-small cell lung cancer (NSCLC) accounting for about 85% of all newly diagnosed cases,

making it the main pathological type of lung cancer.²⁹ Small cell lung cancer is the other main type. In China, lung cancer is the most common cancer and the leading cause of cancer-related deaths. Unfortunately, NSCLC is often diagnosed in advanced stages, leading to poor prognosis.³⁰ In 2012, we first discovered and proposed “ferroptosis”—a cell death mode different from apoptosis, necrosis, and autophagy, which is characterized by abnormal lipid peroxidation and accumulation of reactive oxygen species (ROS).³¹ In ferroptosis cells, mitochondria usually show a decrease in volume, an increase in membrane density, and a reduction or disappearance of cristae. Although ferroptosis may play a dual regulatory role in promoting or inhibiting the occurrence of tumors, small molecular drugs induced ferroptosis may be a potential anti-tumor treatment strategy.³² Ferroptosis is a newly discovered iron dependent programmed death.³³ More and more studies have shown that ferroptosis plays an important role in the progress of tumors, especially NSCLC, and targeting ferroptosis is a potential

therapeutic strategy for lung cancer.³³ To provide new research directions for the treatment of NSCLC, it is important to understand the mechanism and regulatory network of ferroptosis, the role of NSCLC-related regulatory genes (such as KRAS, TP53, EGFR, etc.) in ferroptosis, and the relationship between ferroptosis and chemotherapy, radiotherapy, and immunotherapy. We have revealed that the TRIM11 gene promotes cell proliferation and reduces ROS-induced ferroptosis of NSCLC. Shang et al have identified that TRIM11 suppresses ferritinophagy of pancreatic ductal adenocarcinoma.²⁸

GPX4 is produced on the cell membrane and can directly reduce the level of phospholipid peroxide.³⁴ When the activity of GPX4 decreases, it cannot inhibit the production of ROS in the cell, resulting in the accumulation of lipid peroxide and the production of lethal ROS, thus inducing cell ferroptosis.³⁵ Cystine can be oxidized to cysteine after entering the cell, which can then be combined with glycine to generate glutathione (GSH) under the action of glutamate-cysteine ligase and glutathione synthetase. There are two forms of glutathione: oxidized and reduced,³⁶ which can participate in the regulation of intracellular oxidation balance.³⁷ Glutathione reductase can catalyze their mutual transformation. This study revealed that the regulation of AMPK affected the effects of TRIM11 on ferroptosis of NSCLC. Therefore, TRIM11 might contribute to the inhibition of ferroptosis in NSCLC.

AMPK is an important energy regulator. Under low energy conditions, AMPK phosphorylates specific enzymes and growth control nodes to enhance the efficiency of ATP utilization and maintain the energy supply of the body.³⁸ AMPK plays a crucial role in cell physiology and the pathological development of chronic diseases, including cancer.³⁹ AMPK phosphorylation can inhibit the expression of SCD1, reduce lipid peroxidation, and inhibit the occurrence of ferroptosis.⁴⁰ Ferroptotic cells will produce ROS, and ROS production may also lead to activation of AMPK phosphorylation, and negative feedback can inhibit cell ferroptosis.⁴¹ This study showed that TRIM11 protein interlinks with AMPK protein, and TRIM11 gene induces AMPK expression in NSCLC. Liu et al. showed that TRIM11 binds to and ubiquitinates AMPK subunit.⁴² Therefore, our results revealed that the inhibition of TRIM11 protects NSCLC via the inhibition of AMPK.

In conclusion, our study demonstrated that TRIM11 promotes cell proliferation and reduces ROS-induced ferroptosis in NSCLC by activating AMPK. These findings provide a new understanding of the role of TRIM11 in NSCLC and suggest a novel target for NSCLC treatment. Inhibition of TRIM11 may be a potential strategy for the treatment of early-stage NSCLC and other cancers.

Further studies are needed to explore the therapeutic potential of targeting TRIM11 in cancer treatment.

AUTHOR CONTRIBUTIONS

Zheng Liang designed the experiments. Jian Li and Guoliang Zhang performed the experiments. Jian Li and Guoliang Zhang collected and analyzed the data. Zheng Liang and Menghui Chen drafted manuscript. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors state that there are no financial, personal, or professional conflicts of interests that may hinder this work.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

All patients were informed and signed informed consent voluntarily. This study was approved by the Ethics Committee of The Third Hospital of Shijiazhuang and complied with the guidelines outlined in the declaration of Helsinki were followed. The written consent was received from all participants.

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