TNFAIP3 Suppresses Lung Cancer Progression via Regulating Cell Proliferation and Apoptosis

Han Chen  
the general hospital of northern theater command, Shenyang

Jie Gao  
Institute of translational Medicine, Shanghai university

Yongsheng Yu  
Tongji University School of Medicine

Qian Zhou  
Tongji University School of Medicine

Shan yongqi (✉ YongqiShanpaper@163.com)  
Department of general surgery, The general hospital of northern theater command, Shenyang, China.

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Abstract

**Background:** The ubiquitin-editing enzyme TNF inducible protein 3 (TNFAIP3) is a crucial regulator of inflammation and immunity. It is also involved in tumorigenesis of various cancers such as lymphomas, colorectal tumors and breast cancer. In this study, we aimed to explore the role and regulatory mechanism of TNFAIP3 in lung cancer.

**Methods:** The expression of TNFAIP3 was determined in the Cancer Genome Atlas (TCGA) database. The levels of TNFAIP3 in lung cancer tissues was determined by immunohistochemistry (IHC) assay. TNFAIP3 knockdown and overexpression were performed, followed by further evaluation of cell viability, cell cycle and apoptosis. Cell cycle and apoptosis were observed by using flow cytometry and the key regulatory proteins were detected by western blotting. Colony formation assessment and EdU assay were adopted to check cell proliferation.

**Results:** TNFAIP3 expression was downregulated in lung cancer tissues at both mRNA and protein levels, comparing with that in adjacent non-tumor tissues. Consequently, the colony formation ability of lung cancer cells was enhanced, and the number of EdU positive lung cancer cells was increased. By contrast, elevated TNFAIP3 expression resulted in decreased colony formation ability of lung cancer cells. Mechanistically, TNFAIP3 overexpression rendered cell cycle of lung cancer cells halted at G0/G1 phase and caused apoptosis of lung cancer cells.

**Conclusion:** Our data suggested that TNFAIP3 exhibits tumor suppressive roles in lung cancer.

Background

Lung cancer is the leading cause of cancer-related death worldwide with high mortality and poor prognosis\(^1\). In recent years, lung cancer patients, whose tumors contain mutations or translocations in oncogenes such as EGFR, ALK, ROS1 or RET, are usually administrated with targeted therapies\(^2-5\). Despite the fact that current targeted therapies have dramatically improved the survival and life quality of these patients, many lung tumors cannot be treated with such therapies due to the lack of identifiable driver oncogenes. Thus, to understand the mechanisms regulating lung tumorigenesis is crucial and could be exploited for lung cancer therapy.

TNFAIP3, also known as A20, is an OTU (ovarian tumor protease) family deubiquitylase, functions as a crucial regulator of inflammation and immunity\(^6\). Human genetic studies have demonstrated that polymorphisms and mutations of TNFAIP3 are linked to several human diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis, psoriasis, Crohn’s disease and so on\(^7-9\). The function of TNFAIP3 as an anti-inflammatory factor is primarily realized through inhibiting NF-κB activation\(^6-8\).

The role of TNFAIP3 during tumorigenesis is diversified. Current studies suggest that TNFAIP3 is tightly associated with tumor development and exerts tumor-suppressive or oncogenic function in varied tumor context. The tumor suppressive role of TNFAIP3 has been well characterized in B-cell lymphomas\(^10-12\).
TNFAIP3 inactivation caused by deletion, mutation or promoter methylation happens in lymphomas, and such inactivation of TNFAIP3 leads to increased NF-κB activity and enhanced cell survival\textsuperscript{13}. Besides, TNFAIP3 also acts as a tumor suppressor in other cancers. For example, simultaneous deletion of both intestinal epithelial cells and myeloid TNFAIP3 results in development of colorectal tumors in aged mice\textsuperscript{14}. Hepatocyte-specific TNFAIP3 knockout mice are more susceptible to chemically or high fat-diet-induced hepatocellular carcinoma. It is worth to be noted that, TNFAIP3 also exhibits oncogenic roles in several cancer types, such as promoting basal-like breast cancers metastasis through monoubiquitylating Snail\textsuperscript{15}. Hjelmeland A. B. and Guo Q. et al. proposed that TNFAIP3 is overexpressed in glioma, and inhibiting TNFAIP3 expression compromises the growth and survival of the glioblastoma stem cells\textsuperscript{16,17}. TNFAIP3 expression is also up-regulated in acute lymphoblastic leukemia (ALL) patients, which promotes pathogenesis of ALL\textsuperscript{18}. However, the exact role of TNFAIP3 in lung cancer is not elucidated.

In this study, we have analyzed the expression profile of TNFAIP3 in lung tumor, and deciphered its function during lung cancer development.

**Methods**

**Database Analysis**

GEPIA (http://gepia2.cancer-pku.cn/#help) is an online tool capable of performing gene profiling of tumor patients and healthy donors from the Cancer Genome Atlas (TCGA) database and GTEx database. In this study, we analyzed the expression of TNFAIP3 in patients with lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC) from TCGA database.

**Cell Culture**

The human lung cancer cell lines, A549 and H1299, were purchased from American Type Culture Collection (Rockville, MD, USA). Cells were grown in RPMI1640 medium (Life Technologies, Inc., Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Life Technologies, Inc.), penicillin (100 U/mL) and streptomycin (100 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA) in a humidified incubator at 37 °C with 5% CO\textsubscript{2}.

**Cell Transfection**

The sequence of TNFAIP3 was obtained from the Pubmed website. The lentiviral shTNFAIP3 and the corresponding negative control (shCon) were designed and purchased from Ribobio (Guangzhou, China). The sequences of shTNFAIP3 are as follows: shTNFAIP3-1, 5'-TGTAACTCTTTGGGTTATTAC-3'; shTNFAIP3-2, 5'- TTGGAATCAGGTTCCAATTTC-3'. The shRNA was cloned into pLKO vector, co-transfected to HEK293T cell line with lentiviral helper plasmids pVSVG, pREV and pGAG system. The supernatants containing virus was collected, filtered and used for transfection of A549 and H1299 cells with polybrene. For overexpression of TNFAIP3, full length TNFAIP3 cDNA was cloned into pCMV 4.0
vector. Transfection was conducted with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to manufacturer’s instruction.

**Xenograft Mice Model**

All animal experiments were carried out in agreement with the National Institutes of Health (NIH) requirements for the care and use of laboratory animals and approved by the General Hospital of Northern Theater Command Animal Study Committee (Shenyang, China). BALB/c nude mice (5-week old, 18.0 ± 2.0g) were obtained from Beijing Vital River Laboratory and were randomly divided into indicated groups. Five-week-old male BALB/c-nu/nu athymic nude mice were fed and housed. Tumors were established by subcutaneous injection of parent (Vector group) or TNFAIP3 overexpressed A549 and H1299 cells (TNFAIP3 group) at the concentration of $1 \times 10^7$ cells/mL in 0.2 mL of 1:1 saline/Matrigel (BD Biosciences) mixture. Five mice were assigned to each group. The tumor volume and body weight of the mice were measured every other day. The mice were sacrificed when the tumor size in Vector group reached approximately 1000 mm$^3$. The tumor volume ($V$) was calculated using the following formula: 

$$V = \frac{\text{length} \times \text{width}^2}{2}.$$ 

Mice were anaesthetized by intraperitoneal injection of pentobarbital sodium overdose (100 mg/kg) on the 30th day. Euthanasia was considered to be successful if there was cardiac arrest and no spontaneous breath for 3 min, and then tumors were taken out and weighed. No side effects or mortality were observed in this experiment.

**Lung Tumor Tissue Microarray (TMA)**

The ethics committee of the General Hospital of Northern Theater Command (Shenyang, China) approved this study (approval number GHNT2018127). This study was performed in accordance with the Declaration of Helsinki. A lung tumor tissue microarray (TMA) was used to detect the expression of TNFAIP3 in lung tumor patients. Tumor tissue microarrays, purchased from Shanghai Outdo Biotech Company, and contains 75 samples of lung tumor and adjacent nontumor tissues. The tissue array was treated with standard IHC procedure.

**Immunohistochemistry (IHC) Assay**

The tumors collected from mice were fixed with 4% paraformaldehyde, embedded with paraffin, sectioned to 4 μm pieces on slides. The tissues were treated with the following process: deparaffinization, blockage of endogenous peroxidase with 2% H$_2$O$_2$ incubation, improvement of permeability by 0.1% Triton X-100, incubation with 5% BSA, then incubation with indicated primary antibodies (Abcam, MA, USA) overnight in a 4 °C refrigerator. Next day, after incubation with HRP-labeled secondary antibody for 45 minutes at room temperature, the slides were visualized by DAB kit (Beyotime, Shanghai, China). Nucleus were stained with haematoxylin. Five random areas of each slide were captured, and the percentage of positive staining was quantified and calculated as histograms.

**Western Blotting**
A549 and H1299 cells were plated $1 \times 10^5$/well in 6-well plates, and transfected with shTNFAIP3 or pCMV-TNFAIP3. Cells were lysed in 1× cell lysis buffer (Promega, Madison, WI). Protein concentration was measured using the Bradford method. Equal amounts of protein were isolated by SDS-PAGE and then transferred to nitrocellulose membranes (0.22 mm, Whatman). The membranes were incubated with anti-TNFAIP3, anti-p21, anti-Cyclin D1, anti-PARP, anti-Cleaved PARP or anti-GAPDH primary antibodies (1:500, Santa Cruz Bio-technology, USA). Subsequently, the blots were incubated with HRP-linked secondary antibody. The bands were visualized by ECL chemiluminescence substrate kit (Amersham, Piscataway, NJ) and captured by Gel image system (Bio-rad, Hercules, USA). The blots were quantified and analyzed as a percentage of control groups.

**Colony Formation**

A549 and H1299 cells were trypsinized, counted and seeded in 6-well plates 24 hours after transfection. Cells were cultured for 10 days to form visible clones. The clones were then washed, fixed and stained with crystal violet, captured and counted.

**EdU (5-ethynyl-2'-deoxyuridine) Assay**

Cells were seeded in 24-well plate at a density of $2\times10^4$ cells per well, incubated with diluted EdU solution (50 μM) for 2 hours. Then cells were fixed in 4% paraformaldehyde for 30 minutes at 4 °C and washed three times with PBS. After permeabilized in 2% Triton X-100 for 10 minutes, cells were washed with PBS for 5 minutes and incubated with 100 μL reaction solution for 20 minutes at room temperature. Subsequently, 200 μL diluted DAPI reagent was added in each well for 10 minutes, and washed with PBS. The fluorescence images were captured immediately after staining. The ratio of EdU-positive cells was counted and quantified.

**Flow Cytometry**

Flow cytometry was used to evaluate cell cycle and apoptosis of lung cancer cells after TNFAIP3 knockdown. Lung cancer cells were plated in 6-well plates and transfected with TNFAIP3 overexpression plasmids or empty vector as control, collected 24 hours after the transfection. After double staining with fluorescein isothiocyanate (FITC)-Annexin V and propidium iodide was performed using a FITC Annexin V Apoptosis Detection Kit (BD Biosciences) according to the manufacturer’s recommendations, the cells were analyzed with a flow cytometer (FACScan; BD Biosciences) equipped with Cell Quest software (BD Biosciences). Cells were sorted into viable cells, dead cells, early apoptotic cells, and apoptotic cells, and the relative ratio of early apoptotic cells was compared with that of control transfected cells in each experiment. For cell cycle analysis, cells were stained with propidium oxide for 30 minutes at 37 °C and analyzed with the FACScan flow cytometer. The ratio of G0/G1, S and G2/M phase were calculated.

**Statistics**
All the data are presented as the means ± SD. One-way ANOVA was used to assess the difference between multiple groups. Differences between two groups were analyzed by the Student’s t-test. $p < 0.05$ was considered statistical significance.

**Results**

**TNFAIP3 Expression Is Downregulated in Lung Tumor Tissue**

To identify the role of TNFAIP3 in lung cancer development, we first evaluated its expression at transcription and protein levels in lung tumor patients. According to the analysis of patients bearing lung tumor in TCGA database, TNFAIP3 mRNA level was remarkably suppressed in 483 lung adenocarcinoma (LUAD) cases comparing with that in 347 nontumor tissues (Fig. 1A). Similarly, the analysis of 486 lung squamous cell carcinoma (LUSC) and 338 nontumor cases showed declination of TNFAIP3 mRNA in cancerous tissues (Fig. 1A). After identification of lower TNFAIP3 level in lung tumor via online database, we next adopted lung tumor tissue microarray to detect the protein level of TNFAIP3 by IHC assay. As demonstrated in Figure 1B, the protein level of TNFAIP3 was significantly lower in 75 lung carcinoma tissue samples in comparison with their paired adjacent nontumor tissues. Two randomly selected images of TNFAIP3 staining by IHC were shown in Figure 1C. These results indicated TNFAIP3 as a negative regulator associated with lung tumor formation.

**TNFAIP3 Knockdown Facilitates Proliferation of Lung Cancer Cells**

To confirm if this downregulation of TNFAIP3 is related with lung tumor genesis, we conducted knockdown experiments by using shTNFAIP3, and investigated its effect on lung cancer cell proliferation. The decreased TNFAIP3 protein level in transfection group demonstrated the efficacy of shTNFAIP3-1 and shTNFAIP3-2 in lung cancer cell line A549 and H1299 (Fig. 2A). Colony formation experiment demonstrated notable elevation of colonies after TNFAIP3 knockdown (Fig. 2B). Moreover, the enhancement of DNA synthesis detected by EdU assay also indicated higher proliferation level after TNFAIP3 knockdown (Fig. 2C). These results indicated that the TNFAIP3 was associated with lung tumor cell proliferation.

**TNFAIP3 Overexpression Inhibits Lung Cancer Cell Proliferation via Inducing Cell Cycle Arrest**

Next, we performed exogenous expression of TNFAIP3 via transfection of pCMV-TNFAIP3 plasmid. The efficacy of TNFAIP3 overexpression was testified by elevated protein level (Fig. 3A). Colony formation experiment showed attenuated proliferation of A549 and H1299 cells under TNFAIP3 overexpression (Fig. 3B). Subsequent cell cycle examination demonstrated that TNFAIP3 overexpression decreased S phase ratio and caused G0/G1 phase arrest in lung cancer cells (Fig. 3C), accompanied with the declination of cell cycle inhibitory factor p21 and upregulation of Cyclin D1 (Fig. 3D). These results suggested that impaired proliferation of lung cancer cells under high level of TNFAIP3 could be related with cell cycle arrest.
TNFAIP3 Promotes Lung Cancer Cell Apoptosis

After confirming the role of TNFAIP3 in cell cycle progression, we tried to determine its function in apoptosis of lung cancer cells. We conducted Annexin V/PI dual staining and detected the portion of apoptotic cells in control and TNFAIP3 overexpression groups by flow cytometry. As determined by flow cytometry, TNFAIP3 overexpression led to a significant elevation of cell apoptosis in lung cancer cell line A549 (Fig. 4A) and H1299 (Fig. 4B). The results of further western blotting assay indicated significant elevation of cleaved PARP (Fig. 4C), the biomarker of cell apoptosis, under TNFAIP3 overexpression. These findings suggested enhanced cell apoptosis stimulated by TNFAIP3.

TNFAIP3 Overexpression Impairs Lung Tumor Growth

To further confirm the role of TNFAIP3 in tumorigenesis of lung tumor cells, we subcutaneously implanted parent and TNFAIP3-overexpressed A549 and H1299 cells into the BALB/c-nu/nu mice. We found that the tumor size and weight were notably reduced in the TNFAIP3 overexpression groups, compared with that in the control groups (Fig. 5A and B). In addition, in consistent with cellular experiment, the biomarker of apoptosis, Cleaved Caspase3, was obviously accumulated in tumor tissues under TNFAIP3 overexpression (Fig. 5D), indicating the enhanced cell apoptosis in xenograft tumor.

Discussion

Lung cancer ranks one of the most malignant cancer worldwide with high morbidity and mortality\(^1\). Multiple studies have indicated that the incidence of lung cancer is closely related with activation of oncogenes, including KRAS, EGFR, BRAF, and HER2\(^{19-22}\). Hence, target therapeutic medicines, such as EGFR and MEK inhibitors, were developed and approved to improve the prognosis of lung cancer patients\(^{23-25}\). However, despite the usage of molecular inhibitor targeting these oncogenes, the rapid therapeutic resistance and unsatisfied effectiveness make it urgent to explore novel potential target as adjuvant treatment\(^{26-28}\).

Through bioinformatic analysis of mRNA expression pattern in lung cancer patients from TCGA database, we found that the ubiquitin-editing enzyme TNFAIP3, also known as A20, showed notable lower level in lung tumor tissues comparing with the normal tissues. Moreover, as a ubiquitin-editing enzyme, TNFAIP3 is capable of regulating the stability of proteins at post-transcriptional level, thereby participates in the development and progression of various cancers\(^{29-31}\). However, it is worth to be noted that the function of TNFAIP3 varies, as a tumor enhancer or suppressor, depending on different cancer context\(^{29, 30, 32}\). For example, TNFAIP3 is overexpressed in breast cancer, associated with breast cancer aggressiveness and poor prognosis of breast cancer patients\(^{29}\). TNFAIP3 mediates TNF-\(\alpha\) promoted triple negative breast cancer aggressiveness, and its absence leads to cell apoptosis\(^{33}\). Elevated expression of TNFAIP3 multi-monoubiquitylates Snail1 promotes EMT and metastasis of basal-like breast cancer\(^{34}\). Besides, TNFAIP3 could also mediates cancer cell resistance to chemotherapy and radiotherapy by facilitating DNA damage repair\(^{30}\).
The role of TNFAIP3 as a tumor suppressor was reported in several cancer types, and closely related to restricting NF-κB signals via its deubiquitinase activity. For example, TNFAIP3 was proposed as tumor suppressor in several human B-cell lymphomas, exerts its inhibitory functions by downregulating key proinflammatory signaling pathways, including those controlling NF-κB- and IRF3-dependent gene expression. TNFAIP3 upregulation after TNF-α pre-stimulation causes suppression of ERK/p38 signal transduction during TNF-α re-treatment in colorectal cancer. Low expression of TNFAIP3 was also found in pancreatic cancer tissues. Studies on liver tumor development indicated that TNFAIP3 plays an important role in liver protection and tumor prevention, by limiting chronic liver inflammation, stimulating hepatocyte growth, inhibiting the activation of protein tyrosine kinase 2 and Rac family GTPase 1, and preventing epithelial-mesenchymal transition. Based on the expression profile analysis of TNFAIP3 in TCGA database and lung tumor tissue microarray, we hypothesized that TNFAIP3 plays the role of tumor suppressor in lung tumorigenesis.

Hence, we investigated the function of TNFAIP3 in lung tumor cells with knockdown and exogenous overexpression experiments. The outcomes from colony formation assessment and EdU assay demonstrated that the suppression of TNFAIP3 level promoted lung tumor cell proliferation. Subsequent cell cycle and apoptosis detection revealed that the overexpression of TNFAIP3 could cause G0/G1 cell cycle arrest, S phase impairment and enhanced cell apoptosis. These changes on cell cycle and apoptosis were accompanied with alteration of cell cycle-related p21 and CyclinD1 protein, as well as the elevation of apoptotic biomarker cleaved PARP. In vivo xenograft mice model further showed obviously inhibited tumor growth and elevated apoptotic biomarker Cleaved-Caspase3, thus confirming the tumor suppressor role of TNFAIP3 during lung tumor formation. A recent published study on lung cancer revealed TNFAIP3 as a target gene of miR-605-5p. Overexpression of miR-605-5p promotes the proliferation and invasion of H1299 and H460 cells through targeting the 3'UTR of TNFAIP3, which are consistent with our finding.

**Conclusion**

Our research determined the decreased level of TNFAIP3 in lung tumor, demonstrated the TNFAIP3 overexpression-induced cell apoptosis and inhibition of tumor growth. These findings suggested the potential suppressive role of TNFAIP3 in lung tumorigenesis, and provided evidence for a novel therapy of lung cancer.

**Abbreviations**

TNFAIP3: The ubiquitin-editing enzyme TNF inducible protein 3; IHC: immunohistochemistry; OTU: ovarian tumor protease; LUAD: lung adenocarcinoma; LUSC: lung squamous cell carcinoma; EdU: 5-ethynyl-2'-deoxyuridine; TCGA: the Cancer Genome Atlas

**Declarations**
Ethics approval and consent to participate
The ethics committee of the General Hospital of Northern Theater Command (Shenyang, China) approved this study (approval number GHNT2018127). All experimental procedures in mice complied with all relevant ethical regulations.

Acknowledgments

Not applicable

Authors’ contributions

All authors contributed to the study conception and design. Y.Q. and Z.Q. conceived the project and performed the project planning. C.H. and G.J. performed the main experimental work of mouse models and IHC analysis. C.H. and G.J. participated in the related cell proliferation, colony formation, and xenograft assays. Y.S. participated in plasmid construction. All authors read and approved the final manuscript.

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Availability of data and materials

TNFAIP3 mRNA level datasets were obtained from the GEPIA (http://gepia2.cancer-pku.cn/#help) which is an online tool capable of performing gene profiling of tumor patients and healthy donors from the Cancer Genome Atlas (TCGA) database and GTEx database.

Consent for publication

Yes

Competing interests

The authors declare no conflict of interest.

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