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RNF213 inhibits migration in lung adenocarcinoma cell

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ABSTRACT

Non-small cell lung cancer (NSCLC) is one of the most common malignant tumors, and lung adenocarcinoma (LUAD) accounts for up to 40% of NSCLC. Ring finger protein 213(RNF213) has been demonstrated to suppress several cancers, including glioblastoma and breast cancer. Nonetheless, the role of RNF213 in LUAD has not been investigated. The expression of RNF213 in LUAD tissues was analyzed by western blotting, The Cancer Genome Atlas, Genotype Tissue Expression Project, and Gene Expression Omnibus databases. Prognostic value analysis was performed through the Kaplan-Meier Plotter database. We determined the role of RNF213 in LUAD cells through cell counting kit-8 assay, migration, and invasion assay. The clinical roles of RNF213 were evaluated by immunohistochemical staining assay (IHC) and Kaplan-Meier survival analysis. RNF213 expression was reduced in LUAD, thus affecting the prognosis of LUAD. And RNF213 could suppress the migration and invasion of LUAD cells to prevent tumor development. the expression of RNF213 is positively correlated with the overall survival, providing a novel marker in the prognosis of LUAD patients.

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Introduction

Non-small cell lung cancer (NSCLC) accounts for 85% of all lung cancers and is the leading cause of cancer-related fatality worldwide. Moreover, lung adenocarcinoma (LUAD) is the most common histopathological type of NSCLC (1,2). The therapy of LUAD mainly includes surgical treatment (3), radiotherapy (4), chemotherapy (5), biological immunotherapy (6), molecular targeted therapy (7), and traditional Chinese medicine therapy (8). However, a significant number of patients with LUAD have local deep invasion or extensive metastasis at the time of diagnosis and lose the opportunity for surgery (9). Over the past decade, molecularly targeted drugs and immune checkpoint inhibitors have been introduced into the clinical treatment for treating patients with advanced LUAD, improving the patients survival (10). Unfortunately, these treatments tend to be of limited advantage to certain patients, and a majority of advanced-stage patients succumb to the disease within five years of diagnosis (11). It has been shown that metastasis is a major cause of cancer-related death in LUAD patients (12). Therefore, exploring the molecular mechanism of LUAD progression is essential for identifying new potential prognosis markers.

Ring finger protein 213 (RNF213) is a gene located in the 17q25.3 region of the human chromosome and encodes a protein with a molecular weight of 591kDa (13). Moreover, the ring finger domain is an E3 ligase (14). Recently, studies have confirmed that RNF213 is a crucial ATPase, which consists of two ATPase modules. These two types of components include Walker A and Walker B structures, both of which are necessary for the RNF213 protein to exert ATPase activity. Therefore, when combined with the Walker A motif, ATP leads to the formation of RNF213 hexamer, while the Walker B motif hydrolyzes and dissociates ATP to maintain its stability (15). In summary, the RNF213 protein has both ATPase and ubiquitin ligase activity.

Although prior research has revealed that RNF213 is mainly involved in the pathogenesis of Moyamoya disease (15-17). In recent years, many reports have discovered that the RNF213 gene is involved in the progression of many different tumors. It has been reported that next-generation resequencing detected somatic mutations of RNF213 in metastatic tumors (18). Liu ZX. et al have demonstrated that RNF213 with the highest incidence (>10%) of private germline alterations (including insertions and deletions) in the hereditary diffuse gastric cancer cohort (19,20).In ovarian cancer, ultra-deep targeted sequencing was performed to identify pathogenic mutations and found that RNF213 mutated (21). In addition, head and neck squamous cell carcinoma, pancreatic ductal cancer and lymphoma have been demonstrated as frequent mutations of RNF213 (22-24). Thus, RNF213 plays a promising function in cancer. However, the role of RNF213 in LUAD remains undetermined.

In this work, we investigate that the expression of RNF213 is down-regulated in LUAD, and the low expression of RNF213 is associated with poor prognosis. Our results also indicate that knocking down RNF213 can pro-

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mote the migration and invasion of LUAD cells to prevent tumor development. Further, we investigated the clinical significance of RNF213 in LUAD. Our data reveal that RNF213 may be critical for LUAD progression and may serve as a novel marker in the prognosis of LUAD patients.

Materials and Methods

Data sources

RNF213 expression in LUAD based on the RNA sequencing data of TCGA, GTEx ,UCSC and GEO (GSE33532) project was analyzed by the online tool Sangerbox (http://www.sangerbox.com/tool) (25) and Assistant for Clinical Bioinformatics (https://www.aclbi.com/) (26). Kaplan–Meier Plotter (https://kmplot. com/analysis/) (28) was used to analyze the association of related genes with overall survival (OS) of LUAD patients. R software version 4.0.3. was used for data processing (R Foundation for Statistical Computing, Vienna, Austria).

Clinical samples

The study complied with ethical regulations regarding human participants and had the informed consent of all donors. In this study, 79 cases of LUAD samples were collected from the Fifth Affiliated Hospital of Sun Yat-sen University. And complete clinical follow-up data were available. All the above LUAD tissues were confirmed by histopathology. The study was approved by the Medical Ethics Committee of the Fifth Affiliated Hospital of Sun Yat-sen University (2023-K165-1).

Cell culture

The human cell lines A549, NCI-H1975 and HEK-293T were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All experiments were performed with mycoplasma-free cells. Cells were cultured with F-12K medium, RPMI-1640 medium and DMEM (Thermo Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Thermo Scientific, Waltham, MA, USA).

Cell transfection

All transient ectopic expression vectors were constructed using the PSIN-EF1-puro vector (Invitrogen, Carlsbad, MA, USA). RNF213 were cloned into the pSIN lentivirus vector to generate stable cells overexpressing RNF213, respectively. The pLKO.1-puro vector was inserted with the shRNAs targeting RNF213, and the sequences of these shRNAs are as follows: RNF213shRNA-1: GCTGAAATGGAATCGAGAAATCTC. RNF213shRNA-2: GCCTCAGCTAAGTATTCTGTTCTC.

Western blotting

In brief, cells were lightly washed 3 times with chilled PBS and treated with RIPA lysis buffer (Beyotime, Shanghai, China) including 1% protease inhibitor (Beyotime, Shanghai, China) and phosphatase inhibitor Cocktail (MedChemExpress, Monmouth Junction, NJ, USA). The proteins were run on SDS-PAGE microgel at 80V and 120V, shifted to a PVDF membrane (Millipore, Billerica, MA, USA) for 1.5 hours at 350 mA. The membranes were blocked with 5% skim milk at room temperature for 1 hour. Then the PVDF membrane was incubated with primary antibody at 4°C overnight, and with secondary antibody at room temperature for 1 hour. The specific bands were detected by enhanced chemiluminescence (Vazyme, Nanjing, China).

Cell counting kit-8(CCK-8) assay

CCK-8 assay was used to explore the cell proliferation. Cells in each group were seeded 2000 cells per well in 96-well plates, and left to adhere for 4 hours. Then cells were incubated for 1 d, 2 d, 3 d, or 4 d. At 1 d, 2 d, 3 d, or 4 d, the culture medium was discarded, and CCK-8 detection reagent (10 μl, Tokyo, Japan) was added. After incubated at 37°C for 3 h, the absorbance of cells was measured at 450 nm. The experiments were performed three independent times, and the results are presented as the mean ± SD.

Migration and invasion assays

The LUAD cells migration and invasion ability were detected by transwell assay using 24-well Boyden Chambers (Corning, Corning, NY, USA) with 8 μm pores coated with (invasion) or without (migration) Matrigel. We seeded 5×10^4 A549 and NCI-H1975 cells per well on the transwell inserts and cultured them in 300 μL of serum-free media at 37°C in the top chambers for 12 hours and 24 hours, whereas the lower chamber was filled with DMEM containing 10% FBS. After 12 hours of incubation, the cells were fixed, stained, and examined under a microscope.

Antibodies

Antibodies specific for RNF213 (Thermo Fisher Scientific, Cat#PA5-51902, Waltham, MA, USA), Flag-Tag (Cell Signaling, Cat#14793S, Danvers, MA, USA), β-actin (Proteintech, Cat#81115-1-RR, Rosemont, IL, USA) and GAPDH (Genetex, Cat#GTX100118, Irvine, CA, USA) were purchased from the indicated companies.

Immunohistochemistry staining

LUAD tissues were fastened in 4% paraformaldehyde for 16 h at room temperature and then dehydrated using decreasing ethanol concentrations. The tumor sections were incubated in 3% H_2O_2 solution at room temperature for 20 min and cleaned with PBS 3 times. Then, the cells were probed with monoclonal anti-RNF213 (1:200) at 4°C overnight. After 3 times of cleaning with PBS, histological sections were dealt with goat anti-rabbit at room temperature for 2 hours. Streptavidin/peroxidase complex and diaminobenzidine were used for immunostaining, and hematoxylin was used for counterstaining.

Statistical analysis

All data were attained from three independent experiments and are displayed as the mean ± SD. Graphs were drawn using GraphPad Prism 8.0 software (La Jolla, CA, USA). Survival analyses were performed with the Kaplan–Meier method and the differences were tested by log-rank test. The Statistical Product and Service Solutions (SPSS) 16.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Statistical significance was defined as $P<0.05$.

Results

RNF213 is downregulated and predicts poor survival in LUAD patients

As reported, the gene RNF213 is mutated in multiple

tumor tissues. However, many aspects of RNF213 activity in LUAD remain undetermined. To explore the expression of RNF213 in LUAD, we analyzed the TCGA database and GTEx database analysis and found that the expression of RNF213 in LUAD tissues (n=513) was significantly lower than that in normal lung tissues (n=397) (Figure 1A). To further explore the expression of RNF213 in LUAD, we analyzed the GEO database and found that the expression of RNF213 in LUAD tissues was lower than that in normal lung tissues (Figure 1B). To verify the results of the database, western blotting was employed to identify the expression of RNF213 in 15 cases of LUAD and adjacent tissues. As shown in Figure 1C-F, the RNF213 protein level was lower in LUAD tissues (P<0.0001). Additionally, the Kaplan-Meier Plotter database was used to predict the clinical significance of RNF213 in LUAD. The results proved that the patients with high RNF213 expression had longer overall survival (Figure 1G). Collectively, these results confirm that RNF213 is downregulated in LUAD, and prognostic analysis suggests that the lower the expression level of RNF213, the worse the prognosis of patients.

Mutational analyses of RNF213 in LUAD

It has been reported that RNF213 mutates in many kinds of tumors (24). To explore whether RNF213 mutates in LUAD species, we obtained the RNAseq data, mutant maf data and corresponding clinical information of LUAD from the TCGA data set. The somatic mutation of RNF213 in patients with LUAD was downloaded and visualized by using the maftools software package in R software (29) (Figure 2A). Moreover, we used Oncoplot to display the somatic landscape of LUAD tumor cohort. The results showed that the mutation rate of RNF213 was 4%, mainly missense mutation (Figure 2B-C). In addition, the results displayed that RNF213 mainly had Single-nucleotide polymorphism (SNP) (Figure 2D). SNP site could have

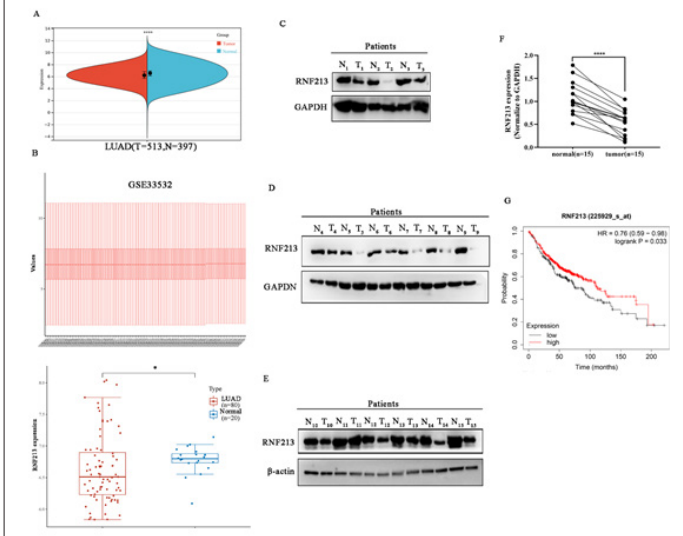

Figure 1. RNF213 is downregulated and predicts poor survival in LUAD patients. (A) RNF213 expression in LUAD tissues and lung tissues according to TCGA dataset. (B) RNF213 expression in LUAD tissues and lung tissues according to GSE33532. (C-F) Expression of RNF213 in LUAD and adjacent normal samples. (G) The Kaplan-Meier curve was plotted to evaluate the overall survival based on RNF213 expression in LUAD tissues from the Kaplan-Meier plotter database. Bars with different characters are statistically different at ***P <0.001 or ****P <0.0001.

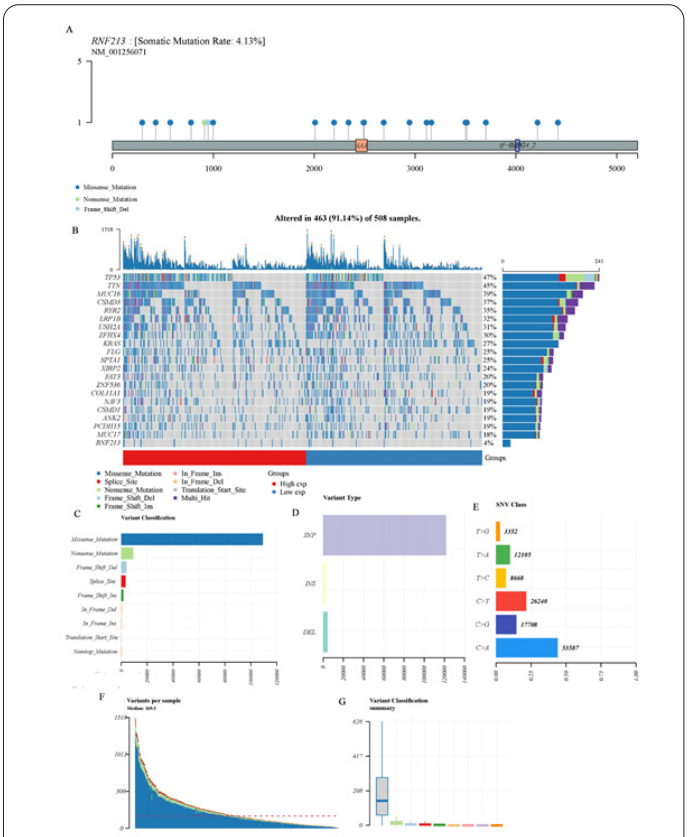

Figure 2. Mutational analyses of RNF213 in LUAD. (A) Lollipop charts of the mutated RNF213 gene. (B) Oncoplot of the somatic landscape of LUAD tumor cohort. (C) the distribution of variants according to variant classification. (D) the distribution of variants according to variant type. (E) the distribution of variants according to SNV class. (F) mutation load of each LUAD sample. (G) variant classification type of LUAD sample.

four different mutation forms including transformation, transversion, insertion or deletion. Then the classification of single nucleotide variants suggested that it was mainly the transformation from C to A (Figure 2E). As shown in Figure F, the mutation load of each LUAD sample was classified, and it was found that missense mutation is the main mutation type (Figure 2G). These results indicate that RNF213 has a mutation in LUAD.

RNF213 has no effect on the proliferation of LUAD cells

To investigate the biological function of RNF213 in LUAD, A549 and NCI-H1975 cells were transfected with shRNAs targeting RNF213. Stable cell lines were selected and were confirmed by western blotting (Figure 3A). Firstly, the CCK-8 assay was used to explore the effect of RNF213 on the proliferation of LUAD cells (Figure 3B-C). In addition, in order to further evaluate the phenomenon, we transfected A549 and NCI-H1975 cells with RNF213 overexpressing plasmid (Figure 3D). Meanwhile, the CCK8 results indicated that the proliferation of cells did not change significantly after overexpressing RNF213 (Figure 3E-F). The results conclude that there is no significant change in the proliferation of LUAD cells after knocking down or overexpressing RNF213.

RNF213 inhibits the invasion and migration of LUAD cells

To further investigate the biological significance of the

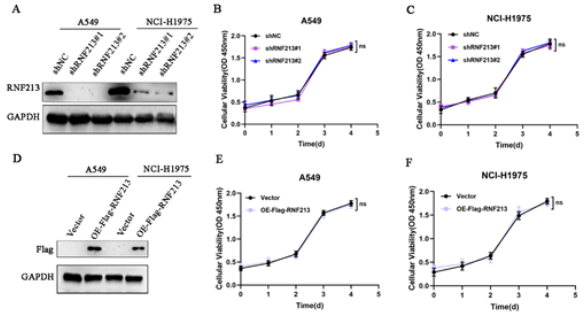

Figure 3. RNF213 has no effect on the proliferation of LUAD cells. (A) RNF213 expression levels in the transfected LUAD cell lines were detected using western blotting. (B-C) CCK-8 assay of RNF213 knockdown in A549 and NCI-H1975 cells. (D) RNF213 expression levels in the transfected LUAD cell lines were detected by western blotting. (E-F) CCK-8 assay of RNF213 overexpressing in A549 and NCI-H1975 cells. Bars with different characters are statistically different at ***P <0.001 or ****P <0.0001.

RNF213 in LUAD, we evaluated the correlation between RNF213 and the proteins of epithelial-mesenchymal transition (EMT). The results determined that RNF213 proteins was associated with epithelial-mesenchymal transition (Figure 4A). Additionally, we performed comprehensive co-expression analysis on the EMT-related genes. The results showed that EMT-related genes such as CRLF2, GPX7, IGFBP2, MGP, and NNMT were negatively correlated with RNF213 in LUAD (Figure 4B). To explore whether RNF213 regulates the migration and invasion of LUAD cells, we then applied a transwell assay. As shown in Figure 4C-F, RNF213 knockout promoted the migration and invasion of A549 and NCI-H1975 cells. Additionally, we transfected RNF213 overexpressing plasmid into A549 and NCI-H1975 cells to further investigate the phenomenon. The results showed that overexpression of RNF213 inhibited the migration and invasion of A549 and NCI-H1975 cells (Figure 4E-H). Taken together, these results indicate that RNF213 suppresses the migration and invasion of LUAD cells.

Assessment of the immunological characteristics of the RNF213 in LUAD

It has been reported that many immune-related genes and cells played a key role in tumors (30,31), we downloaded unified and standardized data sets from UCSC (https://xenabrowser.net/) database. Furthermore, we analyzed the expression data of RNF213 gene and 150 immune regulatory genes of five immune pathways in each LUAD sample and found RNF213 be correlated with 125 immunomodulators (Figure 5A). In addition, we explored the relationship between the RNF213 gene and 60 immune checkpoint pathway genes. The results showed that RNF213 was associated with 47 immune checkpoint genes (Figure 5B). In addition, the immune scores of LUAD patients were calculated according to the expression of RNF213 gene. The results indicated that there was a positive correlation between RNF213 gene expression and immune infiltration (P < 0.0001, r=0.21, Figure 5C). Finally, the B cell, $CD4^+$ T cell, $CD8^+$ T cell, Neutrophil, Macrophage, and DC infiltration scores of each patient in LUAD were evaluated according to the expression of RNF213 gene. The results showed that there was a positive correlation between the expression of B cell, $CD4^+$ T cell, $CD8^+$ T cell, Neutrophil, Macrophage, and DC immune cells and RNF213 expression (Figure 5D-G). In summary, the results suggest that the high expression level of RNF213 recruits more infiltrating immune cells and enhances the anti-tumor ability.

RNF213 as a novel marker in the prognosis of LUAD patients

The above results have indicated that RNF213 could suppress the migration and invasion of LUAD cells and that RNF213 expression is associated with prognosis in LUAD patients. To further investigate the clinical significance of RNF213 in LUAD, RNF213 immunohistochemical staining was performed on these sections from 64 LUAD patients (Figure 6A). The results showed that RNF213 was expressed lower in LUAD tissues compared to adjacent normal tissues (P<0.0001, Figure 6B-C). According to the RFN213 H-score cut-off value, 64 LUAD patients were divided into RNF213 high-expression group and RNF213 low-expression group. The results revealed that patients with high RNF213 expression (n=29) had longer OS than patients with low RNF213 expression (n=35) (P<0.0001, Figure 6D). In conclusion, RNF213 is expressed higher in tissue samples of LUAD patients, and

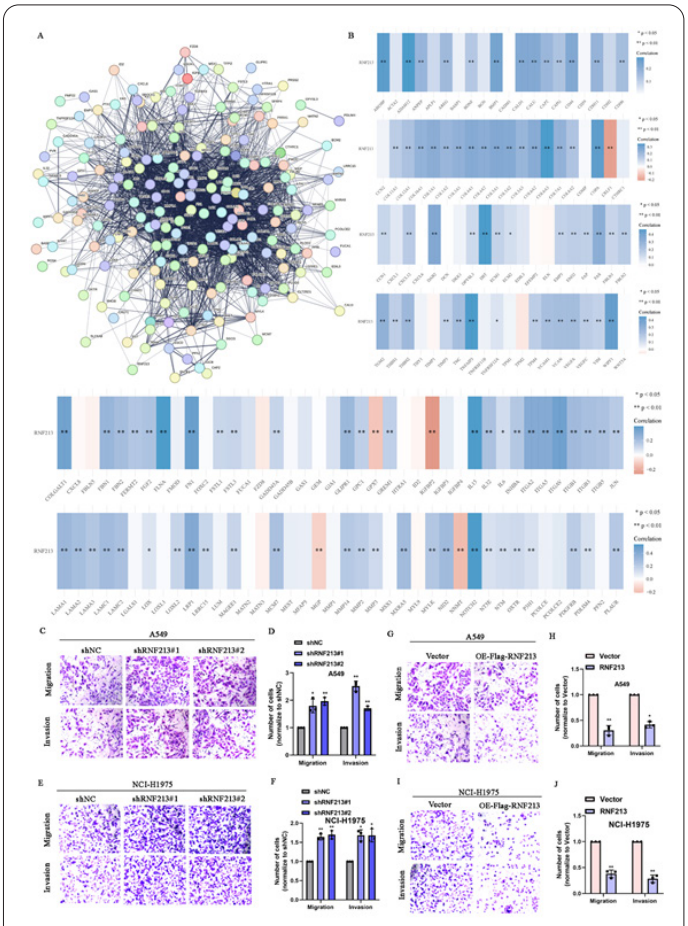

Figure 4. RNF213 inhibits the invasion and migration of LUAD cells. (A) The PPI network of RNF213 and EMT-related proteins was constructed using String. (B) Gene co-expression analysis of RNF213 and EMT-related genes. (C-D) Migration and invasion assay in A549 cells transfected with the RNF213 shRNA. (E-F) Migration and invasion assay in NCI-H1975 cells transfected with the RNF213 shRNA. (G-H) Migration and invasion assay in A549 cells infected with the RNF213 plasmid. (I-J) Migration and invasion assay in NCI-H1975 cells infected with the RNF213 plasmid. Bars with different characters are statistically different at * P < 0.05 or ** P < 0.01.

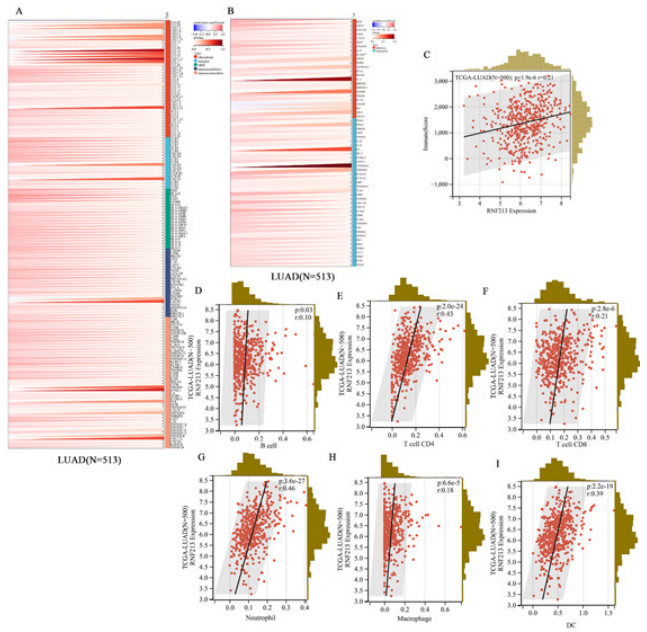

Figure 5. Assessment of the immunological characteristics of the RNF213 in LUAD. (A) Correlation between RNF213 and the immune regulatory genes. (B) Correlation between RNF213 and the inhibitory immune checkpoints. (C) Immune scores of RNF213 expression in LUAD. ($P < 0.0001$, r=0.21). (D) Correlation between RNF213 expression and B cell infiltration scores. ($P < 0.05$, r=0.10). (E) Correlation between RNF213 expression and $CD4^+$ T cell infiltration scores. ($P < 0.0001$, r=0.43). (F) Correlation between RNF213 expression and $CD8^+$ T cell infiltration scores. ($P < 0.0001$, r=0.21). (G) Correlation between RNF213 expression and Neutrophil infiltration scores. ($P < 0.0001$, r=0.46). (H) Correlation between RNF213 expression and Macrophage infiltration scores. ($P < 0.0001$, r=0.18). (I) Correlation between RNF213 expression and DC infiltration scores. ($P < 0.0001$, r=0.39)

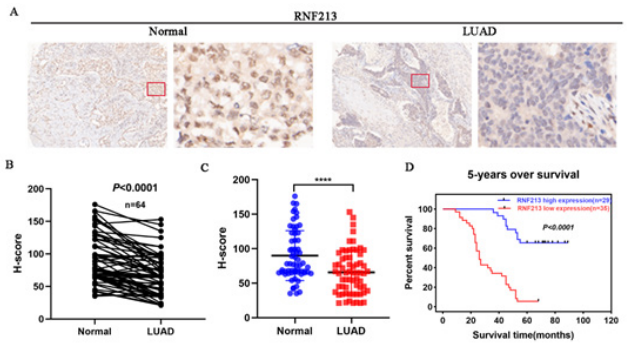

Figure 6. RNF213 as a novel marker in the prognosis of LUAD patients. (A) Representative immunohistochemical (IHC) staining of RNF213 in 64 LUAD tissues. (B-C) Expression of RNF213 in the LUAD and adjacent normal samples used in (A). (D) Kaplan–Meier survival curves showed better overall survival in high RNF213 expression group. Bars with different characters are statistically different at ****$p < 0.0001$.

high expression of RNF213 prolongs the overall survival of LUAD patients.

Discussion

Metastasis plays a key role in the progression of LUAD, which is a major factor affecting the prognosis. In terms of molecular mechanism, it involves a variety of related signaling pathways and numerous molecular components, such as metastasis-associated genes, Wnt and TGF-β pathways (32-34). It is well known that dominant genes in cancer can be divided into driver and suppressor genes (35). Driver genes promote tumor growth and proliferation. In contrast, suppressor genes inhibit the occurrence and development of tumors (36,37). In this study, RNF213 was confirmed to be a tumor suppressor of LUAD. Our data discovered that the expression of RNF213 in tumor tissues was reduced, which was consistent with the results of TCGA database and GTEx database analysis. This phenomenon may be due to the mutation of RNF213 gene in LUAD (38). A host of studies have shown that RNF213 gene mutations occur in multiple tumor tissues. In patients with glioblastomas, RNF213 and SLC26A11 formed fusion genes resulting in an abnormal copy number of RNF213 gene (39). RNA-seq detection revealed that RNF213 and SLC26A11 genes fused to form a new fusion gene RNF213-SlC26A11 in patients with LML (40). However, no corresponding studies for LUAD have been revealed. Therefore, we obtained the RNA seq data of LUAD from the TCGA data set and found that there was a mutation in RNF213, mainly in the form of base C conversion to A.

In this paper, we first demonstrated by Western blotting that RNF213 has a low expression level in LUAD tissue and further demonstrated this result with immunohistochemical analysis. In addition, the survival time of patients with low RNF213 expression was significantly shortened. Conversely, in patients with LUAD, higher RNF213 expression indicates improved clinical outcomes. RNF213 may be a prognostic factor for LUAD. However, the observation that high expression of RNF213 is associated with longer survival is preliminary and needs to be further confirmed. Little research has reported that RNF213 regulates signaling associated with tumorigenesis and development. In breast cancer, the PTP1B/RNF213/α-KGDD signaling is closely related to patient survival time (41). In addition, RNF213 was reported to be significantly down-regulated in glioma tissues and glioma cell lines, and RNF213 was found to inhibit the tumorigenesis of glioblastoma by affecting the MAPK/JNK signaling pathway (42). This study focused on the biological function of RNF213 in LUAD and confirmed that RNF213 inhibits the invasion and migration of LUAD cell. However, the specific pathway through which RNF213 exerts its anti-tumor effect needs to be further explored. We evaluated the immunological characteristics of RNF213 in patients with LUAD and found that RNF213 was positively correlated with a variety of immune cells, suggesting that RNF213 may play a role in tumor suppression by regulating the immune system.

As mentioned above, this study explored the effect and mechanism of RNF213 on the progression of LUAD. The results revealed that RNF213 could inhibit the migration of LUAD cells, which may be conducive to our clearer understanding of the progression mechanism of LUAD. Therefore, we hypothesized that RNF213 played a role in inhibiting tumor development. To verify this hypothesis, tissues from 64 patients with LUAD were obtained for immunohistochemical staining with RNF213. As shown in the results, RNF213 was expressed much lower in LUAD tissues. Importantly, we found that high expression of RNF213 in patients with LUAD was associated with a longer overall survival. Accordingly, the detection of RNF213 in tissues of patients with LUAD may become a

novel molecular marker to predict the prognosis of these patients, thus having clinical significance for the prognosis of patients with LUAD.

In conclusion, this study focused on elucidating the molecular mechanism of the RNF213 gene in LUAD cells. The results revealed that RNF213 could inhibit the migration of LUAD cells, suggesting that RNF213 had a cancer-suppressing effect in LUAD. Furthermore, the expression of RNF213 is positively correlated with the overall survival of patients with LUAD, which has clinical meaning for judging the prognosis of patients with LUAD.

Ethics approval and consent to participate

The authors confirm that all methods were conducted according to the principles of the Declaration of Helsinki. The study was approved by the Medical Ethics Committee of The Fifth Affiliated Hospital of Sun Yat-sen University (2023-K165-1). They confirm that informed consent was obtained from all subjects.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Data availability

The data applied in the bioinformatics analysis were obtained from TCGA, GTEx,UCSC and Kaplan-Meier Plotter database open-access database. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author contribution

XLL and HWZ performed most of the experiments and analyzed data; CRW, ZZL and ZYZ participated in the in vitro study. NC and XDL designed the overall study, and supervised the experiments. XLL wrote the paper. NC revised the paper. All authors read and approved the final manuscript.

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