Overexpression of RING Finger Protein 126 is Associated with Poor Prognosis and Contributes to the Progression of Lung Adenocarcinoma

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Research

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Abstract

Background

More recently, E3 ubiquitin ligases are well-informed to be involved in tumor development, and genetic aberration of this family have been implicated in breast cancer and oral cancer. RING finger protein 126 (RNF126), a newly uncovered E3 ubiquitin ligase, targets multiple proteins to promote their degradation. This study aims to explore the expression pattern and functional role of RNF126 in lung adenocarcinoma (LAD).

Methods

Immunohistochemical staining and real-time PCR were used to evaluate the expression pattern of RNF126 in normal lung tissue and LAD tissues. Western blot, cell proliferation and invasion assays were performed to investigate role of RNF126 in modulating LAD progression. Statistical analyses including Chi-square test, Kaplan-Meier test, Cox regression test, and Student's t-test were conducted for both clinical and experimental data.

Results

We find that both the mRNA and protein expression levels of RNF126 are elevated in LAD tissues, and its expression correlates with clinicopathologic features including tumor size and TNM stage. High expression of RNF126 indicates a poor prognosis of LAD patients. Gene perturbations reveal that RNF126 promotes LAD cells proliferation and xenograft growth. However, RNF126 exerts no significant effect on cell migration and invasion in LAD cells.

Conclusions

Our clinical and cellular data suggest that targeting this molecule could potentially provide advantages for LAD patients with high expression of RNF126.

Introduction

Lung cancer, as the most common cancer and the main cause of cancer-related death worldwide, leads to huge cancer burden nowadays[1]. Among all cases, approximately 85% are classified as non-small cell lung cancer (NSCLC), mainly including lung adenocarcinoma (LAD) and lung squamous carcinoma. Meanwhile, adenocarcinoma, the most common subtype of NSCLC, accounts for more than 50% and is the most aggressive histology type of lung cancer[2]. Even though great improvements have been made in treatment therapies, the survival of patients with LAD has not been largely improved[3]. Therefore, new biomarkers for prognosis and novel targets to promote the therapeutic effect are critically needed.

RING finger protein 126 (RNF126), a ring ubiquitin E3 ligase, contains two distinct domains: a zinc-finger domain near the N-terminus and a C-terminal RING domain[4]. As a E3 ligase, RNF126 typically

sequentially mediates protein ubiquitination, which regulates a multitude of cellular processes during tumorigenesis, including cell cycle progression[5]. More recently, several studies have revealed that RNF126 potentially functions as a tumor promoting enzyme by promoting homologous recombination (HR) and nonhomologous end joining (NHEJ) and by targeting a multiple of proteins for degradation[6]. For example, RNF126 facilitated HR-mediated DNA double-strand break repair through positively regulation of BRCA1 expression by direct interaction of E2F1, in a manner independent of its E3 ligase activity[7]. E2F1, a well-known transcription promotor, involved in a wide range of cellular process, including DNA repair, DNA replication, cell-cycle progression, differentiation, and apoptosis. The overexpression of RNF126 predicted a poor prognosis in invasive breast cancer, and also promoted the proliferation of breast cancer cells by targeting and ubiquitinating CDKN1A for degradation[8]. Similarly, recent studies reported the role of RNF126 on predicting unfavorable prognosis of ovarian cancer [9] and colorectal cancer [10]. However, the expression pattern and clinical significance of RNF126 in lung cancer remains unknown.

In this study, we demonstrated that RNF126 was highly expressed and was an independent predictor of a poor prognosis for LAD patients. We also revealed RNF126 knockdown suppressed proliferation of LAD cells, indicating the potential treatment therapy aimed at inhibiting RNF126. Targeting this E3 ligase might provide benefits for LAD patients with high expression of RNF126.

Methods

Patients and specimen collection

A total of 102 lung adenocarcinoma (LAD) patients were enrolled in this study. 29 human lung adenocarcinoma samples and the matched adjacent normal tissue specimens were obtained from the Yidu Central Hospital of Weifang (Weifang, China). The diagnosis of all patients was depended on histological examination. All patients involved in this study signed consent forms, and the collection of specimens was approved by the Yidu Central Hospital of Weifang Research Ethics Committee.

Immunohistochemical (IHC)

Full samples of 102 LAD tissue and matched adjacent normal lung tissues were performed for IHC staining. Briefly, 4 µm-thick TMA slides of these tissue sections were sequentially subjected to deparaffinization in xylene, rehydration in descending alcohol concentrations, antigen retrieval in a retrieval solution of citrate buffer, blocking with hydrogen peroxide, primary antibody incubation (anti-RNF126 antibody, ab183102), DAB staining and hematoxylin counterstaining[11]. RNF126 staining was evaluated with immunoreactive score (IRS): $IRS = \text{staining intensity} \times \text{percentage of positive cells}$. Staining intensity was scored as 0 for nongranulated; 1 for light yellow; 2 for brownish yellow; 3 for reddish yellow. The percentage of positive cells was marked as 0 for less than 5%; 1 for 5%-10%; 2 for 11%-50%; 3 for 51%-80%; 4 for more than 81%. The RNF126 staining results were independently assessed by two pathologists.

RNA isolation, reverse transcription and RT-qPCR

Total RNA was extracted from LAD and adjacent normal lung tissues using Trizol reagent (Invitrogen, Carlsbad, USA), and then cDNA was prepared using RT-PCR kit (TaKaRa, China) according to the manufacturer's instructions. Quantitative RT-PCR was performed with SYBR Premix Ex Taq II (Takara, China) to detect the expression of RNF126. The method of $2^{-\Delta\Delta Ct}$ was used to calculate the normalized expression of RNF126 with GAPDH as an internal reference genes. Experiments were performed in triplicate for each data point. The primers included in this study were as followings: RNF126 forward: CGCCTGCCGGATTATATCTGT

, reverse: CCGAAAGCAAACGTCCGTAG; GAPDH forward: GGAGCGAGATCCCTCCAAAAT, reverse, GGCTGTTGTCATACTTCTCATGG.

Cell culture and transfection condition

The human LAD cell A549, obtaining from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) was cultured in DMEM medium supplemented with 10% FBS (both from Hyclone, USA), in a humidified atmosphere of 5% CO₂ at 37 °C.

The shRNA (sequencing TGCATGGTTTGTGGCGGAAGA) and plasmid of human RNF126 used in this study were packaged into lentivirus by Yuanmin biotechnology Co., Ltd. (Shanghai, China) using blank vector as control. For the transfection, briefly, 3×10^5 cells were seeded in each well of a six-well plate. Then, the lentiviruses were added to the well with 1ml of DMEM containing no FBS and 5 µg/ml Polybrene (Sigma, USA). 24 hours later, medium containing the virus was removed and replaced with medium containing 10% FBS. Then, expression of RNF126 was validated by Western blot.

Western blot

Total cell extracts were prepared in RIPA buffer supplemented with complete protease inhibitors cocktail (Roche Applied Science) at 4 °C for an hour. The lysates were cleared by centrifugation (12000rpm for 15min), and then the total protein contents were measured by Pierce™ BCA Protein assay kit (Thermo Fisher Scientific, USA). The lysates were isolated by SDS-PAGE, transferred to PVDF membrane filters and probed with anti-RNF126 rabbit antibody (ab183102) and anti-GAPDH mouse antibody (Life Technologies), following by incubating with HRP-conjugated secondary antibodies. Densitometry of obtained signals was semi-quantified with the ImageJ software.

Cell proliferation and viability

The Transient RNF126 knockdown and overexpressed A549 cells were reseeded on 96-well plates at 3000 cells/well. The effect of RNF126 on A549 cells proliferation was determined by MTT assay at daily interval for five days. 20 µL of 5 mg/ml MTT was added to each well and incubated for another 4 hours, then the medium was discarded and 150 µL DMSO was added to dissolve the precipitated formazan. Absorbance was measured at 450 nm.

Cell migration and invasion

Cell migration and invasion were assessed using Transwell chambers (Costar, High Wycombe, UK). For invasion assay, the chamber inserts were pre-coated with Matrigel (BD Biosciences, NJ, USA). Briefly, A549 cells transiently transfected with RNF126-shRNA or RNF126-construct for 24h were re-suspended and added in the upper chamber in serum-free medium. 20% fetal calf serum medium was added to the bottom chamber. After migrating or invading through the chamber membrane at 37 °C with 5% CO₂ for 24 hours, the cells on the membrane were fixed with paraformaldehyde and stained with 0.1% crystal violet for 15 min. The number of cells were counted by microscope and measurements were calculated in triplicate.

Mice study

The experimental protocol was approved by the Medical Experimental Animal Care Commission of Yidu Central Hospital following the laboratory animal welfare [12]. Male BALB/c nu/nu mice (4 weeks old) were purchased from the Shanghai Institute of Materia Medical, and housed under specific pathogen-free conditions as required. Mice were randomly assigned to the control group, knockdown group, and overexpression group. Stable transfected A549 cells (5×10^6 cells) in 200 ul of normal saline was implanted by subcutaneous injection to obtain corresponding subcutaneous tumors. The tumor growth was monitored by calculating tumor volume. Twenty-days later, all enrolled mice were sacrificed to obtain xenografts.

Statistical analysis

The differences between two groups were analyzed with Student's t-test (two-tailed). The differences among multiple groups were analyzed by One-way ANOVA test. Chi-square test was applied for the statistical analyses of cases in groups. Survival curves were performed with the Kaplan-Meier method and log-rank test was used to compare the curves. Cox regression model was conducted to modify potential prognostic variables. $P < 0.05$ was considered statistically significant.

Results

RNF126 is overexpressed and correlates with clinicopathological factors in LAD tissue

To probe the role of RNF126 in cases of lung adenocarcinoma (LAD), we collected 29 LAD samples and the matched adjuvant normal tissues for study. RNF126 expression was detected by IHC (Figure 1A and B). The staining of RNF126 in LAD was much stronger than normal tissues. Meanwhile, figure 1C showed the gene level of RNF126 was significant over-expressed in LAD tissue relative to normal tissue ($p=0.046$). The total of 102 patients involved in the study were divided into low expression group and high expression group according to the IHC staining scores. As summarized in Table 1, RNF126 over-expression was strongly associated with larger tumor diameter and advanced TNM stage.

Prognostic value of RNF126 in LAD patients

Kaplan–Meier plots was established to present the relationship between overall survival and clinicopathological factors in LAD patients, as well as RNF126 expression (Table 2). According to log-rank test, gender ($p = 0.003$), tumor differentiation ($p = 0.001$), LN metastasis ($p = 0.003$) and TNM stage ($p = 0.017$) were statistically significant prognostic factors (Figure 2C, F, G, H). The p value for age and tumor diameter were more than 0.05, indicating these two parameters had no obvious association with overall survival in our included panel of LAD patients. Most importantly, among this panel of 102 LAD patients, Kaplan–Meier plots of positive versus negative RNF126 suggested high RNF126 expression reflected a low cumulative probability and predicted a poor prognosis ($p = 0.043$). We further cross-validated the correlation between *RNF126* gene transcription with patient survival through K-M plotter database (<http://www.kmplot.com/>) [13]. As a result, higher *RNF126* level is correlated with poorer overall survival (Figure 3A, $p < 0.001$) and poorer disease-free survival (Figure 3B, $p = 0.006$). Therefore, RNF126 was identified as a novel candidate gene for prognosis prediction.

In addition, by multivariate Cox regression analysis, elder age (HR=2.511, 95% CI = 1.227-5.140, $p = 0.012$), lymph node metastasis (HR=2.591, 95% CI = 1.314-5.108, $p = 0.006$), advanced TNM stage (HR=2.825, 95% CI = 1.282-6.223, $p = 0.01$) and high RNF126 expression (HR=2.372, 95% CI = 1.192-4.719, $p = 0.014$) were related to poor outcomes (Table 3). We therefore concluded that RNF126 served as an independent prognostic biomarker for LAD.

RNF126 promotes LAD growth both in vitro and in vivo

To determine whether RNF26 was required for cell growth or migration, we depleted or overexpressed RNF126 in A549 LAD cell line (Figure 4A). Meanwhile, RNF126 overexpression inhibited p21 expression, indicating RNF126 might play key role in regulation of cell cycle. When we assayed for cell proliferation using MTT assay, a significant reducing in cell viability in RNF126-knockdown cells and a dramatic increase in RNF126-overexpressed cells were observed as compared to control A549 cells (Figure 4B). However, no differences were observed in cell migration and invasion between control and RNAi-treated or construct-transfected cells (Figure 4C and D; $P = 0.142$ and $P = 0.458$, respectively).

Finally, we conducted xenograft assay by subcutaneous injecting stable-transfected cells into nude mice. By monitoring the tumor growth curve (Figure 4E) and comparing the size of isolated tumors (Figure 4F), we confirmed that overexpressing RNF126 significantly promoted LAD growth while silencing RNF126 resulted in opposite effects. Taken together, our data showed RNF126 played a critical role in LAD growth.

Discussion

Posttranslational modification of protein, including ubiquitination and phosphorylation, plays critical role in activating protein and regulating cellular function. Protein ubiquitination, typically recruiting a catalytic reaction to degradation, is mainly mediated by three enzymes: E1 ubiquitin activating, E2 ubiquitin conjugating, and E3 ubiquitin ligase[14]. Nowadays, E3 ligases have been considered as the potential

novel targets for therapies of certain diseases. Hundreds of E3 ubiquitin ligases are uncovered in mammalian cells to process a variety of protein degrons[15].

RNF126, one of the E3 ligase members, features a zinc-binding RING finger motif which functions as a recognition site for substrates or complexes [16]. RNF126 controls CHER expression through a direct interaction with E2F1, a transcriptional promotor that regulates hundreds of genes including a variety of oncogenes[8]. RNF126 plays a novel role in promoting HR-mediated DNA repair via positively regulating BRCA1 expression through targeting E2F1[7]. P21, a cell-cycle dependent kinase inhibitor, is directed targeted by E3 ligase RNF126 for ubiquitin-mediated degradation, indicating RNF126 promotes cell proliferation via interact with P21[17]. Moreover, RNF126 binds with the epidermal growth factor receptor (EGFR) through a ubiquitin-binding zinc finger domain and promotes ubiquitylation of EGFR in HeLa cells[18].

E3 ubiquitin ligases have been confirmed to be involved in the occurrence of breast cancer, lung cancer and oral cancer[19, 20]. Most recently, several studies highlighted the relevance of RNF126 and breast cancer prognosis and tumorigenesis. RNF126 protein expression elevated in breast cancer tissues, and the high expression of RNF126 predicted a poor prognosis for patients with breast cancer[8]. Knockdown of RNF126 dramatically delayed cell-cycle G1-S progression and decreased cells viability in breast cancer and prostate cancer cell lines[17]. Here, we demonstrated that the gene and protein expression levels of RNF126 were both increased in lung adenocarcinoma (LAD) compared with adjuvant normal tissues. The expression of RNF126 is associated with the overall survival of patients. Critically, multivariate analysis showed high expression of RNF126 is an independent factor for poor prognosis (HR 2.372, 95% CI 1.192–4.719), which is independent from established prognostic factors such as TNM stage and lymph node. In addition, according to *in vitro* and *in vivo* data, knocking down RNF126 significantly attenuated LAD cells proliferation while overexpressing RNF126 promoted LAD growth. Our results also showed that RNF126 negatively control p21 expression in LAD cells, demonstrating the critical role of RNF126 in cell cycle progression although we didn't dig into the detailed underlying mechanisms. Of note, no significant alternation on tumor migration and invasion were observed upon RNF126 knock down or overexpression. Our results indicated that RNF126 was responsible for LAD growth, potentially guiding the development of novel therapies targeted at LAD.

Conclusion

In summary, we have identified RNF126 as a new gene essential for LAD progression. Our results provided a biology understanding of RNF126 function and offers a potential therapy directed at LAD.

Declarations

Statement of Ethics: This research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. Subjects have given their written informed consent and the study protocol was approved by the Yidu Central Hospital of Weifang Research Ethics Committee.

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Conflict of interest: The authors declare no conflict of interest.

Author Contributions: Zhaona Sun conducted statistical analysis and wrote this manuscript; Meiyuan Chen collected clinical specimens and performed IHC experiments; Ziping Li contributed to cellular assays; Hong Zhang designed this project.

Data availability: Data will be available upon request.

Consent for publication: Consent to publish has been obtained from all participants.

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Tables

Table 1. Correlations between RNF126 expression with patients’ clinicopathologic features.

Clinicopathologic features	Cases	RNF126 level		P value
	(n=102)	Low (n=54)	High (n=48)	
Gender				0.895
Female	27	14	13	
Male	75	40	35	
Age				0.427
≤60 yrs	51	25	26	
>60 yrs	51	29	22	
Tumor diameter				<0.001*
≤5.0 cm	66	44	22	
>5.0 cm	36	10	26	
Differentiation				0.962
Well/moderate	64	34	30	
Poor	38	20	18	
Lymph node				0.393
Negative	66	37	29	
Positive	36	17	19	
TNM stage				0.016*
I-II	49	32	17	
III-IV	53	22	31	

Table 2. Kaplan-Meier overall survival analyses.

Clinicopathologic features	OS months (Mean ± S.D.)	5-year OS (%)	P value
Gender			
Female	67.6 ± 4.3	72.6%	0.043*
Male	55.5 ± 3.2	44.2%	
Age			
≤60 yrs	67.0 ± 3.2	65.9%	0.003*
>60 yrs	51.8 ± 3.9	41.5%	
Tumor diameter			
≤5.0 cm	60.6 ± 3.1	63.5%	0.198
>5.0 cm	55.7 ± 4.1	42.0%	
Differentiation			
Well/moderate	58.4 ± 3.3	51.6%	0.672
Poor	60.2 ± 4.5	59.0%	
Lymph node			
Negative	66.3 ± 3.1	68.1%	0.001*
Positive	49.2 ± 4.1	34.0%	
TNM stage			
I-II	69.1 ± 3.7	71.1%	0.003*
III-IV	52.7 ± 3.5	42.7%	
RNF126 level			
Low	66.0 ± 3.7	66.3%	0.017*
High	53.5 ± 3.5	42.8%	

Table 3. Multivariate analysis.

Clinicopathologic features	HR	95% CI	P value
Gender (male vs female)	2.110	0.932-4.780	0.073
Age (>60 yrs vs ≤60 yrs)	2.511	1.227-5.140	0.012
Lymph node (positive vs negative)	2.591	1.314-5.108	0.006*
TNM stage (III-IV vs I-II)	2.825	1.282-6.223	0.010*
RNF126 (high vs low)	2.372	1.192-4.719	0.014*

Figures

Figure 1

Expression of RNF126 in LAD tissue. (A). IHC staining of RNF126 exhibited strong positive expression in LAD tissue compared to matched adjuvant normal tissues. (B). Quantitative RT-PCR showed the gene level of RNF126 in LAD samples elevated significantly. *P < 0.05 compared with normal group.

Figure 2

Overall survival curve of LAD patients in our cohort (A) Kaplan-Meier analysis of the overall survival for all LAD patients. Kaplan-Meier analysis of the overall survival in 102 LAD patients in relation to RNF126 level (B), gender (C), age (D), tumor diameter (E), tumor differentiation (F), LN metastasis (G) and TNM stage (H).

Figure 3

Prognostic significance of *RNF126* transcription as reflected from online database. Kaplan–Meier analyses of *RNF126* transcription for overall survival (A) and disease-free survival (B) of patients generated by K-M plotter database (<http://kmplot.com/>). The overall survival and disease-free survival of the high-*RNF126* group were both significantly worse than that of low-*RNF126* group ($p=1.3e-08$ and $p=0.0056$, respectively).

Figure 4

Effect of RNF126 on the proliferation and metastasis of LAD cells (A) Western blot presented the expression of p21 was downregulated after RNF126 over-expression (overexpression group), while increased after silencing RNF126 by shRNA (knockdown group). (B) MTT assays of A549 cells with RNF126 silencing and overexpression indicated the promoting effect of RNF126 on cellular proliferation. (C, D) Silencing or overexpressing RNF126 showed no significant regulation on cell migration and

invasion as revealed by transwell invasion assays. (E) Stable-transfected cells were subcutaneously injected into nude mice to obtain a xenograft model. Growth curve of xenografts were plotted and reflected a positive effect of RNF126 on tumor growth. (F) The isolated xenografts from sacrificed mice showed a significant larger size in RNF126-overexpressin group while a smaller size in RNF126-knockdown group. *P < 0.05 by One-way ANOVA test.