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RESEARCH

Polycomb Group Oncogene RING1 is Over-expressed in Non-Small Cell Lung Cancer

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Abstract Ring finger protein 1 (RING1) have recently been reported to be related to aggressive tumor features in Prostate Cancer and urothelial carcinoma of the bladder. However, the role of RING1 in non-small-cell lung cancer (NSCLC) tumorigenesis has never been elucidated. So we aimed at investigating the potential role of RING1 in NSCLC. RING1 expression was evaluated by Immunoblot in 8 paired fresh lung cancer tissues and immunohistochemistry on 69 paraffin-embedded sections from 2006 to 2009. Furthermore, flow-cytometry and RNA interference were performed to analyse the role of RING1 in A549 cells. We showed that the expression level of RING1 was significant increased in lung cancer as compared with the adjacent normal tissue. High expression level of RING1 was associated with TNM stage ($P=0.013$), and RING1 was positively related with proliferation marker Ki67 ($P<0.05$). Moreover, RING1 knockdown induces growth suppression of human lung cancer cells through G1/S cell cycle phase arrest in vitro. Kaplan–Meier survival curves showed that high expression level of RING1 was associated with poor prognosis ($P=0.03$). On the basis of these results, we suggested that RING1 protein expression may be a favorable independent prognostic parameter for non-small cell lung cancer.

Keywords Non-small cell lung cancer · RING1 · Prognosis

Abbreviations

NSCLC	Non-small cell lung cancer
PcG	Poly-comb group
CDK2	Cyclin-dependent kinase 2
GWAS	Genome-wide association studies

Introduction

Lung cancer is a major causes of cancer-related death worldwide. Its 5-year survival rate is next to the lowest of all cancers [20]. Non-small cell lung cancer (NSCLC) is the most common type of lung cancer [13]. Despite advances in early detection and standard treatment, the mortality rates of lung cancer have been increasing rapidly in the last three decades. One reason for such a low survival rate is that patients do not receive treatment early enough in disease progression. In order to develop more effective therapies, it is important to obtain a better understanding of the molecular biology of lung cancer.

RING1 is a member of PcG protein that contains the RING finger motif, a specific zinc-binding domain, which is found in many regulatory proteins, and function as transcriptional suppressors [25, 26]. RING1 is firstly reported as a transcriptional repressor, which interacts with the Poly-comb Group Protein Complex and displays tumorigenic activity [24–26]. Although

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published studies have examined the role of RING1 in prostate cancer and bladder cancer [11, 29], so far no study has investigated the role of RING1 expression in the progression of lung cancer.

In the current study, we investigated the clinical significance of RING1 expression in non-small cell lung cancer patients and the correlation between RING1 expression and clinicopathological characteristics by immunohistochemistry in all stages of NSCLC from early to advanced. This study found that the expression level of RING1 was correlated strongly with the TNM stage ($p=0.013$) and the overall survival. In addition, the usefulness of RING1 as a prognostic factor was evaluated by Kaplan–Meier survival curves analysis. These data revealed that RING1 might be a lung cancer-associated molecule with a prognostic value.

Materials and Method

Patients

92 lung cancer tissues were obtained, in which 69 NSCLC surgical specimens (25 adenocarcinomas, 30 squamous cell carcinomas, plus 14 specimens classified as "other", which are 9 adenosquamous carcinoma, 5 large cell carcinoma were evaluated[15, 28]. All NSCLC tissues were collected using protocols approved by the Ethics Committee of Cancer Hospital of Nantong University and written informed consent was obtained from every patient. Patients underwent lung surgical resection without postoperative international standard radio- and/or chemotherapeutic. 69 lung cancer tissues were obtained from the pathology files of the Department of Pathology at Affiliated Hospital of Nantong University from 2006 to 2009 under the auspices of an institutional review board-approved human subjects study protocol. The diagnosis was confirmed histologically in all cases, based mainly on examination of sections stained with H&E. Their ages ranged from 26 to 77 years, with an average age of 62.12 years. The male:female ratio was 39:30. Histological grades were classified to well (grade I),moderately (grade II), and poorly differentiated (grade III). Of the 69 patients examined, 23 were classified as grade I, 26 were classified as grade II, and 20 were classified as grade III. All tumors were from patients newly diagnosed with lung cancer, who had received neither therapy before sample collection nor received postoperative platinum-based chemotherapy. The follow-up time for 69 died patients ranging from 1 to 60 months. Formalin-fixed, paraffin-embedded sections were prepared for all tissues and reviewed by 3 pathologists. Clinical data (patient history, diagnosis, staging and survival) were obtained from the National Cancer Institute "Regina Elena" databases. Survival data were integrated by periodic interviews with their relatives. Tissue specimens were immediately processed after surgical removal. For histological examination, all tumorous and surrounding non-tumorous tissue portions were processed into 10 % buffered formalin-fixed, paraffin-embedded blocks. Protein was analyzed in 8 snap-frozen tumorous and adjacent non-tumorous tissue samples that were stored at −80 °C.

Immunohistochemistry

The sections were deparaffinized using a graded ethanol series and endogenous peroxidase activity was blocked by soaking in 0.3 % hydrogen peroxide. Thereafter, the sections were processed in 10 mM citrate buffer (pH 6.0) and heated to 121 °C in an autoclave for 20 min to retrieve the antigen. After rinsing in phosphate-buffered saline (PBS; pH 7.2), 10 % goat serum was applied for 1 h at room temperature to block any non-specific reactions. The sections were then incubated 4 h with anti-human RING1 monoclonal antibody (diluted 1:100; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-Ki67 mouse monoclonal antibody (diluted 1:100; Santa Cruz Biotechnology). Negative control slides were also processed in parallel using a non-specific immunoglobulin IgG (diluted 1:100; Santa Cruz Biotechnology) at the same concentration as the primary antibody. All slides were processed using the peroxidase-anti-peroxidase method (Dako, Hamburg, Germany). After rinsing in water, the peroxidase sections were counterstained with hematoxylin, dehydrated, and cover-slipped [34] .

Immunohistochemical Evaluation

Stained sections were observed under a microscope. All of the immunostained sections were evaluated in a blinded manner without knowledge of the clinical and pathological parameters of the patients. For assessment of RING1 and Ki67, 5 high-power fields in each specimen were selected randomly, and cell staining was examined under high-power magnification. More than 500 cells were counted to determine the mean percent, which represented the percentage of immunostained cells related to the total number of cells [33]. IHC staining was scored according to the following criteria: The intensity of RING1 cytoplasmic staining was scored as 0 (no staining), 1 (weak), 2 (marked). Percentage scores were assigned as 1- 1–25 %, 2- 26–50 % and 3- 51–100 %. The scores of each tumor sample were multiplied to give a final score of 0 to 6 and the total expression of RING1 was determined as either negative or low expression (-): score <3; positive expression or high expression (+): score ≥3 [18]. In half of the samples, staining was repeated twice to avoid possible technical errors, but similar results were obtained in these samples.

Immunoblot Analysis

Before immunoblotting, cells were washed three times with ice-cold PBS, re-suspended in lysis buffer (50

mMTris-HCl, 120 mM NaCl, 0.5 % Nonidet P-40, 100 mM NaF, 200 lMNa3VO4, and protease inhibitor mixture) or frozen tissues were homogenized in lysis buffer (1 % NP-40, 50 mM/l Tris, pH 7.5, 5 mM/l EDTA, 1 % SDS, 1%sodium deoxycholate, 1 % Triton X-100, 1 mM/l PMSF, 10 mg/ml aprotInin, and 1 mg/ml leupeptin), and then incubated for 20 min at 4 °C while rocking. Lysates were cleared by centrifugation (10 min, 12,000 rpm, 4 °C). 50 μg of total protein was resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Immbilon, Millipore). The membranes were firstly blocked with 5 % nonfat dry milk and then incubated with the primary antibody described above for 2 h at room temperature. After three times of washes, filters were incubated with horseradish peroxidase-conjugated secondary human anti-mouse or anti-rabbit antibodies (1:1,000; pierce) for 1 h at room temperature according to the manufacturer's instructions. Detection of immunocomplexes was performed with an enhanced chemiluminescence system (NEN Life Science Products, Boston, MA) [31].

Cell Cultures and Cell Cycle Analysis

A549, human lung carcinoma cell line, was obtained from the Institute of Cell Biology, Academic Sinica and cultured in DMEM supplemented with 10 % fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in 5 % CO2 at 37 °C. For cell cycle analysis, cells were fixed in 70 % ethanol for 1 h at 4 °C, and then incubated with 1 mg/ml RNase A for 30 min at 37 °C. Subsequently, cells were stained with propidiumiodide (50 μg/ml PI) (Becton Dickinson, San Jose, CA) in PBS, 0.5 % triton-X 100, and analyzed using a Becton Dickinson flow cytometer BD FACSCAN (San Jose, CA) and Cell Quest acquisition and analysis programs [30].

Si-RNA and Transfection

Small interference RNAs (siRNA) were chemically synthesized (GenePharma Co. Ltd). The synthesized oligonucleotides for RNA interference (RNAi) RING1 targeted the sequence: 5′-CCGUGGCUUACUACAAGAA-3′ while a non-specific scrambled si-RNA with a sequence of 5′-UUCUCC GAACGUGUCACGU-3′ was used as a negative control. A549 cells were seeded the day before transfection using the Dulbeccomodified Eagle medium with 10 % FBS but without antibiotics. For transient transfection, the RING1 siRNA vector and the negative control vector were carried out by adding the mixture of si-RNA and the si-RNA transfection reagent (Santa Cruz Biotechnology) and plus si-RNA dilution buffer as suggested by the manufacturer. Transfected cells were used for the subsequent experiments 48 h after transfection[19].

Statistical Analysis

Statistical analysis was performed using the Stat View 5.0 software package. The association between RING1 and Ki67 expression and clinicopathological variables were computed using the $\chi 2$ test. Ki67 and RING1 expression in human Non-small cell lung carcinoma (NSCLC) was studied using the Spearman rank correlation test because the data were not normally distributed. Survival estimates were computed using the Kaplan-Meier method and the log-rank test was used for analysis. $P<0.05$ was considered statistically significant in the statistical analysis.

Result

Increased Expression of RING1 in NSCLC

To determine whether the level of RING1 expression is associated with the progression of NSCLC, comparative analysis

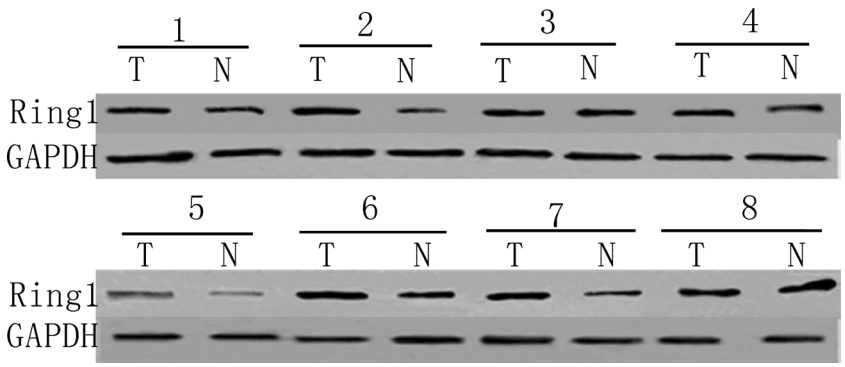

Fig. 1 RING1 is high expressed in NSCLC but low in paired adjacent normal tissues. Immunoblot of eight representative paired samples of NSCLC tissue (*T*) and adjacent normal tissues (*N*) Whole-cell lysates were prepared from tissue specimens obtained from NSCLC and adjacent normal tissues. In all samples tested, RING1 expression levels were significantly higher in NSCLC than in paired adjacent normal tissues. GAPDH was used as a control for protein load and integrity

of RING1 expression was conducted on eight pairs of matched lung cancer tissue and the non-cancerous tissue adjacent to the malignant lesion using Immunoblotting analyses. As shown in Fig. 1, the expression of RING1 protein was dramatically increased expression in tumors tissues compared with the adjacent normal tissue, which showed high Ring1 expression ($P<0.05$; Fig. 1).

Correlation Between Increased Expression of RING1 and Malignancy of NSCLC

To determine whether the expression level of RING1 protein is associated with the histological characteristics of NSCLC, 69 paraffin-embedded, achieved NSCLC clinical specimens, which included 23 cases of stage I (Fig. 2c); 26 cases of stage II (Fig. 2e), 20 cases of stage III (Fig. 2g), were examined by immunohistochemical staining. RING1 expression in lung cancer was scored ≥3 as positive (Fig. 2e). Its percentage of positive cell ranged from 8.04 % to 78.47 %. The mean percentage of positive cell was 46 %. Ki67 expression in lung cancer was scored as positive when strong nuclei staining (Fig. 2d). Its percentage of positive cell ranged from 0.85 %

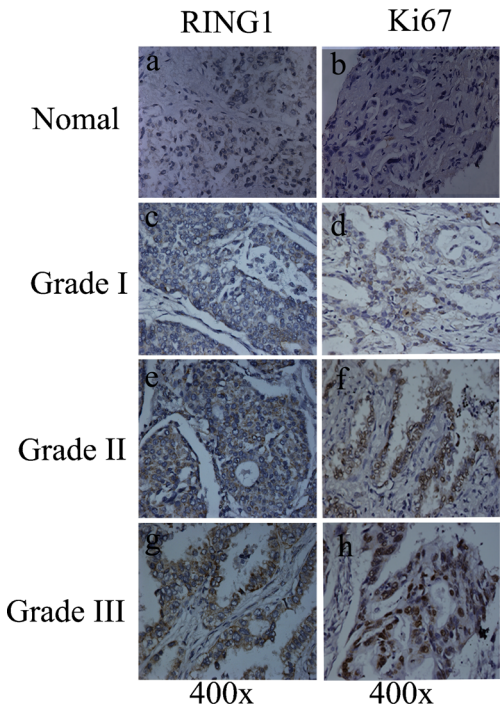

Fig. 2 Immunohistochemical staining of RING1 and Ki67 in adjacent normal tissues (**a** and **b**) and NSCLC tissues (**c**, **d**, **e**, **f**, **g** and **h**). Paraffin-embedded tissue sections were stained with antibodies for RING1 and Ki67 and counterstained with hematoxylin. RING1 negative staining was shown in normal human lung tissue (**a**). RING1 was detected in NSCLC with high expressed, which staining predominant in the cytoplasm (**c**, **e** and **g**). Ki67 negative staining was shown in normal human lung tissue (**b**). Ki67 positive nuclear staining was shown in NSCLC (**d**, **f** and **h**)

Table 1 Ring1 expression and clinicopathological parameters in 69 NSCLC specimens

Parameters	Number of patients	Ring1		p^a
		Negative	Positive	
Age				
<60	26	8	18	0.198
>=60	43	19	24	
Gender				
Male	39	17	22	0.269
Female	30	10	20	
Histologe				
Adenocarcinoma	25	13	12	0.216
Squamous cell carcinoma	30	12	18	
Other	14	9	5	
Differentiation				
Well-moderate	36	12	24	0.569
Poor	33	15	18	
TNM stage				
I	23	8	15	0.013
II	26	6	29	
III	20	13	7	
Tumor status				
I	25	9	16	0.442
II + III	44	18	26	
Nodal status				
N0	26	10	16	0.569
N1+N2+N3	43	17	26	

[a] Statistical analyses were performed by the Pearson $\chi 2$ test. $P<0.05$ was considered significant

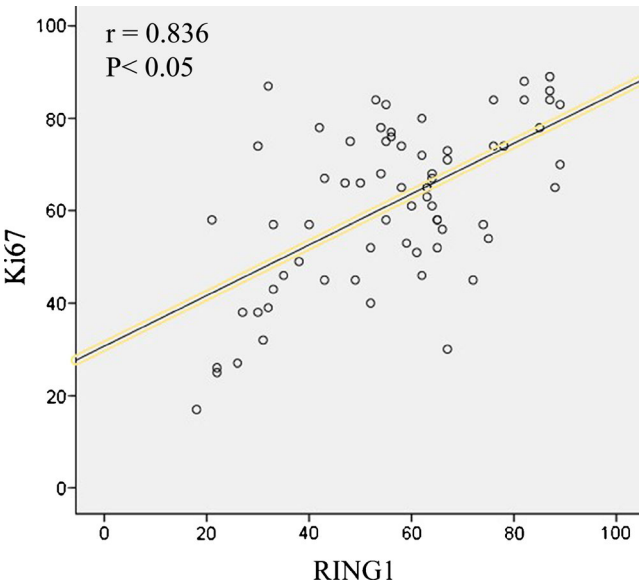

Fig. 3 Relationship between Ki67 proliferation index and RING1 expression in NSCLC. Scatterplot of Ki67 versus RING1 with regression line showing a correlation of them using the Spearman's correlation coefficient

to 86.38 %. The mean percentage of positive cells was 26 %. As shown in Fig. 2, RING1 was found to be up-regulated in NSCLC compared with that in the normal lung tissue (Fig. 2a).

Correlation Between RING1 Expression and Clinicopathological Parameters in Lung Cancer

The clinicopathological data of patients are summarized in Table 1. As shown in Table 1, we evaluated the association of RING1 expression with clinicopathological variables. For statistical analysis, the expression of RING1 in the carcinoma specimens were divided into 2 groups, namely, high and low expressions, according to the percentage of RING1-positive cells and the intensity of RING1 cytoplasmic staining, RING1 expression is not associated with patients' age, gender, histology, tumor differentiation, tumor status and nodal status, but RING1 expression was significantly correlated with TNM stage ($P=0.013$). Furthermore, among most specimens, the proportion of RING1-positive tumor cells was correlated with the proportion of Ki67-positive tumor cells. As continuous variables, RING1 expression was positively associated with Ki67 expression ($r=0.836$, $P<0.05$; Fig. 3).

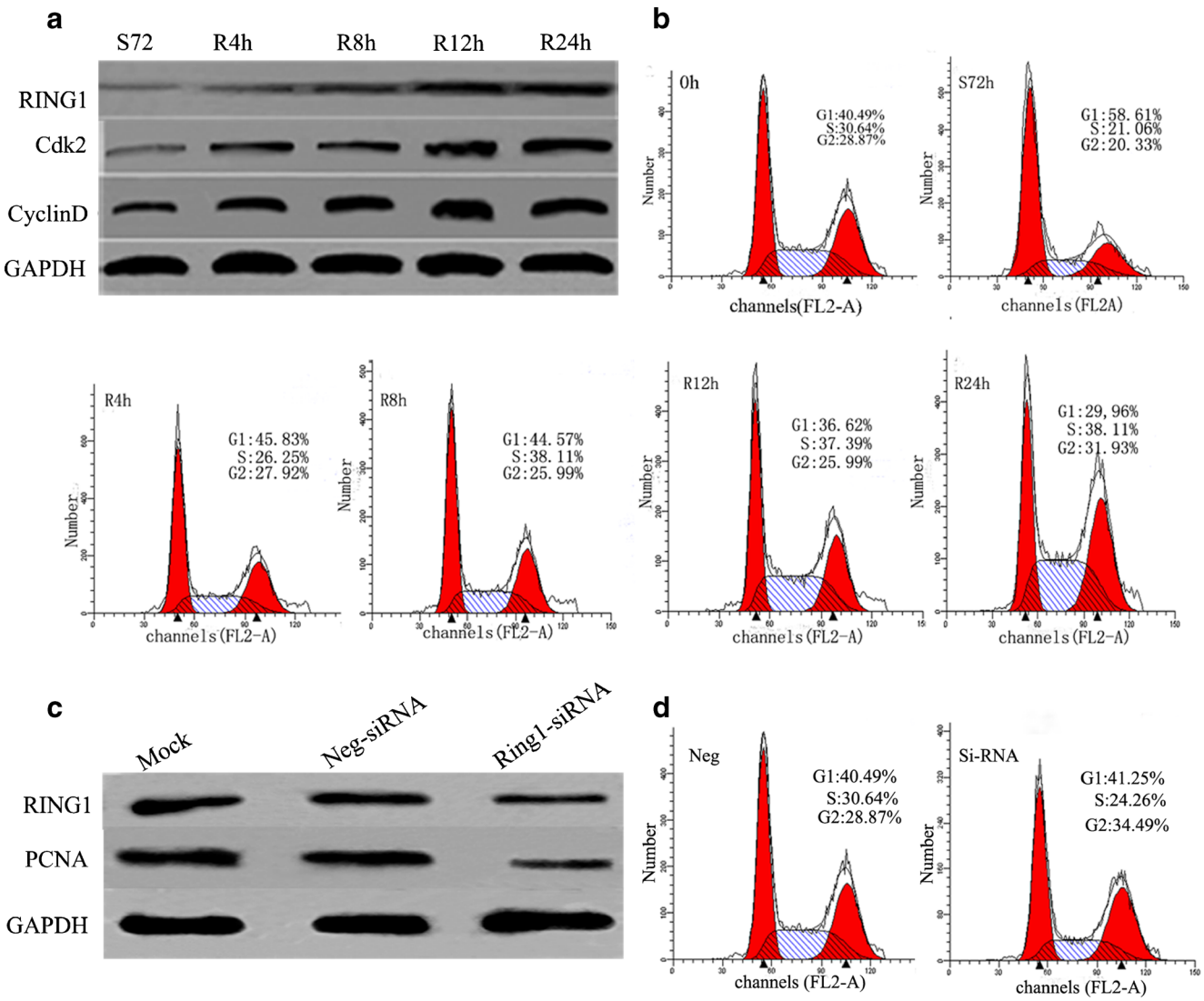

Fig. 4 The expression of RING1 and cell cycle-related molecules in proliferating NSCLC cells. **a** A549 cells were serum starved for 72 h and upon serum releasing, cell lysates were prepared and analyzed by Immunoblot using antibodies directed against RING1, CDK2 and cyclin-D. GAPDH was used as a control for protein load and integrity. **b** Flow-cytometry quantitation of cell cycle progress in A549. The cell after serum starvation for 72 h, then addition of medium containing 10 % FBS for the indicated times. **c** Immunoblot analysis showed that si-RNA treatment of RING1 markedly decreased RING1 and PCNA levels 48 h after si-RNA transfection in A549 cells in comparison with cells transfected with negative control (Neg) and mock treatments. **d** RING1 knockdown resulted in the delay of G_1-S transition and significant arrest in G_1 phase of A549 cells. The data are showed as mean±SD for three experiments

RING1 Was High Expressed in Proliferating NSCLC Cells

Based on the published study of EZH2, which is related to cell cycle. we predict that RING1 may also be related to cell cycle regulation in NSCLC. So we further detected the expression of RING1 during cell cycle progression in NSCLC cells. A549 cells were arrested in G1 phase by serum deprivation for 72 h. After serum-addition, the cells were released from G1 phase and reentered into S phase (Fig. 4b) [14]. Western blot results displayed that the expression of RING1 was increased as early as 4 h after serum-stimulation, meanwhile, the expression of CDK2 and Cyclin-D was also up-regulated (Fig. 4a). Moreover, Cell cycle analysis showed a significantly reduced population in the S phase whereas a remarkable G1 cells increased after transfection of RING1 si-RNA into the A549 cell line, as compared with negative control cells (Fig. 4c and d). These results highlight the important role of RING1 in regulating NSCLC cell proliferation.

Correlation Between RING1 and Patients' Survival

We carried out Kaplan–Meier analysis to study the correlation between RING1 and patients' survival. Survival analysis was restricted to 69 patients with follow-up data and results of RING1 by IHC. By using the Kaplan-Meier analysis, patients with RING1 high expression are significantly associated with short overall survival ($P = 0.004$, Fig.5).

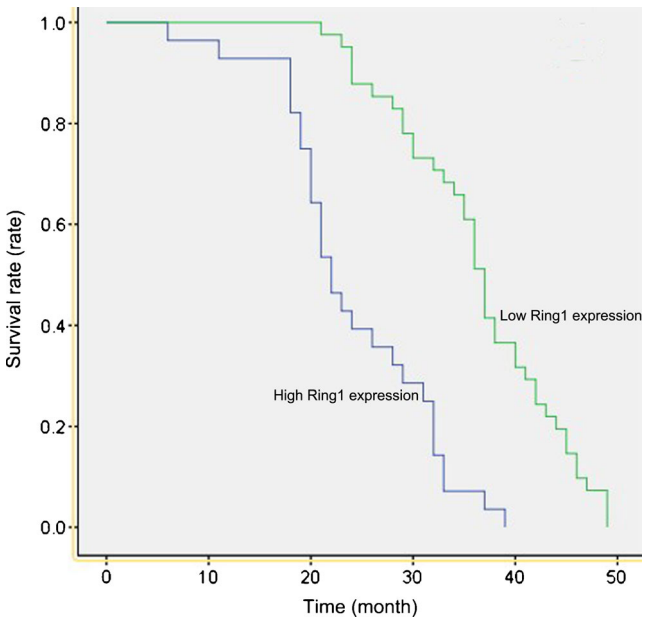

Fig. 5 Kaplan–Meier survival curves for high RING1expression versus low RING1 expression in 69 patients of NSCLC showed a highly significant separation ($P = 0.004$, log rank test)

Discussion

PcGs, together with Trithorax group (TrG) proteins, are best known for their role in controlling *Drosopbila* development and for the regulation of the expression of homeotic(Hox) genes [1, 8]. In fact, the deletion of individual PcG genes in Drosopbila results in strong homeotic transformations during larvae development consistent with the mis-expression of Hox genes [8].

Recent studies have demonstrated that PcG proteins function in multiprotein complexes. Two of the best characterized PcG complexes are the Poly-come Repressive Complexes (PRC) PRC1 and PRC2. PRC1 is a very large complex of more than 10 subunits including the oncoprotein BMI as well as the other PcG proteins, such as RING1, HPC, HPH and SCML[23]. PRC2 is a smaller complex containing the PcG proteins EZH2, EED, SUZ12 and the histone binding protein RbAp46. The PRC2 complex possesses histone methytransferase(HMT) activity, specific for lysine(K) residues K27 of histone H ([6, 23];.R. [3]), which maybe related with the development of carcinomas. For example, Watanabe reported that deregulation of histone lysine methyltransferases contributes to oncogenic transformation in human bronchoepithelial cells [32], and Kondo Y revealed that alterations of histone modifications was related to gene silencing in hepatocellular carcinomas [16]. Recently, member of PRC2 complex, especially of EZH2, is often reported in various cancers ([9, 17, 27]; W. [4]). The role of PcG genes in the maintenance of cell identity is underscored by the fact that several PcG genes can be classified as oncogenes and tumor suppressor genes [2, 26]. For instance, over-expression of HPC2, RING1, and BMI-1 in experimental model systems resulted in cellular transformation of cell lines, or produced lymphomas in mutant mice [7, 10, 12, 25, 26]. However, as a member of PRC1, in spit of RING1 interacts with multiple poly-comb-group proteins and displays tumorigenic activity [24–26], little study has been done on the member of PRC1 complex RING1 in NSCLC.

In the current study, we detected RING1 protein expression in NSCLC tissue specimens and found differential expression of this protein in NSCLC and normal lung tissues. We also found that high expression of RING1 protein was significantly associated with poor patient survival compared to that of the patients with low expression. We investigated RING1 expression and its role in NSCLC, significant correlations were observed between RING1 expression and TNM stage. Recently, RING1 expression had been reported in prostate cancer, breast cancer and bladder cancer. In prostate cancer, low-RING1 protein have been shown to relate to tumor angiogenesis, leading to poor clinical outcomes. However, RING1 expression in bladder cancer and breast cancer are over-expressed and over-expressed RING1 in bladder cancer is related with poor outcome. [5, 11, 22, 29], which supports our results. So far, PcG genes are capable of producing cancer

in humans is unclear, Hongyan Qin reported that over-expression of RING1 together with KyoT2 in cells inhibited transactivation of RBP-J by NIC [21]. Moreover, we further demonstrated that RING1 was important for NSCLC cell proliferation, down-regulation of RING1 expression by si-RNA in tumor cell lines led to reducing cell growth.

In summary, RING1, which is a member of the PcG protein family, acts as a transcriptional repressor and is implicated in various malignancies. Our results showed that high RING1 expression in NSCLC correlates with unfavorable prognosis and shorter survival rate. Aberrant RING1 expression may be involved in NSCLC and predict unfavorable clinical behavior. RING1 can be used potentially as a therapeutic target, which would represent a major breakthrough in the treatment of advanced NSCLC.

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Conflict of interest None.

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