

RASSF4 is downregulated in nonsmall cell lung cancer and inhibits cancer cell proliferation and invasion

Yong Han¹ · Qianze Dong¹ · Jie Hao¹ · Lin Fu¹ · Xu Han¹ · Xiaoying Zheng¹ · Enhua Wang¹

Received: 2 September 2015 / Accepted: 28 October 2015 / Published online: 2 November 2015
© International Society of Oncology and BioMarkers (ISOBM) 2015

Abstract RASSF4 has been implicated as a tumor suppressor in several human cancers. Its clinical significance and biological characteristics in human nonsmall cell lung cancer (NSCLC) have not been explored yet. In this study, we explored expression pattern of RASSF4 in 89 NSCLC specimens. The results showed that RASSF4 was downregulated in 36/89 NSCLC tissues compared with normal tissue. RASSF4 downregulation significantly associated with advanced TNM stage, positive nodal status, and poor prognosis. We examined RASSF4 protein expression in normal lung epithelial cell line and lung cancer lines. We found that RASSF4 expression was downregulated in four of seven lung cancer cell lines compared with normal bronchial epithelial cells. RASSF4 plasmid transfection was performed in H460 and A549 cell lines. RASSF4 overexpression inhibited proliferation, colony formation, and invading ability. In addition, we identified that RASSF4 could inhibit cell cycle progression with downregulation of cyclin D1. Expression of invasion-related protein MMP2, MMP9 was also decreased. In conclusion, the present study suggested that RASSF4 serves as an important tumor suppressor in NSCLC.

Keywords RASSF4 · Nonsmall cell lung cancer · Proliferation · Invasion

Introduction

Lung cancer is the leading cause of death among malignant tumors worldwide [1–3]. Although targeted therapies have been established, mutations such as EGFR and KRAS causing activation of these gene products are identified only in a limited number of patients. Many complex genetic, epigenetic, and microenvironmental factors play important roles in growth and invasion of tumor cells [4–7]. Thus, identification of these oncogenes/tumor suppressors and elucidation of their mechanism is an important mission for development of new treatment.

RASSF4 is a RASSF family protein. To date, RASSF family proteins such as RASSF1 and RASSF2 have been shown to directly interact with Ras proteins with the characteristics of effectors [8–11]. These proteins can induce cell death in a Ras-dependent manner. Moreover, these proteins are all frequently downregulated during tumor development by promoter methylation [12]. Several studies have shown that RASSF4 is downregulated in human cancers, suggesting involvement of RASSF4 in carcinogenesis [13]. However, there was no report concerning protein expression pattern and clinical significance of RASSF4 in human nonsmall cell lung cancer. Its biological roles in lung cancer cells also remain unexplored.

Here, we investigated RASSF4 protein expression in NSCLCs and analyzed its correlation with clinicopathological factors. We also examined the function of RASSF4 as a tumor suppressor in lung cancer cell lines

Electronic supplementary material The online version of this article (doi:10.1007/s13277-015-4343-9) contains supplementary material, which is available to authorized users.

✉ Enhua Wang
wangenhuacmu@hotmail.com

¹ Department of Pathology, First Affiliated Hospital and College of Basic Medical Science, China Medical University, Shenyang 110001, China

and provide evidence that RASSF4 inhibits lung cancer cell proliferation and invasion.

Materials and methods

Tissue samples

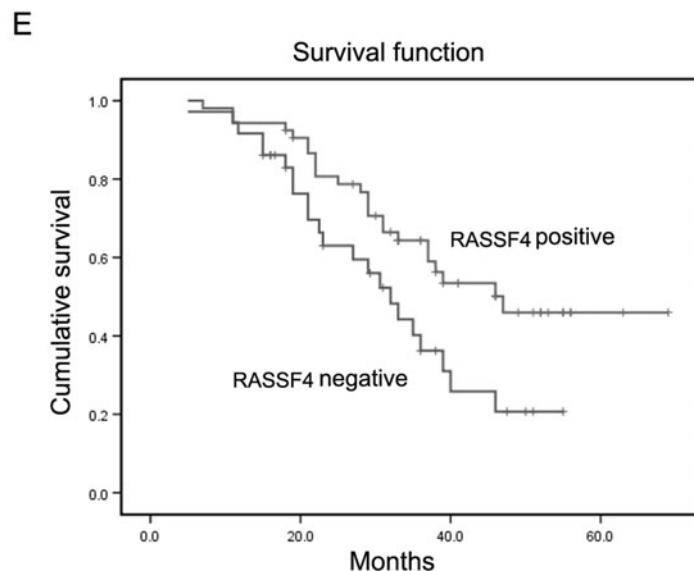
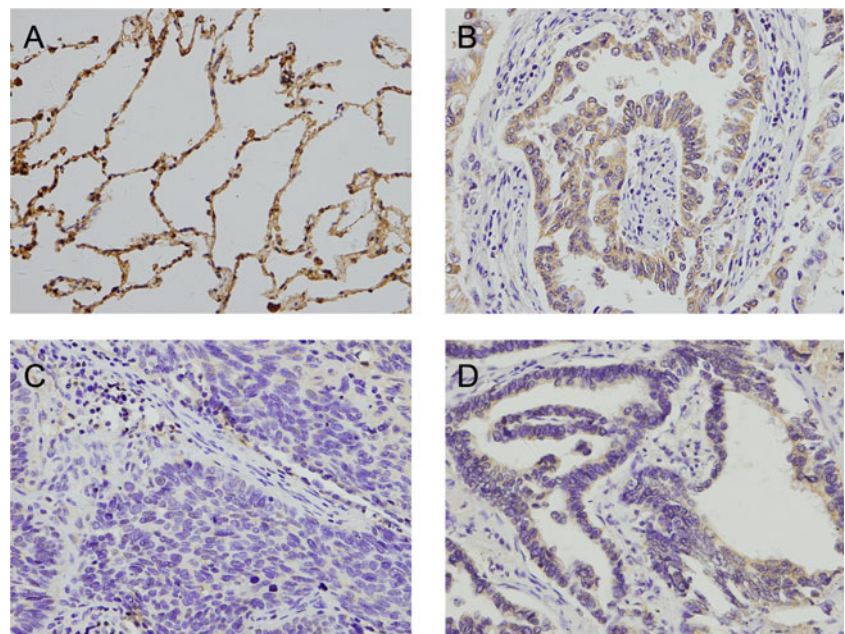
The study was approved by the ethical committee of China Medical University. A total of 89 cases of NSCLC were retrieved from the Pathology Archive of the First Affiliated Hospital of China Medical University from 2007 to 2010. Clinicopathological information about the patients was obtained from patient records. All of the enrolled patients

underwent curative surgical resection without having prior chemotherapy or radiation therapy.

Immunohistochemistry

Tumor specimens were fixed with neutral formalin, embedded in paraffin, and 4- μ m thick sections were prepared. The sections were deparaffinized in xylene, rehydrated in graded alcohol series. Antigen retrieval was performed using 0.01-M citrate buffer for 2-min boil. Hydrogen peroxide was applied to block peroxidase, and then the slides were incubated with normal goat serum. The primary antibody for RASSF4 (1:400, Proteintech, USA) was incubated overnight at 4 °C. Sections were stained in parallel with nonimmune IgG to

Fig. 1 Expression of RASSF4 in nonsmall cell lung cancers. **a** Positive cytoplasmic staining of RASSF4 in normal alveolar cells. **b** Positive staining of RASSF4 in a case of lung adenocarcinoma. **c** Negative staining of RASSF4 in lung squamous cell carcinoma. **d** Negative RASSF4 staining in a case of lung adenocarcinoma (magnification $\times 200$). **e** Survival analyses of patients with RASSF4 expression and those without. The overall survival was significantly lower in patients with RASSF4-negative NSCLCs than in patients with RASSF4-positive NSCLCs



provide a negative control. The expression of RASSF4 was then detected by the EliVision plus kit (Maixin, Fuzhou, China). DAB visualization was then performed, and the slides were counterstained with hematoxylin.

Two independent investigators examined all tumor slides randomly. Five views were examined per slide, and 100 cells were observed per view at $\times 400$ magnification. Immunostaining of RASSF4 was scored following a semi-quantitative scale by evaluating in representative tumor areas, the intensity, and percentage of cells. Cytoplasmic staining was considered as positive. The intensity of RASSF4 staining score was indicated as 0 (none), 1 (weak), and 2 (strong). Percentage scores were indicated as 1 (1–25 %), 2 (26–50 %), 3 (51–75 %), and 4 (76–100 %). The scores of each tumor sample were multiplied to give a final score of 0 to 8, and the total expression of RASSF4 was determined as low expression with score ≤ 4 and high expression (+) with score >4 .

Cell culture and transfection

Human NSCLC cell lines NHBE, H460, A549, H1299, H157, H3255, and small cell lung cancer (SCLC) cell line H446 were purchased from ATCC. The cells were cultured using RPMI-1640 (Gibco, Invitrogen, NY, USA) with 10 % fetal bovine serum in an incubator at 37 °C with 5 % CO₂. For transfections, pCMV6-RASSF4 plasmid and the control empty vector pCMV6 were purchased from Origene

(Origene, Rockville, MD, USA). Cells were transfected using Attractene reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RASSF4 small interfering RNA (siRNA) and negative control siRNA were purchased from Dharmacon (GE healthcare, USA). siRNA transfection was performed using Dharmafect1 reagent (GE healthcare, USA).

Western blotting

Total proteins from cells were extracted in lysis buffer and quantified using the Bradford method. Thirty-microgram protein was separated by SDS-PAGE. Samples were transferred to PVDF membranes (Millipore, Billerica, MA, USA) and incubated overnight at 4 °C with antibody against RASSF4 (1:800, Sigma, USA), cyclin D1, MMP2, MMP9 (1:1000, Cell Signaling, USA), and GAPDH (1:500, Santa Cruz, USA). After incubation with HRP-coupled anti-mouse or rabbit IgG antibody (1:1000 dilution, Cell Signaling Technology, USA) at 37 °C for 2 h, target proteins on PVDF membrane were visualized using Pierce ECL kit and captured using a DNR BioImaging System (DNR, Jerusalem, Israel).

Colony formation and MTT assays

Colony formation assay Forty-eight hours after transfection, cells were plated into 6-cm culture dishes (about 1000 per dish).

Table 1 Distribution of RASSF4 status in NSCLC according to clinicopathological characteristics

Characteristics	Number of patients	RASSF4 downregulation	RASSF4 positive expression	<i>p</i> value
Age				
<60	43	17	26	0.8650
≥ 60	46	19	27	
Gender				
Male	50	21	29	0.7358
Female	39	15	24	
Differentiation				
Well	33	12	21	0.5466
Moderate–poor	56	24	32	
Histology				
Adenocarcinoma	53	20	33	0.5268
Squamous cell carcinoma	36	16	20	
TNM stage				
I	40	10	30	0.0073
II+III	49	26	23	
Tumor status				
T1	32	12	20	0.6710
T2–T4	57	24	33	
Nodal metastasis				
Negative	53	16	37	0.0167
Positive	36	20	16	

Table 2 Univariate and multivariate analysis for predictive factors in patients with NSCLC

Factors	Univariate		Multivariate	
	Hazard ratio (95 % CI)	<i>p</i> value	Hazard ratio (95 % CI)	<i>p</i> value
Histology	1.564 (0.852–2.870)	0.1492	2.180 (1.113–4.271)	0.0231
Differentiation	1.560 (0.840–2.900)	0.1595	1.559 (0.959–2.535)	0.0735
TNM Stage	2.138 (1.451–3.151)	0.0001	1.906 (1.228–2.958)	0.0040
RASSF4 status	0.505 (0.282–0.904)	0.0214	0.728 (0.384–1.383)	0.3325

After 2 weeks, plates were washed with PBS, and Giemsa staining was performed to visualize colony. The colonies with more than 50 cells were counted using a microscope.

MTT assay Cells were plated in 96-well plates (approximately 3000 cells per well) and cultured for 5 days. Twenty microliters of 5 mg/ml MTT solution was added to the well. After incubation for 4 h, the medium was removed, and remaining MTT formazan was dissolved in 150 μ l of DMSO. Solution was measured at 490 nm.

Matrigel invasion assay

Cell invasion assay was performed using a 24-well Transwell chamber. The inserts were coated with 18- μ l Matrigel (1:4 dilution, BD Bioscience, USA). After the transfection, cells were trypsinized and re-suspended in 100 μ l of serum-free medium, which were transferred to the upper chamber. Total 600- μ l medium containing 10 % FBS was added to lower chamber. After 18-h incubation, noninvaded cells on the upper chamber were removed using cotton tips, and the invaded cells were stained using hematoxylin. Invading cell number was counted in five high power fields under the microscope.

Statistical analysis

SPSS version 16 for Windows was used for all statistical analyses. A χ^2 test was used to examine possible correlations between RASSF4 expression and clinicopathologic factors. Student's *t* test was used to compare densitometry data on focus numbers between control and SIX1-transfected cells. All *p* values are based on a two-sided statistical analysis, and *p*<0.05 was considered to indicate statistical significance.

Results

Expression pattern and clinical significance of RASSF4 in NSCLC

We investigated RASSF4 expression in 89 NSCLC tissue specimens and 20 normal lung tissue specimens by

immunohistochemistry. In normal lung tissues, positive cytoplasmic staining was observed in normal bronchial epithelial cells and alveolar cells (Fig. 1a). As for lung cancer tissues, 53 cases showed strong cytoplasmic RASSF4 staining and 36 cases (40.5 %) showed downregulated RASSF4 staining compared with normal tissue (Fig. 1c, d). RASSF4 protein was mainly located in the cytoplasmic compartment of tumor cells. We examined the correlation between RASSF4 status and clinical factors. We found that RASSF4 downregulation significantly associated with advanced TNM stage (Table 1, *p*=0.0073) and positive nodal status (*p*=0.0167). No difference was observed in the RASSF4 status according to the age, gender, differentiation, and tumor size (Table 1).

Furthermore, Kaplan-Meier survival analysis showed a significantly lower overall survival in patients with negative RASSF4 compared with those with positive expression (*p*<0.05, log rank test; Fig. 1e). In addition, univariate analysis showed that TNM stage and RASSF4 status were both significant prognostic factors (TNM stage: hazard ratio 2.138, *p*<0.001; RASSF4 status: hazard ratio 0.505,

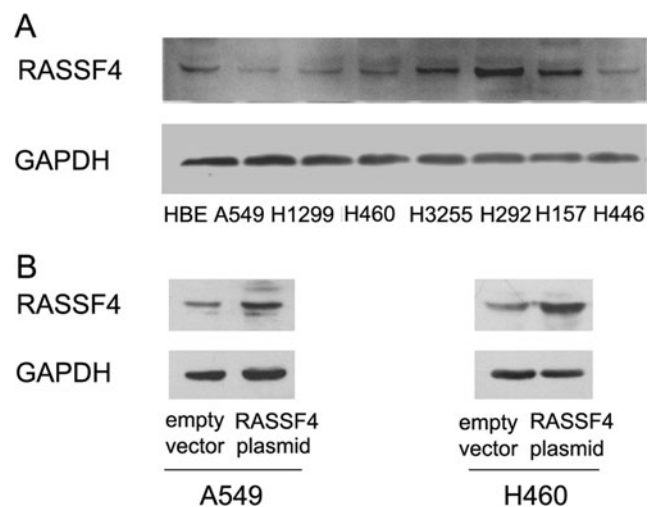


Fig. 2 RASSF4 expression in lung cancer cell lines and its transfection efficiency. **a** Endogenous expression of RASSF4 was examined in HBE and lung cancer cell lines by western blot. Lung cancer cell lines have significant downregulated RASSF4 expression. **b** Western blot analysis showed that pCMV6-RASSF4 plasmid markedly increases its levels in H460 and A549 cells compared with control

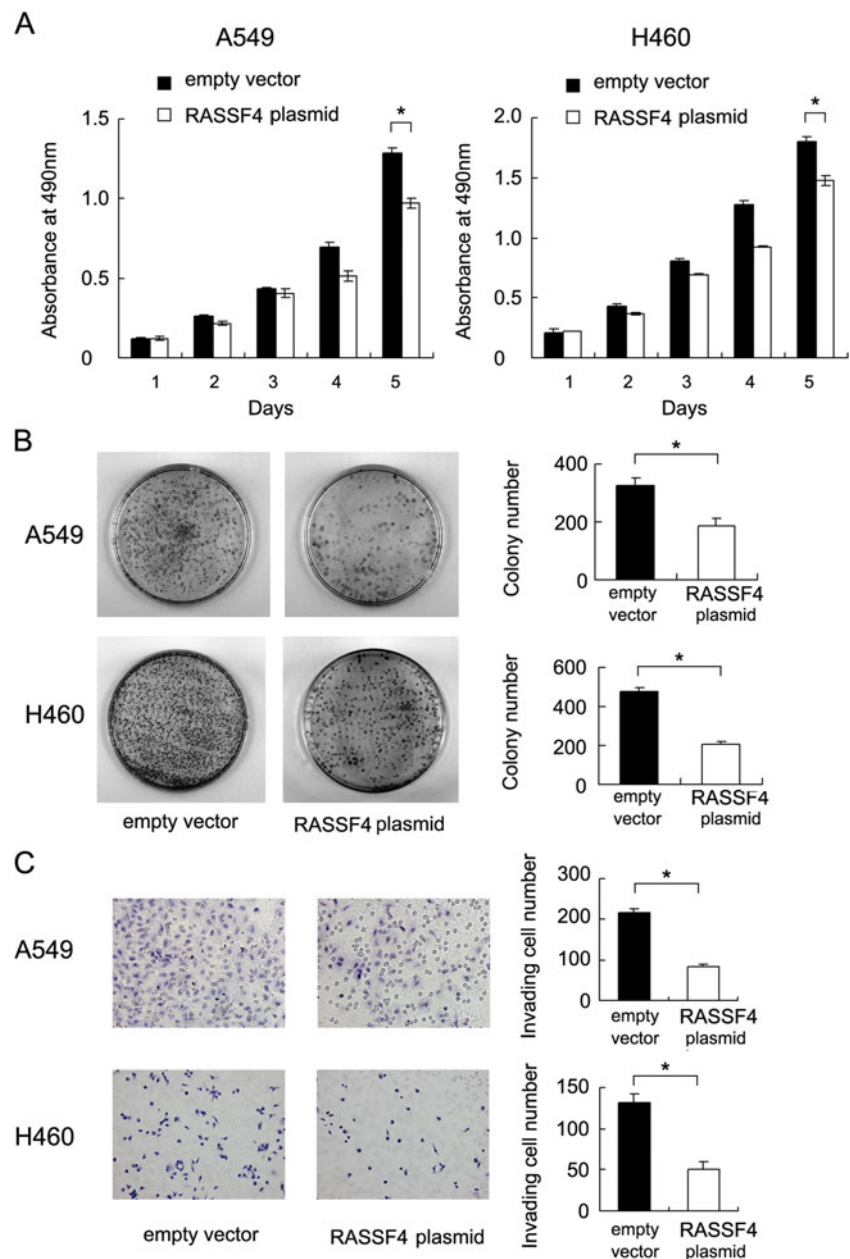
$p=0.0214$), and multivariate analysis using a Cox regression model indicated that TNM stage was an independent, unfavorable prognostic factor (Table 2).

RASSF4 is downregulated in lung cancer cell lines and inhibits cell proliferation and invasion

Relative expression level of RASSF4 was analyzed by western blot in a panel of lung cancer cell lines. In accordance with tissue samples, the RASSF4 protein expression was remarkably decreased in NSCLC cell lines, especially in H460, A549, H1299, and H446, compared with normal HBE cell line (Fig. 2a). A549 and H460 cell lines were selected for RASSF4 transfection. We upregulated

RASSF4 expression using RASSF4 plasmid, and transfection efficiency was confirmed by western blot analysis (Fig. 2b). RASSF4 overexpression in A549 and H460 cells greatly inhibited the proliferation rate (day 5, $p<0.05$) and the potential of colony formation ($p<0.05$) (Fig. 3a, b). To characterize the effect of RASSF4 on cell invasion, matrigel invasion assay was performed in A549 and H460 cells. As shown in Fig. 3c, significant reduced invading ability was observed in cells with RASSF4 transfection compared with empty controls. In addition, we performed siRNA depletion in H157 cell line with relatively high RASSF4 expression. As shown in supplementary Fig. 1, RASSF4 depletion significantly upregulated cancer cell proliferation rate and invading ability.

Fig. 3 RASSF4 restoration inhibits cell proliferation and invasion. **a** MTT assay in H460 and A549 cells transfected with RASSF4 plasmid. Time-dependent decrease in cell proliferation after RASSF4 transfection compared with control. **b** Colony formation assay was performed in cells transfected with RASSF4 siRNA and cells transfected with control. A marked decrease in colony formation is seen in the groups with RASSF4 restoration. **c** Matrigel invasion assay showed that RASSF4 transfection decreased cell invasion in A549 and H460 cell lines. $*p<0.05$



RASSF4 inhibits cell cycle and regulates cyclin D1, and MMP2 and MMP9 expression

The aforementioned results indicate that RASSF4 leads to decreased cellular proliferation and invasion. We further checked the role of RASSF4 on cell cycle progression. As shown in Fig. 4a, RASSF4 overexpression inhibited G1-S transition in H460 and A549 cell lines. To underline the possible mechanisms, we examined a panel of growth and invasion-related proteins. As shown in Fig. 4b, RASSF4 transfection significantly inhibited cell cycle protein cyclin D1 and invasion-related protein MMP2 and MMP9.

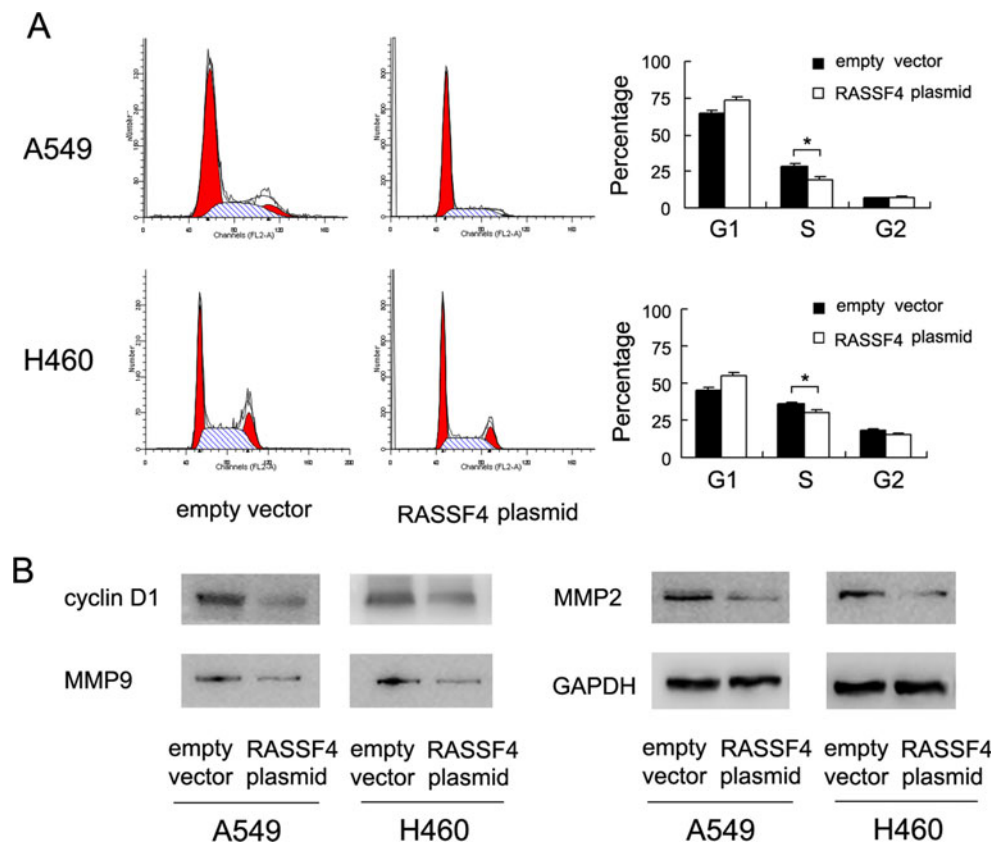
Discussion

Epigenetically inactivation of RASSF4, which is caused by methylation and/or deletions, has been investigated in a number of cancers. Reduced RASSF4 mRNA expression and hypermethylated promoter region of RASSF4 was found in head and neck squamous cell carcinoma and nasopharyngeal carcinoma [14, 15]. Overexpression of RASSF4 induces Ras-dependent apoptosis in 293-T cells and inhibits the growth of human tumor cell lines [16]. However, there was no study concerning protein expression of RASSF4 in lung cancer tissues and its correlation with clinicopathological parameters. In

addition, biological roles of RASSF4 in human nonsmall cell lung cancer cells remain elusive. In this study, we demonstrated that RASSF4 protein expression was significantly decreased in 38.6 % NSCLC tissues, which was significantly correlated with lymph node metastasis and TNM stage, suggesting RASSF4 as a putative tumor suppressor in NSCLC. Importantly, we showed that loss of RASSF4 correlated poor patient prognosis. To date, this is the first report concerning protein expression pattern and clinical significance of RASSF4 in human cancers. We also examined RASSF4 expression in several lung cancer cell lines. RASSF4 expression was significantly lower in four of seven cancer cell lines compared with HBE, which was consistent with immunohistochemical findings showing RASSF4 as a tumor suppressor.

Using RASSF4 plasmid, we demonstrated that its restoration in NSCLC cell lines with low endogenous expression significantly inhibited proliferation and cell cycle progression. Previous reports showed evidence supporting RASSF4 as a tumor suppressor. It has been reported that RASSF4 overexpression induced cell death and inhibited survival in MCF-7 cell line [16]. Our result was in accord with these studies, suggesting the role of RASSF4 as a tumor suppressor as in lung cancer cells. To date, the effect of RASSF4 on cell cycle and cell invasion has not been investigated. The mechanism of RASSF4 as a tumor suppressor also has not been elucidated. Thus, we checked cell cycle progression and related proteins

Fig. 4 RASSF4 transfection inhibits cell cycle progression, with cyclin D1 and MMP9 downregulation. **a** Cell cycle analysis showed that RASSF4 transfection decreased cell percentage in S phase and increased the cell percentage in G1 phase. **b** Western blot analysis showed that RASSF4 restoration could decrease the protein expression of cyclin D1, MMP2, and MMP9. * $p < 0.05$



and found that RASSF4 overexpression inhibited S phase percentage and cyclin D1 expression. Cyclin D1 play a critical role during cell cycle progression at G1-S checkpoint. Many studies have demonstrated that cyclin D1 was overexpressed in nonsmall cell lung cancer and correlated with malignant progression and poor prognosis [17, 18]. These results suggested that RASSF4 could regulate cell proliferation through modulation of cyclin D1 status. The role of RASSF4 on cell invasion has not been reported previously. In the present study, we found that RASSF4 inhibited invading ability of lung cancer cell lines. RASSF4 restoration decreased MMP2 and MMP9 levels, which are important mediators of invasion in many types of cancers [19–22]. Thus, the role of RASSF4 on invasion inhibition may be due to its role on MMP2 and MMP9 downregulation.

In conclusion, this study demonstrated downregulation of RASSF4 protein expression in NSCLC and its correlation with TNM stage, nodal status, and poor prognosis. Our results demonstrated that RASSF4 restoration in lung cancer cells could inhibit proliferation and invasion, possibly through regulation of cyclin D1 and MMP family proteins. Given these findings, RASSF4 serves as an important tumor suppressor in nonsmall cell lung cancer.

Acknowledgments The study was supported by the National Natural Science Foundation of China (No. 81302022).

Compliance with ethical standards

Conflicts of interest None.

References

- Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics, 2007. *CA Cancer J Clin.* 2007;57(1):43–66.
- Minna JD, Roth JA, Gazdar AF. Focus on lung cancer. *Cancer Cell.* 2002;1(1):49–52.
- Schiller JH, Harrington D, Belani CP, Langer C, Sandler A, Krook J, et al. Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer. *N Engl J Med.* 2002;346(2):92–8.
- Dong QZ, Wang Y, Dong XJ, Li ZX, Tang ZP, Cui QZ, et al., CIP2A is Overexpressed in Non-Small Cell Lung Cancer and Correlates with Poor Prognosis. *Ann Surg Oncol.*
- Dong QZ, Zhao Y, Liu Y, Wang Y, Zhang PX, Jiang GY, et al. Overexpression of SCC-S2 correlates with lymph node metastasis and poor prognosis in patients with non-small-cell lung cancer. *Cancer Sci.* 2010;101(6):1562–9.
- Fidler IJ, Kripke ML. Genomic analysis of primary tumors does not address the prevalence of metastatic cells in the population. *Nat Genet.* 2003;34(1):23. **author reply 25.**
- van 't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, et al. Gene expression profiling predicts clinical outcome of breast cancer. *Nature.* 2002;415(6871):530–6.
- Liu L, Amy V, Liu G, McKeenan WL. Novel complex integrating mitochondria and the microtubular cytoskeleton with chromosome remodeling and tumor suppressor RASSF1 deduced by in silico homology analysis, interaction cloning in yeast, and colocalization in cultured cells. *In Vitro Cell Dev Biol Anim.* 2002;38(10):582–94.
- Vos MD, Ellis CA, Bell A, Birrer MJ, Clark GJ. Ras uses the novel tumor suppressor RASSF1 as an effector to mediate apoptosis. *J Biol Chem.* 2000;275(46):35669–72.
- Akino K, Toyota M, Suzuki H, Mita H, Sasaki Y, Ohe-Toyota M, et al. The Ras effector RASSF2 is a novel tumor-suppressor gene in human colorectal cancer. *Gastroenterology.* 2005;129(1):156–69.
- Vos MD, Ellis CA, Elam C, Ulku AS, Taylor BJ, Clark GJ. RASSF2 is a novel K-Ras-specific effector and potential tumor suppressor. *J Biol Chem.* 2003;278(30):28045–51.
- Vos MD, Martinez A, Ellis CA, Vallecorsa T, Clark GJ. The proapoptotic Ras effector Nore1 may serve as a Ras-regulated tumor suppressor in the lung. *J Biol Chem.* 2003;278(24):21938–43.
- Michifuri Y, Hirohashi Y, Torigoe T, Miyazaki A, Fujino J, Tamura Y, et al. Small proline-rich protein-1B is overexpressed in human oral squamous cell cancer stem-like cells and is related to their growth through activation of MAP kinase signal. *Biochem Biophys Res Commun.* 2013;439(1):96–102.
- Chow LS, Lo KW, Kwong J, Wong AY, Huang DP. Aberrant methylation of RASSF4/AD037 in nasopharyngeal carcinoma. *Oncol Rep.* 2004;12(4):781–7.
- Steinmann K, Sandner A, Schagdarsurengin U, Dammann RH. Frequent promoter hypermethylation of tumor-related genes in head and neck squamous cell carcinoma. *Oncol Rep.* 2009;22(6):1519–26.
- Eckfeld K, Hesson L, Vos MD, Bieche I, Latif F, Clark GJ. RASSF4/AD037 is a potential ras effector/tumor suppressor of the RASSF family. *Cancer Res.* 2004;64(23):8688–93.
- Liu J, Liao Q, Zhang Y, Sun S, Zhong C, Liu X. Cyclin D1 G870A polymorphism and lung cancer risk: a meta-analysis. *Tumour Biol.* 2012;33(5):1467–76.
- Liu Y, Wang L, Lin XY, Wang J, Yu JH, Miao Y, et al. The transcription factor DEC1 (BHLHE40/STRA13/SHARP-2) is negatively associated with TNM stage in non-small-cell lung cancer and inhibits the proliferation through cyclin D1 in A549 and BE1 cells. *Tumour Biol.* 2013;34(3):1641–50.
- Dong QZ, Wang Y, Tang ZP, Fu L, Li QC, Wang ED, et al. Derlin-1 is overexpressed in non-small cell lung cancer and promotes cancer cell invasion via EGFR-ERK-mediated up-regulation of MMP-2 and MMP-9. *Am J Pathol.* 2013;182(3):954–64.
- Jian H, Zhao Y, Liu B, Lu S. SEMA4b inhibits MMP9 to prevent metastasis of non-small cell lung cancer. *Tumour Biol.* 2014;35(11):11051–6.
- Song H, Tian Z, Qin Y, Yao G, Fu S, Geng J. Astrocyte elevated gene-1 activates MMP9 to increase invasiveness of colorectal cancer. *Tumour Biol.* 2014;35(7):6679–85.
- Feng X, Miao G, Han Y, Xu Y. CARMA3 is overexpressed in human glioma and promotes cell invasion through MMP9 regulation in A172 cell line. *Tumour Biol.* 2014;35(1):149–54.