

***RAB38* is a potential prognostic factor for tumor recurrence in non-small cell lung cancer**

JIA-JUAN HSIEH^{1*}, MING-MO HOU^{2*}, JOHN WEN-CHENG CHANG²,
YUNG-CHI SHEN¹, HSIN-YI CHENG² and TODD HSU¹

¹Department of Bioscience and Biotechnology and Center of Excellence for The Oceans, National Taiwan Ocean University, Keelung 20224; ²Division of Hematology-Oncology, Chang Gung Memorial Hospital, Chang Gung University College of Medicine, Taoyuan 33305, Taiwan, R.O.C.

Received September 26, 2018; Accepted April 9, 2019

DOI: 10.3892/ol.2019.10547

Abstract. Ras-related protein Rab-38 (*RAB38*) is a member of the Ras small G protein family that regulates intracellular vesicular trafficking. Although the expression of *RAB38* is reportedly deregulated in several types of cancer, its role in tumor biology remains to be elucidated. In the present study, the expression of *RAB38* was analyzed in tumor specimens from patients with non-small cell lung cancer (NSCLC) with tumor recurrence within 4 years (Group R), and those remaining disease-free following initial surgery (Group NR), by reverse transcription-semi-quantitative PCR and subsequent semi-quantification using ImageJ v4.0 software. The results revealed that the expression of *RAB38* in Group R and NR specimens was positively associated with tumor recurrence; a high expression level was also associated with poor survival rate in these patients. Using NSCLC cell lines, it was demonstrated that tumor cells with mutations in the active epidermal growth factor receptor (*EGFR*) gene expressed higher levels of *RAB38* compared with those with the wild-type gene by reverse transcription-PCR and western blot analysis. Furthermore, following specific *RAB38* gene knockdown by short hairpin RNA transfection, *EGFR* mutants exhibited markedly reduced invasiveness when compared with cells transfected with empty vector controls by Matrigel Transwell assays. These results suggest that *RAB38* is an important

prognostic factor in NSCLC, and may serve a critical role in NSCLC-associated tumor metastasis.

Introduction

Lung cancer is the leading cause of cancer-associated mortality worldwide. Non-small cell lung cancer (NSCLC) accounts for >80% of all cases of lung cancer, and the majority of patients present with advanced disease at diagnosis (1). Despite recent advances in multi-modal therapy, the overall 5-year survival rate for NSCLC remains poor, ranging between 8 and 12%. Surgery continues to be the primary treatment option for localized NSCLC. However, the results of surgical treatment remain unsatisfactory, with 35-50% of patients experiencing disease relapse within 5 years.

RAB proteins are members of the Ras superfamily of GTPases that regulate intracellular trafficking (2). It has been revealed that a point mutation in the GTP-binding domain of *RAB38* is responsible for human Hermansky-Pudlak syndrome, which is characterized by oculocutaneous albinism, bleeding diathesis and pulmonary fibrosis (3). Studies have investigated the potential role in regulating tumor progression (4); several RAB proteins have been identified in transcriptomic studies, exhibiting deregulated expression in malignant cells compared with normal tissues (5,6). However, the exact functions of these proteins in tumor biology remain to be fully elucidated. Accumulating evidence has demonstrated that RABs are overexpressed or upregulated in several types of cancer, including hepatoma, tongue, breast, pancreatic and prostate cancer (7-12); by contrast, the downregulation of certain *RAB* genes as a result of hypermethylation has been identified in colon, gastric and endometrial cancer (13,14).

RAB38 is a recently identified member of the RAB family of proteins and its expression is tissue-specific. *RAB38* is predominantly expressed in melanocytes and melanoma tissues, but not in other normal tissues, serving as a melanocyte differentiation antigen (15). In gliomas, the expression levels of *RAB38* have been associated with disease progression (16). However, the biological effects of *RAB38* in other types of cancer remain unclear.

Utilizing two microarray platforms, a four-gene signature, including *LCN2*, *PTHLH*, *RAB38* and *FJX1*, was identified

Correspondence to: Professor Todd Hsu, Department of Bioscience and Biotechnology and Center of Excellence for The Oceans, National Taiwan Ocean University, 2 Beining Road, Keelung 20224, Taiwan, R.O.C.
E-mail: toddhsu@mail.ntou.edu.tw

*Contributed equally

Abbreviations: NSCLC, non-small cell lung cancer; DFS, disease-free survival; OS, overall survival

Key words: Ras-related protein Rab-38, prognosis, non-small cell lung cancer

as being significantly associated with tumor recurrence in patients with NSCLC (17). In the present study, the association between the expression of *RAB38* and survival rate was further evaluated in patients with NSCLC. The results demonstrated that the expression of *RAB38* was negatively associated with patient survival rate, and that knockdown of the expression of *RAB38* attenuated tumor invasion *in vitro*. These results suggest that *RAB38* may be used as an important prognostic predictor in NSCLC.

Materials and methods

Sample acquisition. Tumor tissues were retrospectively acquired from the Tissue Bank of Chang Gung Memorial Hospital (Taoyuan, Taiwan) following the standard procedure, and written informed consent was obtained from all patients. The tissue sections were reviewed by a pathologist, and only those comprising >50% tumor area were used (confirmed by hematoxylin and eosin staining). A total of 60 patients were enrolled in the present study and frozen specimens were available from all patients. The present study was approved by the Institutional Review Board of Chang Gung Memorial Hospital.

RNA extraction and semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis. The patients were divided into two groups: Group R comprised patients who had tumor recurrence within 4 years following surgery, and Group NR comprised patients who remained disease-free 4 years following initial surgery. A total of 40 Group R and 20 Group NR cases of NSCLC were included. Following mechanical tissue disruption, total tumor RNA was extracted using the RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. Subsequently, 2–5 µg of total RNA was converted to cDNA using the SuperScript[®] III kit (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. The *RAB38* and control *GAPDH* genes were amplified by PCR. The primers were as follows: *RAB38*, forward 5'-AGGCTGCGC TTCCCTGGTCA-3' and reverse 5'-CCACGCCAGGTGCGC CAATC-3'; *GAPDH*, forward 5'-GACAACAGCCTCAAG ATCATCA-3' and reverse 5'-GGTCCACCACTGACACGT TG-3'. PCR amplification was performed in a total volume of 20 µl, containing 2–5 µg of cDNA. Following denaturation at 94°C for 5 min, a total of 30 thermal cycles were performed at 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec. The amplified samples were mixed with gel loading dye and subjected to electrophoresis using a 1% agarose gel, containing 0.5 µg/ml ethidium bromide in 1X tris-borate-EDTA buffer. The gel was visualized and analyzed using ChemiCapt 3000 software (version 5.03; Vilber Lourmat, Marne-la-Vallée, France), and the expression levels of the PCR products were quantified using Image J v4.0 software (National Institutes of Health). The relative semi-quantitative RT-PCR density of *RAB38* to *GAPDH* was quantified. High expression was defined as a ratio of *RAB38* to *GAPDH* ≥0.5.

Cell culture and stable knockdown. The following human lung cancer cell lines were purchased from the American Type Culture Collection: HCC827, which expresses high levels of *RAB38* and mutated epidermal growth factor receptor (EGFR:

Table I. Demographic data of 60 patients with non-small cell lung cancer (R=40; NR=20).

Variable	Group R, n (%)	Group NR, n (%)	P-value ^a
Age (years)	69.5 (40-89)	71.5 (31-90)	0.469
Gender (male:female)	27:13	14:6	0.844
Stage			0.011
I	16 (40.0)	14 (70.0)	
II	7 (17.5)	5 (25.0)	
III	17 (42.5)	1 (5.0)	
Histology			0.585
AC	25 (62.5)	10 (50.0)	
ASC	2 (5.0)	2 (10.0)	
SqCC	13 (32.5)	8 (40.0)	

^aPearson's χ^2 analysis. Group R, tumor recurrence within 4 years; Group NR, disease-free following initial surgery; AC, adenocarcinoma; ASC, adenosquamous carcinoma; SqCC, squamous cell carcinoma.

deletion, E746-A750) RNA, and A549, which expresses low levels of *RAB38* and non-mutated EGFR RNA. The cells were maintained in RPMI 1640 (complete medium; Invitrogen; Thermo Fisher Scientific, Inc.). The Lentiviral Expression Vector system (dual function of TOOLSilent shRNA vector; Biotools Co., Ltd., New Taipei City, Taiwan) was used to establish stable *RAB38*-knockdown sublines with Lipofectamine[®] 3000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) (target sequence, 5'-AATTCAAAAAAGCAATGAGTGTGACCTACTCTTGAACATTAGGTCACACTCATTGCG-3'). The stable transfectants were selected using G418 for 2 weeks. The basal expression of *RAB38* and knockdown efficiency were determined by RT-PCR, as described above.

Western blot analysis. The expression levels of the *RAB38* were also detected by western blot analysis. The protein was extracted using ice-cold lysis buffer (20 mM Tris-base pH8.0, 150 mM NaCl, 1% NP-40) supplemented with a cocktail of protease inhibitor (cat. no. ab65621; Abcam) and the concentration of protein was determined using a Bradford protein assay. Subsequently, the protein (35 µg) was separated by a 4–15% gradient SDS-PAGE and transferred onto a nitrocellulose membrane. The blotted membrane was washed with tris buffered saline (TBS) for 5 min at room temperature and incubated in blocking buffer (1X TBS containing 5% non-fat milk) for 1 h at room temperature. The membrane was then incubated overnight at 4°C with a monoclonal antibody direct against human β -actin (1:10,000 dilution, cat. no. ab8224; Abcam, Cambridge, MA, USA) and *RAB38* (1:100 dilution, cat. no. GTX51060; GeneTex, Inc., Irvine, CA, USA). Following washing three times for 5 min with 1X TBS containing 0.1% Tween-20, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (1:5,000 dilution; *RAB38* cat. no. ab97051; β -actin cat. no. 97023; Abcam) for 2 h at room temperature. The antigen-antibody interaction was

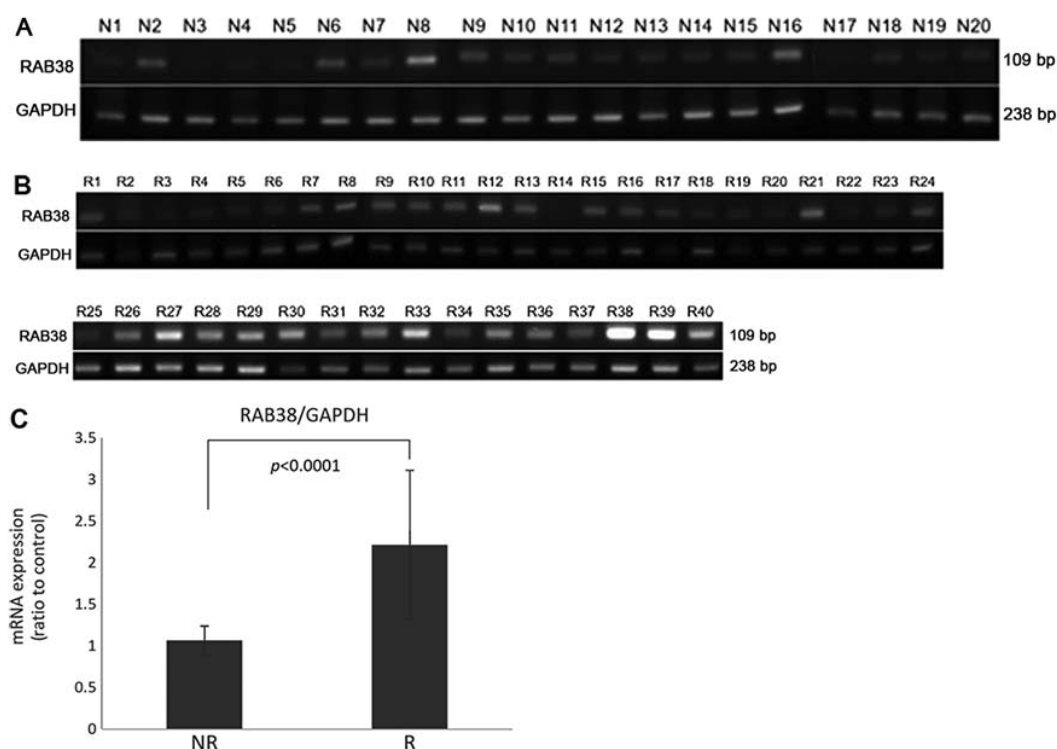


Figure 1. Expression of *RAB38* in non-small cell lung cancer tumor samples. The RNA expression levels of *RAB38* were significantly higher in (A) 20 Group NR samples compared with those in (B) 40 Group R samples ($P < 0.0001$), as identified by reverse transcription-qPCR. (C) Quantification of PCR products following electrophoresis was conducted using Image J v4.0 software. Two independent experiments were performed. qPCR, quantitative polymerase chain reaction; RAB38, Ras-related protein Rab-38.

traced with a chemiluminescence detection system. The blot signals were detected using a digital image system. These data were quantified by densitometric analysis with Image J v4.0 software, and the relative expression level was normalized by the internal standard β -actin.

In vitro invasiveness. The transfected cells were seeded into the upper wells of a BioCoat Matrigel invasion chamber (Corning, Bedford, MA, USA) in serum-free medium containing 0.1% BSA (Corning). The lower wells were filled with complete medium. Following incubation for 48 h at 37°C, a cotton swab was applied to remove the non-invaded cells in the upper chamber; those that had migrated through the filter pores were fixed and stained with 0.5% (w/v) crystal violet (cat. no. C6158; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in methanol. Images were captured using a light microscope (IX70, Olympus Corporation, Tokyo, Japan), and analyzed with PAX-IT image analysis software (Midwest Information Systems, Villa Park, IL, USA).

Statistical analysis. Semiquantitative results are presented as the mean \pm standard deviation. Differences in the expression of *RAB38* were analyzed using Student's t-test. Categorical data were analyzed by Pearson's χ^2 method. Disease-free survival (DFS) was defined as the period of time following primary surgical treatment until recurrence of NSCLC. Overall survival (OS) was defined as the date of diagnosis until mortality, or the most recent follow-up. The DFS and OS were estimated using Kaplan-Meier survival analysis, and the log-rank test was used to assess the difference between these two groups. For validation

of the value of prognosticator, univariate and multivariate Cox proportional hazards analyses were also performed. Statistical analyses were performed with IBM SPSS version 20.0 software (IBM Corp., Armonk, NY, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Patient characteristics. A total of 60 patient samples were assessed. The patients' characteristics are shown in Table I. The mean patient age was 69.5 years in Group R and 71.5 years in Group NR. The sex ratio (male/female) was 2.08 in Group R and 2.33 in Group NR. The percentages of adenocarcinoma, adenosquamous cell carcinoma and squamous cell carcinoma were 62.5, 5.0 and 32.5% in Group R and 50.0, 10.0 and 40.0% in Group NR, respectively. There was no difference between the R and NR groups, with the exception that there was a higher number of cases of cancer stage III in Group R. In Group R, the stage distribution of I, II and III was 40.0, 17.5 and 42.5%; whereas in Group NR the stage distribution of I, II and III was 70.0, 25.0 and 5.0%.

Expression of *RAB38* is higher in Group R samples compared with those in Group NR. In the present study, the expression of levels of *RAB38* in 40 Group R (Fig. 1A) and 20 Group NR (Fig. 1B) frozen tumor tissues were assessed using semi-quantitative RT-PCR analysis. The results revealed that the expression levels of *RAB38* were significantly higher in Group R samples compared with those in Group NR samples ($P < 0.0001$; Fig. 1C).

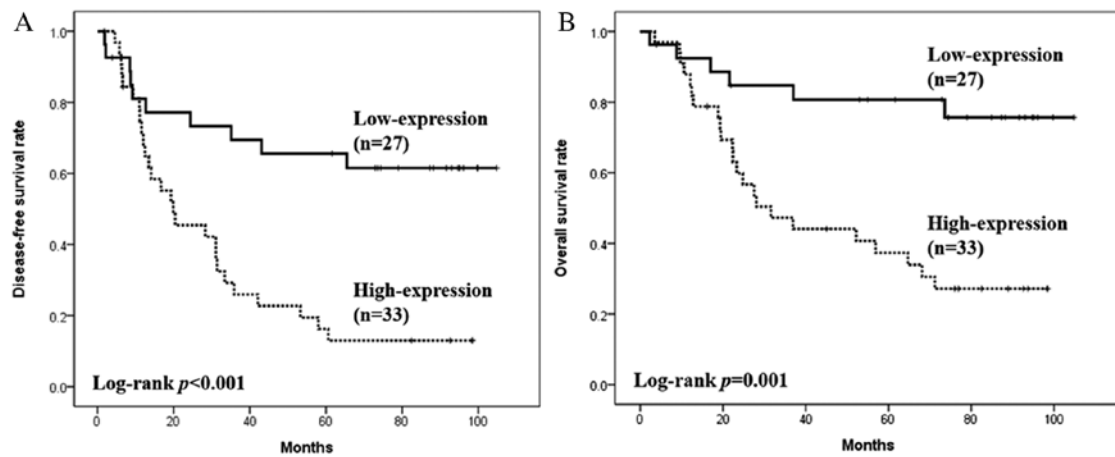


Figure 2. Effects of *RAB38* on patient survival rates. (A) The median DFS was 19.9 months (95% CI=4.2-35.6) and was not reached in patients whose tumors expressed high and low *RAB38*, respectively (HR=0.28, 95% CI=0.133-0.589, $P<0.001$). (B) The median OS was 31.6 months (95% CI=15.0-48.2) and was not reached in patients whose tumors expressed high and low *RAB38*, respectively (HR=0.23, 95% CI=0.094-0.576, $P=0.001$). DFS, disease-free survival; OS, overall survival; CI, confidence interval; HR, hazard ratio; *RAB38*, Ras-related protein Rab-38.

RAB38 may be a prognostic factor in NSCLC. The expression level of the *RAB38* was significantly associated with DFS and OS in patients with NSCLC. As shown in Fig. 2A, the median DFS was not reached in the *RAB38* low expression group, whereas in the *RAB38* high expression group, the DFS was 19.9 months [95% confidence interval (CI)=4.2-35.6 months; $P<0.001$]. The median OS was not reached in the *RAB38* low expression group, whereas the OS of the *RAB38* high expression group was 31.6 months (95% CI=15.0-48.2 months; $P=0.001$; Fig. 2B).

Consistent with previous results (17), the expression level of *RAB38* was correlated with tumor recurrence. While 29 of the 33 patients (87.8%) with high expression levels of *RAB38* developed recurrence, it was apparent in only 11 of the 27 patients (40.7%) with low expression levels of *RAB38* ($P=0.001$). In Group R, the mRNA expression level of *RAB38* was associated with poor OS, although this was not statistically significant (median OS, 37.0 vs. 27.5 months; $P=0.335$). Although the higher number of stage III cases in Group R may lead to a higher recurrence rate and poor prognosis in patients with a high expression of *RAB38*, poor prognosis was also observed in stage I cases (Fig. 3) in tumors with high expression of *RAB38* (median OS, not reached vs. 68.1 months, $P=0.011$). The results of the univariate and multivariate analyses are shown in Table II. The univariate analysis revealed that only cancer stage ($P=0.004$) and the expression level of *RAB38* ($P=0.002$) were prognostic factors for NSCLC. Cox multivariate analysis indicated that the expression level of *RAB38* ($P=0.010$) was a more robust prognostic factor than stage status ($P=0.053$).

Expression of RAB38 is associated with the invasiveness of lung cancer cells. The expression of *RAB38* was further analyzed in two human NSCLC cell lines. *RAB38* was expressed at a higher level in HCC827 cells harboring an active *EGFR* mutation, compared with that in A549 cells expressing wild-type *EGFR* (Fig. 4A). Additionally, *RAB38* silencing in the HCC827 cells (with a reduction of 20-30% according to RT-PCR and western blot data, Fig. 4B) substantially inhibited

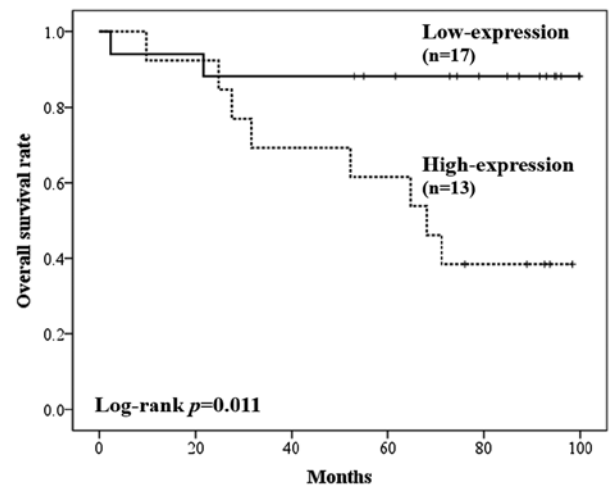


Figure 3. OS in patients with stage I cancer expressing differential *RAB38*. OS was analyzed using Kaplan-Meier survival analysis. The median OS was 68.1 months (95% CI=45.8-90.4) and was not reached in patients whose tumors expressed high and low *RAB38*, respectively ($P=0.011$). OS, overall survival; *RAB38*, Ras-related protein Rab-38; CI, confidence interval.

Matrigel invasiveness (with a reduction of 60%, Fig. 4C and D), indicating that the expression of *RAB38* may be positively associated with lung cancer metastasis.

Discussion

In the present study, the gene expression of *RAB38* was evaluated in patients with NSCLC; it was demonstrated that high levels of *RAB38* were more frequently detected in stage III NSCLC, and that this was significantly associated with tumor recurrence and poorer OS. However, the mRNA expression level of *RAB38* was also associated with poor OS in patients with stage I NSCLC. The small sample size may have contributed to this observation, thus further investigation with a larger sample number is required to confirm these conclusions. To the best of our knowledge, this is the first report describing the prognostic role of *RAB38* in human NSCLC.

Table II. Univariate and multivariate overall survival analyses of 60 (R=40, NR=20) patients with non-small cell lung cancer using the Cox proportional hazards model.

Variable	Univariate analysis			Multivariate analysis		
	HR	95% CI	P-value ^a	HR	95% CI	P-value ^a
Age (years)						
≤65	0.635	0.271-1.487	0.295			
>65	1					
Gender						
Male	0.810	0.382-1.717	0.582			
Female	1					
Histology			0.919			
AC	0.855	0.396-1.846	0.691			
ASC	0.982	0.217-4.432	0.891			
SqCC	1					
Stage			0.004			0.053
III	4.227	1.813-9.856	0.001	2.718	1.137-6.497	0.025
II	2.029	0.735-5.596	0.172	2.495	0.890-6.994	0.082
I	1			1		
RAB38						
High	4.29	1.736-10.603	0.002	3.650	1.367-9.743	0.010
Low	1			1		

^aCox analysis. HR, hazard ratio; CI, confidence interval; AC, adenocarcinoma; ASC, adenosquamous carcinoma; SqCC, squamous cell carcinoma; RAB38, Ras-related protein Rab-38.

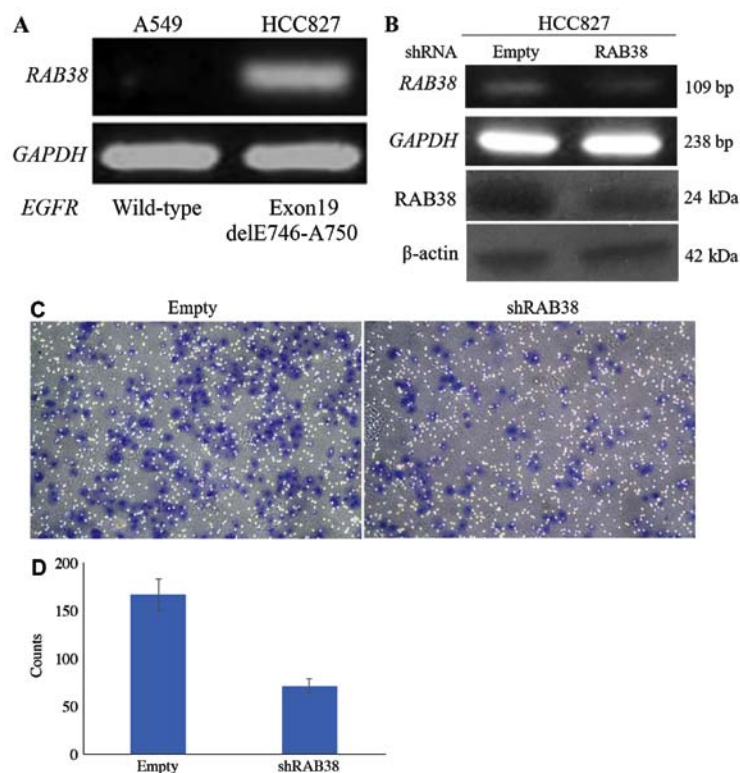


Figure 4. Upregulation of *RAB38* in non-small cell lung cancer with an active epidermal growth factor receptor mutation. (A) Expression of *RAB38* in A549 and HCC827 cells was detected using RT-PCR analysis. HCC827 cells were transfected with shRNA targeting *RAB38* or empty vector. (B) Knockdown efficiency was determined by RT-PCR and western blot analyses. (C) *In vitro* invasiveness of gene-modified HCC827 cells was performed with a Matrigel invasion chamber. Original magnification, x100. (D) *RAB38* knockdown significantly inhibited tumor Matrigel invasiveness compared with that in the negative, empty vector controls. A total of three independent experiments were performed. *RAB38*, Ras-related protein Rab-38; RT-PCR, reverse transcription-polymerase chain reaction; shRNA, short hairpin RNA.

Although RAB proteins have been reported to be over-expressed in numerous types of cancer, the functionality of these proteins in cancer progression remains to be clarified. The tissue-specific expression pattern of *RAB38* has previously been demonstrated. Jäger *et al* (18) reported that high RT-PCR signals were detected in cultured melanocytes and adrenal gland tissues; whereas weak to moderate signals were observed in the testes, kidney, uterus, prostate and pancreas. *RAB38* mRNA was expressed in 80-90% of melanoma cases, but showed minimal expression in non-melanocytic malignancies. Although not detectable in lung tissues, the expression of *RAB38* was observed in one of four lung cancer cell lines assessed, suggesting that it may serve a role in lung tumorigenesis (18). This study further demonstrated that *RAB38* is strongly immunogenic, leading to spontaneous antibody responses in a significant proportion of patients with melanoma, and indicated that it may serve as a potential therapeutic target (19).

Chen *et al* (20) established a paired human lung adenocarcinoma cell line from primary tumor site tissues and metastatic lymph nodes and analyzed the gene expression profiles by microarray. The expression of *RAB38* was markedly increased in tumor cells derived from metastatic lymph nodes compared with those from the primary tumor site. The present study revealed that gene silencing markedly reduced tumor cell invasion in HCC827 cells. Collectively, these findings suggest that *RAB38* may be involved in lung cancer metastasis.

By contrast, in searching for potential tumor suppressor genes in lung cancer, Wu *et al* (21) reported that *RAB37* was frequently downregulated in tumor tissues, compared with that in normal non-cancerous tissues, by promoter methylation, and that the low expression of *RAB37* was associated with tumor metastasis. In addition, the expression of *RAB37* was shown to be inversely correlated with cell motility in lung cancer cell lines (22), indicating the differential functionality of RAB proteins in lung cancer metastasis.

Crosstalk between integrin and growth factor receptor pathways has been reported to serve a prominent role in tumor progression (23,24). β 1-integrin is required for EGFR signaling, and the silencing of β 1-integrin substantially impairs the EGF-induced activation of EGFR in lung cancer cells (25). Ectopic expression of *RAB25* was demonstrated to increase the expression of β 1-integrin and subsequent activation of EGFR in breast cancer cells *in vitro*, and to increase tumorigenesis and pulmonary metastasis in ovarian cancer cells *in vivo* (26). The present study demonstrated that *RAB38* was upregulated in cases of NSCLC associated with an active *EGFR* mutation, compared with those associated with wild-type *EGFR*, suggesting that the expression of *RAB38* may be associated with *EGFR* status. However, only two cell lines were analyzed, and further investigation is required to confirm this association. Furthermore, the collection of additional tumor specimens is required to determine this association in patient tissues. Investigation of the functional analysis of *RAB38* in NSCLC, *in vitro* and *in vivo*, is currently in progress.

In conclusion, the present study demonstrated that *RAB38* is an important prognostic factor and potential therapeutic target in NSCLC.

Acknowledgements

The authors would like to thank Professor Alex YC Chang (Johns Hopkins Singapore) for data evaluation and as a consultant for manuscript writing. The authors also wish to thank the Laboratory of Dr Cheng-Hsu Wang (Chang Gung Memorial Hospital, Keelung branch) for their technical support.

Funding

The present study was supported by grants to JC (grant nos. CMRPG380761 and CMRPG3B0081~3) and MH (grant no. CMRPG3F1261) from Chang Gung Memorial Hospital, Taiwan.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

JH and HC performed data analyses and wrote the manuscript. YS and MH collected the dataset, and contributed to data analyses and manuscript revision. JC and TH conceived and designed the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

In the original creation of the datasets, the Institutional Review Board of Chang Gung Memorial Hospital approved the study and informed consent to participate was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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