RagD gene expression and *NRF2* mutations in lung squamous cell carcinomas

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Abstract. *RagD* is a member of the small G protein family, which encodes a recently discovered activator of the mTOR pathway. *In vitro*, *RagD* plays an important role in the proliferation of *NRF2* gene (*NFE2L2*) mutated cancer cells. We hypothesized that tumor *RagD* expression may be correlated with the mutation status of *NRF2* in lung cancers. *RagD* mRNA levels were analyzed by quantitative real-time polymerase chain reaction (qPCR) in 90 surgically-treated lung squamous cell cancer cases, including 14 *NRF2* mutation cases, and normalized by *β-actin* mRNA levels. Mean *RagD*/*β-actin* mRNA levels of lung squamous cell carcinoma patients did not differ with age (≤65 vs. >65), Brinkman index (<400 vs. ≥400) or gender. *RagD*/*β-actin* mRNA levels were significantly higher in stage III samples (3.204±3.623) compared to stage I samples (1.357±1.560) (P=0.0039). In addition, higher *RagD*/*β-actin* mRNA levels were identified in *NRF2* mutant samples (3.107±3.633) compared to wild-type samples (1.774±2.301) (P=0.074). These results suggest that *RagD* induction by *NRF2* activation plays a role in the proliferation of lung squamous cell cancers.

Introduction

Despite recent improvements in diagnosis, lung cancer is a major cause of mortality from malignant diseases due to its high incidence, malignant behavior and lack of major advancements in treatment strategy (1). Although there have been advances in understanding the biology of lung cancer and introduction of new chemotherapeutic agents for treatment, the 5-year survival rate remains less than 15% (2). Recently, progression in understanding oncogenic kinase signaling pathways has provided more successful targets for developing effective therapeutic strategies (3), which may improve the outcome of lung cancer.

A potential therapeutic target is the mammalian target of rapamysin (mTOR) pathway, which plays a central role in regulating cell functions, including proliferation, growth, survival, mobility and angiogenesis (4,5). Dysregulation of the mTOR pathway has been reported in lung cancers (6,7). A member of the small G protein family, *RagD*, which encodes a recently discovered activator of the mTOR pathway (8), was significantly upregulated in cells expressing mutant *NRF2* (9). It has been demonstrated that mutations of the *NRF2* gene (*NFE2L2*) are associated with primary lung cancer (10-13). It has also been revealed that patients with lung tumors containing the *NRF2* gene mutation display a poorer prognosis compared to patients with non-mutant tumors (11,12). Additionally, *NRF2* gene somatic mutation is more common in lung squamous cell carcinomas (11).

Although we have revealed the *NRF2* gene mutation status in lung cancer (11), the correlation between *NRF2* gene mutation and *RagD* expression status in lung cancer has not been reported. To determine the *RagD* mRNA expression status, we performed quantitative real-time polymerase chain reaction (qPCR) using a LightCycler (Roche Diagnostics GmbH, Mannheim, Germany). The findings were compared to the clinicopathological features of lung squamous cell carcinomas.

Patients and methods

Patients. The study group included 90 lung squamous cell carcinoma patients who had undergone surgery at the Department of Oncology, Immunology and Surgery, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan. All tumor samples were immediately frozen and stored at -80˚C until assayed. Written informed consent was obtained from each patient prior to the study. The study was approved by the Institutional Review Board of Nagaya City University Graduate School of Medicine.

The clinical and pathological characteristics of the 90 lung squamous cell carcinoma patients are shown in Table I. Among the 90 patients, 83 were male and the mean age was 66.8 years (range, 49-80 years). A total of 30 patients had lymph node metastasis and 47 cases were pathological stage I,

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19 were stage II and 24 were stage III. All patient samples were sequenced for the *NRF2* gene (11) and 14 cases were positive for the *NRF2* gene mutation.

PCR assays for NRF2. Total RNA was extracted from lung cancer tissues using an Isogen kit (Nippon Gene Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. RNA concentration was determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Rockland, DE, USA). Approximately 5 cases were excluded from each assay due to the an insufficient number of tumor cells to effectively extract tumor RNA. RNA (1 μg) was reverse transcribed using a First-Strand cDNA synthesis kit with 0.5 μg oligo$(dT)_{16}$ (Roche Diagnostics GmbH) according to the manufacturer's instructions. The reaction mixture was incubated at 25˚C for 15 min, 42˚C for 60 min, 99˚C for 5 min and then at 4˚C for 5 min. The cDNA concentration was also determined using a NanoDrop ND-1000 Spectrophotometer. Approximately 200 ng of each cDNA was used for PCR analysis. To ensure the accuracy of mRNA extraction and reverse transcription, all samples were subjected to PCR amplification using a *β-actin* primers kit (Nihon Gene Research Laboratory, Miyagi, Japan) and a LightCycler FastStart DNA Master HybProbe kit (Roche Diagnostics GmbH). The RT-PCR assay reactions were performed using a LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics GmbH) in a 20 μl reaction volume. The primer sequences for the *RagD* gene were as follows: forward, 5'-GACAAAGTTCCTGGCTCTCG-3' and reverse, 5'-AGCACTCTAGGGGTCCCATT-3' (210 bp). Cycling conditions consisted of an initial denaturation period at 95˚C for 10 min, followed by 40 cycles at 95˚C for 10 sec, 62˚C for 10 sec and 72˚C for 9 sec.

Statistical analysis. Statistical analyses were conducted using the Mann-Whitney U test for unpaired samples and the Wilcoxon's singed rank test for paired samples. Linear relationships between variables were determined by means of simple linear regression. Correlation coefficients were determined by rank correlation using the Spearman's rho test and Chi-squared test. The overall survival of lung cancer patients was examined using the Kaplan-Meier analysis, and differences were examined using the log-rank test. The Stat-View software package (Abacus Concepts Inc., Berkeley, CA, USA) was used for all statistical analyses and $P<0.05$ was considered to indicate a statistically significant difference.

Results

NRF2 gene mutation in lung cancer. Previously, we investigated the *NRF2* gene mutation status in the N-terminal domain by direct sequencing (11). A total of 291 non-small cell lung cancer (NSCLC) patients, including 148 lung squamous cell carcinoma patients, were investigated and 16 were identified to express *NRF2* gene mutations. All of the mutations were identified in male patients with lung squamous cell carcinomas.

RagD mRNA levels in lung cancer patients. In this study, we investigated 90 lung squamous cell carcinoma patients, including 14 *NRF2* mutant patients, in order to examine their

Table I. Clinicopathological data of 90 lung squamous cell carcinoma patients.

Characteristics	Number of patients (%)	*RagD* gene status: *RagD/β-actin* mRNA levels	P-value
Age			
Mean ± SD (years)	66.8±8.0		
≤65	37 (41.1)	1.532±2.477	0.1683
>65	53 (58.9)	2.294±2.617	
Gender			
Male	83 (92.2)	2.070±2.648	0.2622
Female	7 (7.8)	0.929±1.011	
Pathological stage			
I	47 (52.2)	1.357±1.560	0.0039[a]
II	19 (21.1)	1.979±2.559	
III	24 (26.7)	3.204±3.623	
Lymph node metastasis			
N0	60 (66.7)	1.810±2.106	NS
N1	14 (15.6)	2.000±3.116	
N2	16 (17.8)	2.606±3.593	
BI status			
<400	4 (4.4)	1.625±0.624	0.7789
≥400	86 (95.6)	1.998±2.630	
Differentiation			
Well	22 (24.4)	2.214±2.938	NS
Moderate	45 (50.0)	2.204±2.696	
Poor	21 (23.3)	1.367±1.921	
NRF2 mutation			
Mutant	14 (15.6)	3.107±3.633	0.0747
Wild-type	76 (84.4)	1.774±2.301	

[a]P-value is comparison of stage I and III. N0, negative lymph node metastasis; BI, Brinkman index; NS, not significant.

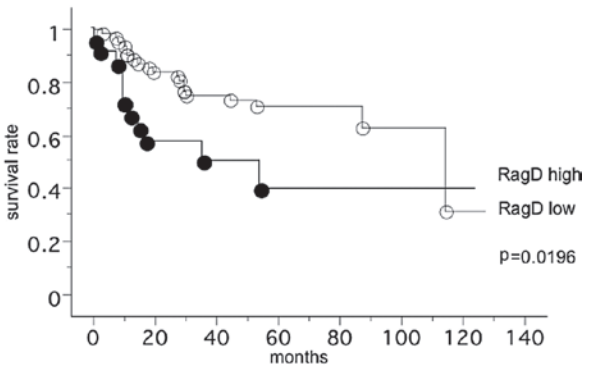

Figure 1. Overall survival was studied in reference to the *RagD/β-actin* mRNA level whether it was more or less than 2.14. Survival of the patients with high *RagD/β-actin* mRNA (11/21 resulted in mortlity; mean survival, 33.6 months) was significantly less than the patients with low MRP3/*β-actin* mRNA level (19/69 resulted in mortlity; mean survival, 85.0 months) (log-rank test, P=0.0196).

RagD/*β-actin* levels (Table I). We revealed that the mean *RagD*/*β-actin* level in lung cancer tissues was 2.138±2.698 and did not correlate with age (R^2=0.17; P=0.2487). Additionally, *RagD*/*β-actin* mRNA levels were not correlated with age (≤65 vs. >65 years; P=0.1683), Brinkman index (<400 vs. ≥400; P=0.7789), lymph node metastasis, tumor invasion status or pathological differentiation status. *RagD*/*β-actin* mRNA level was correlated with pathological stage, and there was a tendency towards higher *RagD*/*β-actin* mRNA level in higher pathological stages (stage I, 1.357±1.560; stage II, 1.979±2.599; stage III, 3.204±3.623). *RagD*/*β-actin* mRNA level was significantly higher in stage III cases compared to stage I cases (P=0.0039). In addition, significantly higher levels of *RagD*/*β-actin* mRNA were demonstrated in *NRF2* mutant cases (3.107±3.633) compared to *NRF2* wild-type cases (1.774±2.301) (P=0.0747).

The overall survival of 90 lung squamous cell carcinoma patients, with follow-up until December 31, 2010, was studied in reference to the *RagD*/*β-actin* mRNA level. The survival of patients with high *RagD*/*β-actin* mRNA levels (11/21 mortalities; mean survival, 33.6 months) was significantly less compared to patients with low *RagD*/*β-actin* mRNA levels (19/68 mortalities; mean survival, 85.0 months) (log-rank test, P=0.0196) (Fig. 1). However, multivariate analysis demonstrated *RagD* mRNA was not an independent prognostic factor.

Discussion

In this study, we identified that *RagD* mRNA levels were correlated with advanced stage lung squamous cell carcinomas. We also demonstrated that high *RagD* mRNA levels correlated with poor prognosis using univariate analysis. Although the sample size was small, there was a tendency towards higher *RagD* mRNA levels in *NRF2* mutant lung squamous cell carcinoma patients.

RagD is a member of the small G protein family gene, which encodes a recently discovered positive regulator of the mTOR pathway (8,14,15), and is upregulated in *NRF2* mutant cell lines. It has been demonstrated that *RagD* knockdown reduces the activation of mTOR signaling and *NRF2* down-regulation reduces *RagD* expression. Therefore, *RagD* plays an important role in the proliferation of *NRF2*-mutant cancer cells (9). However, the putative promoter region of the *RagD* gene contains no ARE sequence, and chromatin immunoprecipitate sequence analyses has revealed that *RagD* is not a direct target of *NRF2* (9). An additional regulatory mediator may link the *NRF2* gene and *RagD*.

The *NRF2* gene is a master transcriptional activator of genes encoding a number of cytoprotective enzymes that are induced in response to environmental and endogenously derived oxidative/electrophilic agents (16-18). A previous study demonstrated that RNAi-mediated silencing of *NRF2* gene expression in NSCLC inhibited tumor growth (19). A *NRF2* gene promoter polymorphism has been identified and was suggested to correlate with carcinogenesis (20). The correlation between *NRF2* mutations and *RagD* mRNA levels of lung squamous cell carcinomas suggests a role of *NRF2* in tumor growth. Constitutive expression of *NRF2* may provide a survival advantage to invasive and metastatic cancer cells. *NFR2* may adapt these cancer cells to the micro-environment by increasing chemoresistance under hypoxic conditions (21,22).

Higher *RagD* mRNA levels were correlated with poor prognosis, however, this may be due to the correlation with pathological stages. Our previous study demonstrated that mutant *NRF2* had poor prognosis (11), which confirmed results from other experiments (10,12). In addition, previous studies revealed that mTOR expression was a prognostic biomarker for poor survival of lung cancers (6,7,23). A longer follow-up period and larger cohort are required to analyze *RagD* expression as a prognostic biomarker for lung cancers.

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