Increased *NRF2* gene (*NFE2L2*) copy number correlates with mutations in lung squamous cell carcinomas

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Abstract. Nuclear factor (erythroid-derived 2)-like 2 (NRF2) is a transcription factor belonging to the cap 'n' collar subfamily of the basic-leucine zipper (bZIP) family of transcription factors, which plays a significant role in adaptive responses to oxidative stress. Previously, we reported that *NRF2* gene (*NFE2L2*) mutations correlate with poor prognosis of lung squamous cell carcinomas. We therefore hypothesized that an increased *NRF2* gene copy number may correlate with clinicopathological features in lung cancer patients. In this study, the increased copy number of the *NRF2* gene was analyzed by real-time polymerase chain reaction (real-time-PCR) amplifications in 90 surgically-treated non-small cell lung cancer (NSCLC) cases. In total, 16 *NRF2* mutation cases were included. An increased *NRF2* gene copy number was found in 7 (7.8%) lung squamous cell carcinoma patients. Increased *NRF2* copy number status significantly correlated with mutation status (mutant, 31.25% vs. wild-type, 2.7%; p=0.0017). The mean *NRF2* gene copy number was significantly higher in mutant (2.478±0.668) compared to wild-type *NRF2* (1.917±0.737) (p=0.0048). However, the copy number did not correlate with smoking status (p=0.3741), gender (p=0.1545), age (≥65 vs. <65, p=0.1237) and pathological stage. Although an increased *NRF2* copy number correlates with mutations in squamous cell carcinoma, the percentage of the increased copy number was low; therefore, another mechanism may exist for the activation of *NRF2* mutations in cancer.

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 the lack of major advancements in treatment strategy (1). Lung cancer was the leading indication for respiratory surgery (42.2%) in 1998 in Japan (2). More than 15,000 patients underwent surgery at Japanese institutions in 1998 (2). The clinical behavior of lung cancer is largely associated with its stage. The cure of the disease by surgery is only achieved in cases presenting with an early stage of lung cancer (3).

Nuclear factor (erythroid-derived 2)-like 2 (NRF2) is a transcription factor belonging to the cap 'n' collar subfamily of the basic-leucine zipper (bZIP) family of transcription factors, which plays a significant role in adaptive responses to oxidative stress (4). At the expression level, NRF2 is expressed widely in various human tissues (5), including lung cancer (6). It has been indicated that the overexpression of NRF2 in premalignant cells may enable the cancer cells to survive in an oxidizing tumor environment. Subsequently, the cancer cells alter their metabolic processes, mitochondrial dysfunction and activation of oncogenic signals. It has been shown that patients with lung tumors containing the *NRF2* gene (*NFE2L2*) mutation display a poorer prognosis than patients with non-mutant tumors (7,8). It has been reported that mutations of the *NRF2* gene have been associated with primary lung cancer (6-9). *NRF2* gene somatic mutation is more common in lung squamous cell carcinomas (7). Recently, *NRF2* overexpression was investigated in lung cancer (10-12), and the NRF protein has also been shown to be highly expressed in squamous cell carcinomas of the lung (6).

Although we have previously reported the status of the *NRF2* gene (*NFE2L2*) mutations in lung cancers (7), the association of *NRF2* gene copy number status and Japanese lung cancer has not been previously reported. Therefore, in the present study, in order to determine the *NRF2* gene copy number status in Japanese lung squamous cell carcinomas, we investigated the copy number by real-time polymerase chain reaction (real-time-PCR) amplifications. 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

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Surgery, Nagoya City University Hospital, Nagoya, Japan. All tumor samples were immediately frozen and stored at -80˚C until assayed.

The clinical and pathological characteristics of the 90 lung squamous cell carcinoma patients were as follows: 43 cases at stage I, 20 at stage II and 27 at stage III. The mean age was 66.7 years (range, 36-85). Among the 90 lung cancer patients, 35 had lymph node metastasis, 83 were male and 16 had *NRF2* gene mutations. The samples from these patients had previously been sequenced for the *NRF2* gene (*NFE2L2*) (7).

PCR assays for NRF2. Genomic DNA was extracted from lung cancer tissues using the Wizard SV Genomic DNA Purification System (Promega, Madison, WI, USA) according to the manufacturer's instructions. DNA concentration was determined by a NanoDrop spectrophotometer (NanoDrop Technologies, Inc. Rockland, DE, USA) and adjusted to a concentration of 2.5 ng/ml. We then used 5 μl of each DNA for PCR assays. *NRF2* copy number was analyzed by quantitative real-time PCR, performed using a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) by using the QuantiTect SYBR-Green kit (Qiagen Inc., Valencia, CA, USA) (13-15). *NRF2* primers used for amplification were: 5'GGTTTCTTCGGCTACGTTT-3' and 5'TAACTCAGGAATGGATAATAGCTC-3'. Total DNA content was estimated by assaying *Line-1* elements for each sample using the primers, 5'-AAAGCCGCTCAACTACATGG-3' and 5'-TGCTTTGAATGCGTCCCAGAG-3'. The cycling conditions were as follows: initial denaturation at 95˚C for 15 min, followed by 40 cycles at 94˚C for 15 sec, 56˚C for 30 sec and 72˚C for 34 sec. Copy numbers >3 were considered as increased, according to previous reports (13,16,17).

Statistical analysis. Statistical analyses were performed using the Mann-Whitney U-test for unpaired samples and Wilcoxon's signed rank test for paired samples. Linear relationships between variables were determined by means of a simple linear regression. Correlation co-efficients were determined by rank correlation using the Spearman's test and χ^2 test. The overall survival of the lung cancer patients was examined by the Kaplan-Meier method, and differences were examined by the Log-rank test. All analyses were performed using the Stat-View software package (Abacus Concepts Inc., Berkeley, CA, USA), and a p-value <0.05 was considered to indicate a statistically significant difference.

Results

NRF2 gene mutation in Japanese lung cancer. We investigated the *NRF2* gene (*NFE2L2*) mutation status in the N-terminal domain by direct sequencing as previously described (7). In total, 13 out of 109 squamous cell carcinoma patients had *NRF2* gene mutations. In addition, we analyzed 39 squamous cell carcinoma patients and 3 had *NRF2* gene mutations. These 3 were known mutations (T80A, W24C, R34Q). In total, 16 out of 148 (10.8%) had *NRF2* gene mutations (Fig. 1). All of the mutations were found in squamous cell carcinomas. The *NRF2* gene mutations were clustered on exon 2 and resulted in amino acid changes in either the DLG or the ETGE motif of the regulatory Neh2 domain (7).

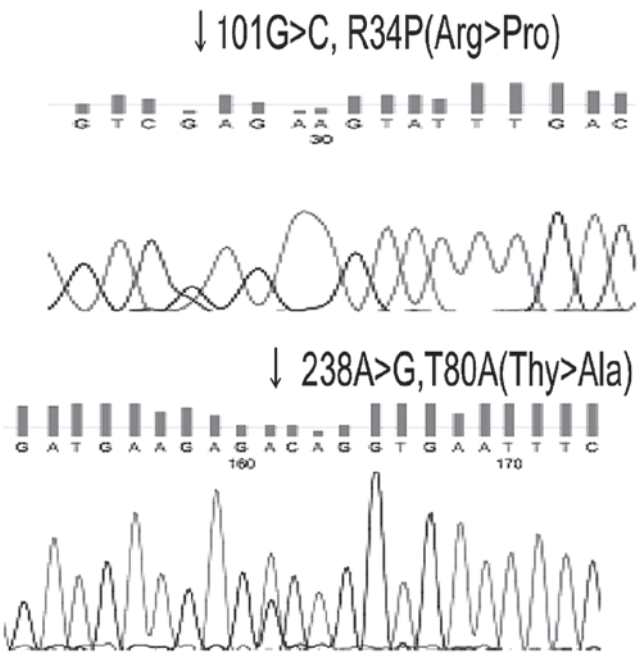

Figure 1. Results from the direct sequencing of the *NRF2* gene. Upper panel, 101 G to A transversion resulted in 34 arginine (Arg) to proline (Pro) conversion (R34P mutation). Lower panel, 238 A to G conversion resulted in thyreonine (Thy) to alanine (Ala) conversion (T80A mutation).

NRF2 gene status in Japanese lung cancer patients. Using the primer sets for the *NRF2* gene, from 90 lung cancer patients, 7 patients had more than 3 copies of the *NRF2* gene and the clinicopathological background is shown in Table I. *NRF2* gene copy number status significantly correlated with gene mutations (mutant, 31.25% vs. wild-type, 2.7%; p=0.0017). However, the copy number status did not correlate with smoking status (Brinkman index <400 vs. ≥400; p=0.4443), pathological stage (stage I vs. stage II and III, p=0.4375), lymph node metastasis (positive vs. negative, p=0.4242) or age (<65 vs. ≥65, p=0.9999). *NRF2* gene copy number significantly correlated with gene mutations (mutant, 2.478±0.668 vs. wild-type, 1.917±0.737; p=0.0048). However, the mean *NRF2* copy number did not correlate with smoking status (Brinkman index <400, 2.239±0.499 vs. ≥400, 1.998±0.593; p=0.3741), lymph node metastasis (negative, 2.074±0.567 vs. positive, 1.926±0.980; p=0.1695), age (≥65, 2.083±0.782 vs. <65, 1.96±0.732; p=0.1237) or pathological stage.

The overall survival of the 90 lung squamous cell carcinoma patients, with follow-up through to December 31, 2010, was studied in reference to the *NRF2* gene status. The survival of the patient with an increased copy number of the *NRF2* gene (n=7) and the patient with a normal copy number of *NRF2* (n=83) was not significantly different (Log-rank test, p=0.6582).

Discussion

In our analysis, an increased *NRF2* gene copy number was found in 7.8% of Japanese lung squamous cell carcinomas. The *NRF2* gene statuses correlated with *NRF2* mutations, indicating that the mutations were activating mutations.

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

Factors	NRF2 gene status		P-value
	Number of patients	Copy number	
Mean age (years)	66.7±8.4	90	
Pathological stage			NS
I	43 (47.8%)	2.035±0.490	
II	20 (22.2%)	2.165±1.033	
III	27 (30.0%)	1.877±0.859	
Lymph node metastasis			0.1695
N0	55 (61.1%)	2.074±0.567	
N+	35 (38.9%)	1.926±0.980	
BI status			0.3741
<400	7 (7.8%)	2.239±0.499	
≥400	83 (92.2%)	1.998±0.593	
Differentiation			
Well	27 (30.0%)	1.955±0.817	0.8671
Moderate/poor or other	63 (70.0%)	2.042±0.729	
NRF2 mutations			0.0048
Mutant	16 (17.8%)	2.478±0.668	
Wild-type	74 (82.2%)	1.917±0.737	
Age, years			0.1237
≥65	41 (45.6%)	2.083±0.782	
<65	49 (54.4%)	1.960±0.732	
Gender			0.1545
Male	3 (92.2%)	1.984±0.751	
Female	7 (7.8%)	2.404±0.720	

*NS, not significant. N0, lymph node metastasis-negative; N+, lymph node metastasis-positive; BI, Brinkman index.

NRF2 (*NFE2L2*) is a master transcriptional activator of genes encoding many cytoprotective enzymes that are induced in response to environmental and endogenously derived oxidative/electrophilic agents (18-20). A previous report showed that the RNAi-mediated silencing of *NRF2* gene expression in non-small cell lung cancer inhibited tumor growth (21). A *NRF2* gene promoter polymorphism has been identified and has been suggested to correlate with carcinogenesis (22). The association of *NRF2* mutation and increased copy number in lung squamous cell carcinomas suggests a role of *NRF2* in tumorigenesis. The constitutive expression of *NRF2* may provide a survival advantage to invasive and metastatic cancer cells, by adaptation to the microenvironment and evolution of chemoresistance in cancer cells under hypoxic conditions (23,24). A previous study showed that the degree of cisplatin (CDDP)-induced DNA crosslinking and the number of cells undergoing apoptosis were increased significantly in A549 cells transfected with *NRF2*-siRNA (25). The expression of multidrug resistance-associated proteins, the drug efflux proteins, has also been shown to be significantly reduced in *NRF2*-silenced A549 cells (25). Another study also showed that the inhibition of NRF2 function restored CDDP sensitivity in human ovarian cancer cells (26).

However, in our analysis, more than half of *NRF2* gene mutations did not have an increased *NRF2* gene copy number. The prognosis analysis between *NRF2* gene copy number statuses was not significantly different, which suggests that another mechanism may exist for the activation of *NRF2* mutations in cancer. As previously reported, *in vitro,* wild-type NRF2 was efficiency polyubiqitinated while mutant NRF2 proteins were only weakly polyubiquitinated after treatment with MG132 (8). Wild-type NRF2 protein decreased rapidly, whereas mutant NRF2 proteins were degraded more slowly, having half-lives of approximately twice that of wild-type (8). In addition, mutant NRF2 proteins were significantly more active than wild-type NRF2 by analyzing luciferase activity (8). In addition, the prognostic value of NRF2 expression in lung cancers was controversial. Merikallio *et al* showed that NRF2 protein expression was correlated with poor prognosis using multi-variate analysis (10). Kim *et al* showed that high NRF2 expression was not correlated with poor prognosis in stage I lung cancers (27). Interaction between other genes, such as *Keap1*, would be also important for control of NRF2 expression in NSCLC (28).

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