

***MRP3* gene expression correlates with *NRF2* mutations in lung squamous cell carcinomas**

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Abstract. The expression of multidrug-resistant protein-3 (MRP3), a membrane-bound transporter, has been linked to drug resistance in non-small cell lung cancers (NSCLCs). Recently, we reported that *NRF2* gene (*NFE2L2*) mutations are correlated with poor prognosis for lung squamous cell carcinomas. We hypothesized that tumor *MRP3* expression may be correlated with the mutation status of upstream regulators, including *NRF2*, in NSCLC patients. *MRP3* mRNA levels were analyzed by quantitative real-time polymerase chain reaction (qPCR) in 67 surgically-treated lung squamous cell cancer cases from the Nagoya City University Hospital and normalized by β -actin mRNA levels. Fourteen *NRF2* mutation cases were included. The mean *MRP3*/ β -actin mRNA levels did not differ according to age (≤ 65 vs. >65 years), Brinkman index (≤ 400 vs. >400) or the pathological stage of the lung squamous cell carcinoma. *MRP3*/ β -actin mRNA levels were significantly higher in men (1.200 ± 1.524) than in women (0.179 ± 0.083 ; $p=0.0036$). In addition, the *MRP3*/ β -actin mRNA levels were significantly higher in *NRF2* mutants (2.598 ± 2.373) than in wild-type *NRF2* mutants (0.734 ± 0.820 ; $p=0.0002$). These data support the hypothesis that the expression of MRP3 is induced by *NRF2* activation in lung squamous cell cancers.

Introduction

Despite recent improvements in diagnosis, lung cancer is a major cause of death due to its high incidence, malignant behavior and lack of major advancements in treatment strategy (1). Lung cancer was the leading indication for respiratory surgery (47.5%) in 2009 in Japan (2). Over 31,000 patients underwent surgery at Japanese institutions in 2009 (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 representing an early stage of lung cancer (3).

NRF2 is a transcription factor belonging to the cap'n/collar subfamily of the basic-leucine zipper (bZIP) family of transcription factors. *NRF2* plays a significant role in the adaptive responses to oxidative stress (4). *NRF2* is expressed widely in various human tissues (5), including lung cancer tissue (6). The overexpression of *NRF2* in premalignant cells enables the cancer cells to survive in an oxidizing tumor environment. Subsequently, the cancer cells alter the metabolism process and induce mitochondrial dysfunction and the activation of oncogenic signals. It has been shown that patients with lung tumors containing the *NRF2* gene (*NFE2L2*) mutation have a poorer prognosis than patients with non-mutant tumors (7,8). Moreover, the mutations of the *NRF2* gene have been associated with primary lung cancer (6-9). The *NRF2* gene somatic mutation is more common in lung squamous cell carcinomas (7). Multidrug-resistant proteins (MRPs) are members of the ATP-binding cassette superfamily that facilitate detoxification by transporting toxic compounds, including chemotherapeutic drugs, out of cells (10-12). Analysis of the *MRP3* promoter revealed the presence of multiple putative electrophile responsive element (EpRE) sequences that suggested the possible regulation of this gene by *NRF2* (13).

Although we have reported the *NRF2* gene mutation status in lung cancers (7), the association of the *NRF2* gene mutation and *MRP3* expression status in Japanese lung cancer has not been previously reported. To determine the *MRP3* mRNA expression status, we used quantitative real-time PCR (qPCR) using LightCycler. The findings were compared with the clinicopathological features of the lung squamous carcinomas.

Patients and methods

Patients. The study group included 67 lung squamous cell carcinoma patients who had undergone surgery at the Department of Surgery, Nagoya City University Hospital. Tumor samples were immediately frozen and stored at -80°C until assayed. Consent was obtained from the patients and the study was approved by the Ethics Committee of Nagoya City University Hospital.

The clinical and pathological characteristics of the 67 lung squamous cell carcinoma patients were as follows: 30 cases at

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Table I. *NRF2* mutations in Japanese lung cancers.

Nucleotide mutation	Amino acid change	Histology	Gender	Age (years)
238 A-G	T80A (Thr>Ala)	SCC	Male	68
72G-C	W24C (Trp>Cys)	SCC	Male	69
95 T-G	V32G (Val>Gly)	SCC	Male	74
100C-G	R34G (Arg>Gly)	SCC	Male	74
101G-C	R34P (Arg>Pro)	SCC	Male	63
101G-A	R34Q (Arg>Gln)	SCC	Male	65
101G-A	R34Q (Arg>Gln)	SCC	Male	66
101G-A	R34Q (Arg>Gln)	SCC	Male	79
230A-G	D77G (Asp>Gly)	SCC	Male	76
235G-C	E79Q (Glu>Gln)	SCC	Male	77
85 G-T	D29Y (Asp>Tyr)	SCC	Male	58
235G-A	E79K (Glu>Lys)	SCC	Male	77
101G-A	R34Q (Arg>Gln)	SCC	Male	67
237G-C	E79D (Glu>Asp)	SCC	Male	73
101G-A	R34Q (Arg>Gln)	SCC	Male	64
235G-A	E79K (Glu>Lys)	SCC	Male	77

SCC, squamous cell carcinoma.

stage I, 17 at stage II and 20 at stage III. The mean age was 66.8 years (range, 49-79). Among the 67 lung cancer patients, 28 had lymph node metastasis, 62 were male and 14 had *NRF2* gene mutations. The samples from these patients had been sequenced for the *NRF2* gene (7).

PCR assays for *NRF2*. Total RNA was extracted from lung cancer tissues using an Isogen kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. RNA concentrations were determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Rockland, DE, USA). Five cases were excluded from each assay, since the tumor cells were too few to sufficiently extract tumor RNA. The RNA (1 μ g) was reverse-transcribed using a First strand cDNA synthesis kit with 0.5 μ g oligo (dT)₁₆ (Roche Diagnostics GmbH, Mannheim, Germany) 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 determined using the Nano Drop ND-1000 Spectrophotometer. Approximately 200 ng of each cDNA was used for PCR analysis. To ensure the fidelity of mRNA extraction and reverse transcription, the samples were subjected to PCR amplification with a β -actin primers kit (Nihon Gene Laboratory, Miyagi, Japan) using a LightCycler-FastStart DNA Master HybProbe kit (Roche Diagnostics GmbH). The PCR assay reactions were performed using LightCycler FastStart DNA Master SYBR-Green I kit (Roche Diagnostics GmbH) in a 20- μ l reaction volume. The primer sequences for *MRP3* gene were as follows: forward: 5-ACAGGGGATGCAGTATCTGG-3 (at exon 14) and reverse: 5-CCTGGCTCCTTTCACGTAG-3 (at exon 16). The cycling conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 10 sec, annealing at 61°C for 10 sec and extension at 72°C for 7 sec.

Statistical analysis. Statistical analysis was 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 simple linear regression. Correlation coefficients were determined by rank correlation using Spearman's and χ^2 tests. The overall survival of the lung cancer patients was examined by Kaplan-Meier methods and differences were examined by the log-rank test. Analyses were carried out using the Stat-View software package (Abacus Concepts, Inc., Berkeley, CA, USA). $P < 0.05$ was considered to indicate a statistically significant result.

Results

***NRF2* gene mutation in Japanese lung cancer patients.** The *NRF2* gene mutation status and N-terminal domain were investigated by direct sequencing as previously reported (7). A total of 291 non-small cell lung cancer (NSCLC) cases, including 148 squamous cell carcinoma patients, were investigated. Sixteen had *NRF2* gene mutations (Table I). Patients with mutations were male with squamous cell carcinomas. Fifteen were smokers and 4 were stage I. 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).

***MRP3* mRNA levels in Japanese lung cancer patients.** The levels of *MRP3*/ β -actin were investigated in the 67 squamous cell carcinoma patients, including 14 *NRF2* mutant patients. The mean *MRP3*/ β -actin level in the lung cancer tissue was 1.124 \pm 1.490 and did not correlate with age ($R^2=0.038$, $p=0.1123$). The *MRP3*/ β -actin mRNA levels did not correlate with age (≤ 65 vs. >65 years; $p=0.1080$) or smoking status (Brinkman index ≤ 400 vs. >400 ; $p=0.4741$). The *MRP3*/ β -actin mRNA levels also did not correlate with lymph node

Table II. Clinicopathological data of the 67 lung squamous cell carcinoma patients.

Variables	No. of patients	<i>MRP3</i> gene status	
		<i>MRP3</i> / β - <i>actin</i> mRNA levels	p-value
Mean age (years) 66.8 \pm 7.7	67		
Pathological stages			N.S.
I	30 (44.8%)	0.824 \pm 0.887	
II	17 (25.4%)	1.079 \pm 1.055	
III	20 (29.9%)	1.611 \pm 2.277	
Lymph node metastasis			N.S.
N0	39 (58.2%)	0.992 \pm 1.107	
N1	13 (19.4%)	1.608 \pm 2.430	
N2	15 (22.4%)	1.047 \pm 1.527	
BI status			0.4741
\leq 400	5 (7.5%)	0.855 \pm 0.993	
$>$ 400	61 (92.5%)	1.145 \pm 1.527	
Differentiation			N.S.
Well	27 (40.3%)	0.726 \pm 0.631	
Moderate	33 (49.3%)	1.568 \pm 1.955	
Poor	16 (23.9%)	0.654 \pm 0.561	
<i>NRF2</i> mutations			0.0002
Mutant	14 (20.9%)	2.598 \pm 2.373	
Wild-type	53 (79.1%)	0.734 \pm 0.820	
Age (years)			0.1080
\leq 65	29 (43.3%)	0.700 \pm 0.723	
$>$ 65	38 (56.7%)	1.447 \pm 1.882	
Gender			0.0036
Male	62 (92.5%)	1.200 \pm 1.524	
Female	5 (7.5%)	0.179 \pm 0.083	

MRP3, multidrug-resistant protein-3; N.S., not significant; N0, lymph node metastasis-negative; N1, peripheral nodes; N2, mediastinal nodes; N.S., not significant; BI, Brinkman index.

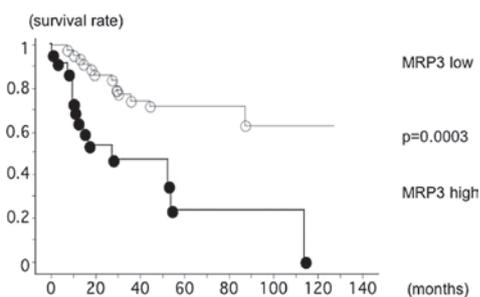


Figure 1. Overall survival was studied with reference to the *MRP3*/ β -*actin* mRNA level, whether it was $>$ 1.124 (high) or not. The survival of the patients with high *MRP3*/ β -*actin* mRNA (14 out of 22 were deceased, mean survival 45.6 months) was significantly worse than that of the patients with low *MRP3*/ β -*actin* mRNA levels (13 out of 45 were deceased, mean survival 69.0 months; log-rank test, $p=0.0003$). *MRP3*, multidrug-resistant protein-3.

metastasis, tumor invasion status and pathological differentiation status. Although the *MRP3*/ β -*actin* mRNA levels did not correlate with pathological stage, there was a tendency towards higher *MRP3*/ β -*actin* mRNA levels at higher pathological stages (stage I, 0.824 \pm 0.887; stage II, 1.079 \pm 1.055; stage III,

1.611 \pm 2.277). *MRP3*/ β -*actin* mRNA levels were significantly higher in the male cases (1.200 \pm 1.524) than in the female cases (0.179 \pm 0.083; $p=0.0036$).

The overall survival of the 67 lung squamous cell carcinoma patients, with follow-up through to December 31, 2010, was studied with reference to whether the patient's *MRP3*/ β -*actin* mRNA level was $>$ 1.124 (high) or not. The survival of patients with high levels of *MRP3*/ β -*actin* mRNA (14 out of 22 were deceased, mean survival 45.6 months) was significantly worse than that of patients with low *MRP3*/ β -*actin* mRNA levels (13 out of 45 were deceased, mean survival 69.0 months; log-rank test, $p=0.0003$; Fig. 1).

Discussion

In this study, we found that *MRP3* mRNA levels correlated with *NRF2* mutations. Although the *MRP3* mRNA levels also correlated with gender, this was due to the *NRF2* mutations all being identified in male squamous cell carcinoma patients. High *MRP3* mRNA levels also correlated with poor prognosis.

The *NRF2* gene is a master transcriptional activator of genes encoding numerous cytoprotective enzymes that are induced

in response to environmental and endogenously-derived oxidative/electrophilic agents (14-16). A previous study has shown that the RNAi-mediated silencing of *NRF2* gene expression in NSCLC inhibited tumor growth (17). *NRF2* gene promoter polymorphism has been identified and was suggested to correlate with carcinogenesis (18). The association between *NRF2* mutation and the *MRP3* mRNA levels of lung squamous cell carcinomas suggests a role for *NRF2* in chemoresistance. The constitutive expression of *NRF2* has been reported to provide a survival advantage to invasive and metastatic cancer cells by adaptation to the microenvironment and the evolution of chemoresistance in cancer cells under hypoxia (19,20). The degree of CDDP-induced DNA crosslinking and the number of apoptotic cells have been revealed to increase significantly in A549 cells transfected with *NRF2*-siRNA (21). The expression levels of multidrug resistance-associated proteins, the drug efflux proteins, have also been reported to be significantly reduced in *NRF2*-silenced A549 cells (21). A previous study revealed that inhibition of the *NRF2* function restored CDDP sensitivity in human ovarian cancer cells (22).

Previous analysis of the human *MRP3* gene revealed four putative *NRF2*-binding sites (EpREs) (12,13). These findings suggest that the activation of *NRF2* contributes to the induction of *MRP3*. *In vitro*, ChIP-analysis demonstrated an increased *NRF2* binding to the -805 bp EpRE following treatment with a *NRF2* activator. In NSCLC cell lines, the total basal levels of *MRP3* mRNA and *NRF2* protein were reported to be concordant (13). It has been shown that wild-type *NRF2* proteins decrease rapidly, whereas mutant *NRF2* proteins are degraded more slowly, having half-lives of approximately twice those of the wild-type proteins (8). In addition, mutant *NRF2* proteins have been reported to be significantly more active than wild-type *NRF2* when analyzed by luciferase activity (8).

Higher *MRP3* mRNA levels correlated with poor prognosis, however, this may be due to the correlation with *NRF2* mutations. Our previous study results revealed that patients with *NRF2* mutations had poor prognosis (7), consistent with other reports (6,8). Since we usually perform adjuvant chemotherapy for advanced lung cancer cases, the chemosensitivity may affect the results. In addition, although not significant, higher *MRP3* mRNA levels were observed at higher pathological stages in our analysis.

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