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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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).

**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 χ² 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).

**MPR3 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±1.490 and did not correlate with age (R²=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.

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

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

SCC, squamous cell carcinoma.

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**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 µg) was reverse-transcribed using a First strand cDNA synthesis kit with 0.5 µg 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-µl reaction volume. The primer sequences for MRP3 gene were as follows: forward: 5'-ACAGGGGATGCAGTATCTGG-3' (at exon 14) and reverse: 5'-CCCTGGCTCTCTTCAGTGA-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.
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±0.887; stage II, 1.079±1.055; stage III, 1.611±2.277). MRP3/β-actin mRNA levels were significantly higher in the male cases (1.200±1.524) than in the female cases (0.179±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 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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References


