Increased *FGFR1* copy number in lung squamous cell carcinomas

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Abstract. The basic fibroblast growth factor (bFGF), via activation of its receptor, FGFR1, has been postulated to be an important inducer of host stromal response and angiogenesis. More recently, *FGFR1* amplifications were investigated using large-scale single nucleotide polymorphism arrays in lung cancer. We hypothesized that *FGFR1* overexpression may be correlated with the clinicopathological features of lung cancers. The increased copy number of the *FGFR1* gene was analyzed by real-time polymerase chain reaction amplifications in 100 surgically treated non-small cell lung cancer cases from Nagoya City University Hospital. Sixty-five squamous cell carcinoma cases were included. An increased *FGFR1* gene copy number was found in 32 (32%) lung cancer patients. The increased *FGFR1* copy number status significantly correlated with gender (females 13.8% vs. males 39.4%, p=0.0173), smoking status (never smoker 4.2% vs. smoker 40.8%, p=0.0004) and pathological subtypes (squamous cell carcinoma 41.5% vs. non-squamous cell carcinoma 14.3%, p=0.0066). However, within the squamous cell carcinomas the *FGFR1* copy number status did not significantly correlate with gender, smoking status, pathological stages and differentiation status of the lung cancers. Thus, the *FGFR1* copy number is common within squamous cell carcinoma.

Introduction

Despite improvements in diagnosis, lung cancer is a major cause of death from malignant diseases, 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 (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 representing an early stage of lung cancer (3).

The fibroblast growth factor (FGF), or basic FGF (bFGF), and its transmembrane tyrosine kinase receptors (the FGFRs) are a large, complex family of signaling molecules involved in several physiological processes, and the dysregulation of these molecules has been associated with cancer development (4,5). bFGF belongs to a family of ubiquitously expressed ligands that bind to the extracellular domain of FGFRs, initiating a signal transduction cascade that promotes cell proliferation, mortality and angiogenesis (4-6). As with some other angiogenic pathways, the bFGF pathway has been shown to be activated in lung cancers. Elevated levels of FGFR1 proteins have been detected in non-small cell lung cancer (NSCLC) cell lines (7,8). Several reports have discussed the expression of the FGFR1 protein in NSCLC tumors (9-12). Recently, *FGFR1* amplifications were investigated using large-scale single nucleotide polymorphism (SNP) arrays in lung cancer (13). *FGFR1* somatic mutation is very rare in lung cancers (14); however, the FGFR1 protein is highly expressed in squamous cell carcinomas of the lung (15).

Although *FGFR1* gene expression has also been investigated in lung cancer cell lines (16), the association between *FGFR1* gene status and lung cancer has not previously been reported. To determine the *FGFR1* copy number status in lung carcinoma for screening purposes, we investigated the *FGFR1* copy number by real-time polymerase chain reaction (PCR) amplifications. The findings were compared to the clinicopathological features of lung cancer.

Patients and methods

Patients. The study group included 100 lung cancer patients who had undergone surgery at the Department of Surgery II, Nagoya City University Medical School between 2001 and 2008. All tumor samples were immediately frozen and stored at -80˚C until assayed.

The clinicopathological characteristics of the 100 lung cancer patients were as follows: 61 cases at stage I, 19 at stage II and 20 at stages III-IV. The mean age was 66.3 years (range, 29-86). Among the 100 lung cancer patients, 24 were non-

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smokers, 71 were male and 65 were diagnosed as squamous cell carcinoma. The samples from these patients had previously been sequenced for epidermal growth factor receptor (*EGFR)* mutations (17-19).

PCR assays for FGFR. 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 using a NanoDrop spectrophotometer (NanoDrop Technologies, Ind., Rockland, DE, USA) and adjusted to a concentration of 2.5 ng/ml. We then used 5 μl of each DNA for PCR assays. *FGFR1* copy number was analyzed by quantitative real-time PCR, performed on a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using a QuantiTect SYBR-Green kit (Qiagen Inc., Valencia, CA, USA) (20,21). *FGFR1* primers used for amplification were 5'-CAACATCTTCA CAGCCACTT-3' and 5'-AGACTGGTCTTAGGCAAACC-3'. Total DNA content was estimated by assaying Line-1 elements for each sample using the primers, 5'-AAAGCCGCTCAA CTACATGG-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.

Statistical methods. Statistical analyses were carried out using the Mann-Whitney U test for unpaired samples and Wilcoxon's singed rank test for paired samples. Linear relationships between variables were determined by means of simple linear regression. Correlation co-efficients were determined by rank correlation using Spearman's test and the Chi-square test. The overall survival of 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 of <0.05 was considered to indicate a statistically significant difference.

Results

FGFR1 gene status in lung cancer patients. Using the primers sets for the *FGFR1* gene, from 100 lung cancer patients, 32 patients had more than 4 copies of the *FGFR1* gene. The clinicopathological background of the patients is shown in Table I. *FGFR1* gene copy number status significantly correlated with gender (males 39.4% vs. females 13.7%; p=0.0173), tobacco-smoking (non-smoker 4.1% vs. smoker 40.8%; p=0.0004) and pathological subtypes (squamous cell carcinoma 41.5% vs. non-squamous cell carcinoma 14.3%; p=0.0066), but not with pathological stages (stage I vs. II-IV; p=0.9999), nor age (<65 vs. >65 years; p=0.3963). The overall survival of the 100 lung cancer patients from Nagoya City University, with follow-up through December 31, 2010, was studied in reference to the *FGFR1* gene status. The survival status of the patients with an increased *FGFR1* gene copy number (n=32, 6 were no longer alive) and the patients with a normal copy number of *FGFR1* (n=68, 20 were no longer alive)) was not significantly different (log-rank test, p=0.2458) (Fig. 1). *EGFR* mutations at the kinase domain were detected from 12 lung cancers; however, the mutations and *FGFR1* increased copy number was totally exclusive.

Table I. Clinicopathological data of the 100 lung cancer patients.

Factors	*FGFR1* gene status		
	Amplified patients	Normal patients	p-value
Mean age (66.3±10.4 years)	68.3±6.7	65.4±11.7	0.1965
Stage			
I	20 (62.5%)	41 (60.3%)	0.9999
II-IV	12 (37.5%)	27 (39.7%)	
Lymph node metastasis			
N0	24 (75.0%)	43 (63.2%)	0.2652
N^+	8 (25.0%)	25 (36.8%)	
Smoking status			
Never smoker	1 (3.1%)	23 (33.8%)	0.0004
Smoker	31 (96.9%)	45 (66.2%)	
Differentiation			
Well	9 (33.3%)	12 (23.1%)	0.4218
Moderate, poor or other	18 (66.7%)	40 (76.9%)	
Pathological subtypes			
Squamous	27 (84.4%)	38 (55.9%)	0.0066
Non-squamous	5 (15.6%)	30 (44.1%)	
Age (years)			
<65	12 (37.5%)	32 (47.1%)	0.3963
≥65	20 (62.5%)	36 (52.9%)	
Gender			
Male	28 (87.5%)	43 (63.2%)	0.0173
Female	4 (12.5%)	25 (36.8%)	

N^+, lymph node metastasis-positive.

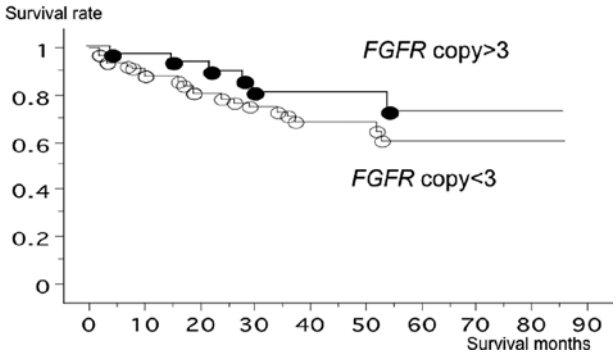

Figure 1. The survival status of the patients with an increased *FGFR1* gene copy number (n=32, 6 were no longer alive) and the patients with a normal copy number of *FGFR1* (n=68, 20 were no longer alive) was not significantly different (log-rank test, p=0.2458).

Discussion

In this study, we found an increased *FGFR1* gene copy number in 32% of lung cancers. The *FGFR1* gene status correlated with smoking status and squamous histology of the lung cancers.

Behrens *et al* demonstrated that the immunohistochemical pattern of cytoplasmic FGFR1 expression was significantly higher in lung cancer patients who were smokers (12). These differences highlight the potential differential role of FGFR1 in the pathogenesis of both smoking- and non-smoking-related lung cancers. There is some evidence that links the bFGF pathway with smoking in lung diseases. It has been suggested that the bFGF pathway plays a role in regulating airway wall remodeling, especially in individuals with smoking-induced chronic obstructive peripheral disease, which is related to inflammation of the small airway (22). Kranenburg *et al* conducted an immunohistochemical analysis of bronchial specimens obtained from patients with chronic obstructive peripheral disease and showed that FGFR1 immunolocalized in the cytoplasm of bronchial epithelial cells and in other cell components of the bronchial wall (23). Marek *et al* demonstrated that among NSCLC cell lines, FGFR-dependent autocrine signaling was observed in H226, H520 and H1703 squamous cell carcinomas (24). By contrast, in the study by Rikova *et al,* only 3 of 41 NSCLC cell lines showed evidence of activated *FGFR1* (25); however, the study was composed of approximately 75% adenocarcinomas (25). Thus, FGFR may be a better target for squamous cell histologies of NSCLC that frequently exhibit a high degree of insensitivity to EGFR tyrosine kinase inhibitors (24). In our study, *EGFR* mutations and *FGFR1* increased copy number were exclusive. The RO4383596 FGFR inhibitor has been shown to be sensitive to H226 and H520 cell lines, but not gefitinib (24).

Accumulating evidence has shown that members of the FGF family together with their transmembrane tyrosine kinase receptors (FGFR) may act as autocrine as well as paracrine (angiogenic) growth factors in many, if not all, solid tumors (26). FGFs act as mitogens and some members induce cell migration, angiogenesis, neurite outgrowth and cell survival (27). Strong indications for an important role of FGF/FGFR signals in malignant growth and possibly malignant transformation have been published for several epithelial solid tumors (26,28). However, the prognostic value of FGFR1 in lung cancers is controversial. Takanami *et al* showed that FGFR1 protein expression correlated with poor prognosis using univariate analysis, but not multivariate analysis (10). Volm *et al* also showed that high FGFR1 expression correlated with poor prognosis using univariate analysis, but not multivariate analysis (11). Actually, FGFR1 has been shown to belong to the most frequently expressed RTK molecules in early stage NSCLC (29).

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