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Expression of fibroblast growth factor 9 is associated with poor prognosis in patients with resected non-small cell lung cancer

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ABSTRACT

Objectives: Fibroblast growth factor (FGF) 9 is a member of the FGF family, which modulates cell proliferation, differentiation, and motility. Recent studies show that the activation of FGF signals including FGF9 is associated with the pathogenesis of several cancers; however, its clinicopathological and biological significance in non-small cell lung cancer (NSCLC) is unclear. The purpose of this study was to clarify the characteristics of NSCLC with FGF9 expression.
Materials and Methods: We evaluated the expression of *FGF9* in resected NSCLC specimens and corresponding non-tumorous lung tissue samples using cDNA microarray and evaluated its clinicopathological characteristics.
Results: Nine out of 90 NSCLC specimens (10%) had "high" *FGF9* expression compared with corresponding non-cancerous lung tissues. Histologically, of the 9 NSCLC specimens with high *FGF9* expression, 5 were adenocarcinoma, whereas none were squamous cell carcinoma. *FGF9* expression was not associated with sex, smoking history, or clinical stage. However, in patients with high and low *FGF9* expression, the postoperative recurrence rates were 78% and 24% ($p = 0.033$), respectively. Overall survival was significantly shorter in patients with high *FGF9* expression than in those with low *FGF9* expression ($p < 0.001$).
Conclusion: Our data indicate that FGF9 may be a novel unfavorable prognostic indicator and a candidate therapeutic target of NSCLC.

1. Introduction

Despite recent improvements in diagnosis and treatment, lung cancer is one of the leading causes of cancer-related death worldwide, and long-term survival remains very poor [1]. Non-small cell lung cancer (NSCLC) accounts for approximately 85% of all lung malignancies. Approximately 40% of NSCLCs are at an advanced stage at the time of diagnosis. The overall survival rate is only approximately 15% at 5 years, and the recurrence rates remain high [2]. Therefore, further characterization of key molecular alterations in NSCLC is anticipated.

The fibroblast growth factor (FGF) family consists of at least 23 polypeptides that have important functions in embryonic development, tissue repair, and tumorigenesis [3–5]. FGF receptors (FGFRs) are encoded by 4 genes (*FGFR1–FGFR4*), and numerous isoforms exist due to alternative RNA splicing [6]. Binding of FGF to FGFR leads to a conformational shift in the FGFR structure, resulting in intermolecular transphosphorylation of the intracellular tyrosine kinase domain and carboxy-terminal tail. Subsequent downstream signaling occurs through 4 main pathways: the RAS–RAF–MAP kinase pathway, the PI3K–AKT pathway, the signal transducer and activator of transcription pathway, and the phospholipase pathway [5,7].

The proliferation of a subset of NSCLC cells is reported to be dependent on FGF/FGFR signaling. Several studies suggest that specific FGFs and FGFRs, such as FGF2, FGF9, FGFR1, and FGFR2, are expressed in NSCLC cell lines and patient-derived lung cancer tissues [8]. Additionally, inhibition of FGF/FGFR signals by shRNA, anti-FGF2 antibody, or an FGFR-tyrosine kinase inhibitor (TKI) has been reported to suppress the proliferation of cancer cells [8,9].

In recent years, activation of FGF/FGFR signals through FGF9 has been reported in some cancers. Leushacke et al. reported that the expression level of *FGF9* mRNA was high in a subset of advanced colon cancers and that *FGF9* overexpression was negatively correlated with patient survival [10]. In prostate cancer, Teishima et al. reported that the 3-year biochemical relapse-free survival rate was

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significantly lower for patients with FGF9-positive cancer than for those with FGF9-negative cancer [11].

FGF9 is expressed in epithelium primordium during the developmental stage of the lung and plays an important role in the differentiation and proliferation of the epithelium and interstitium; however, its expression is repressed in the mature lung [12]. In lung cancer, by using immunohistochemistry, Wang et al. demonstrated that FGF9 was highly expressed in adenocarcinoma patients and that alteration of FGF9 function might reduce the development of lung adenocarcinoma [13]. However, data confirming the relationship between FGF9 expression and prognosis in lung cancer is not available.

To date, many prognostic indicators in NSCLC patients have been identified. However, the impact of FGF9 on the prognosis of NSCLC patients is not reported. The purpose of this study was to characterize NSCLC with FGF9 expression and to evaluate the effect of FGF9 on the prognosis of NSCLC patients.

2. Materials and methods

2.1. Study population

A total of 95 patients underwent surgical resection under the diagnosis of NSCLC at the Department of Thoracic Surgery, Keio University Hospital, from 2001 to 2006. Approval for institutional review of all samples was obtained in accordance with the requirements of Keio University Institutional Review Board (Institutional Review Board #16-90-1).

2.2. cDNA microarray analysis

We used GeneChip Human Genome 2.0 Array (Affymetrix, Inc., Santa Clara, CA) to monitor the expression profiles of the samples. Total RNA was extracted from tumor tissues and paired normal lung tissues using RNeasy Mini Kit (QIAGEN Hilden, Germany) after treatment with TRIzol (Life Technologies, Carlsbad, CA). The labeled cRNA was prepared using standard Affymetrix protocols. The signal intensities of the probe sets were normalized using the Affymetrix Power Tools RMA method implemented using Resolver software (Rosetta Biosoftware, Seattle, WA), and log ratio values to the average of normal samples were calculated for each sample by using Resolver.

2.3. Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

Total RNA was collected using RNeasy Mini Kit (QIAGEN). RNA was reverse transcribed to cDNA using TaqMan Reverse Transcription Reagents (N8080234, Life Technologies). Quantitative analysis was performed using an ABI Prism 7000 Sequence Detection System (Life Technologies).

For qRT-PCR analysis, we used human FGF9 (#4413146, Life Technologies) and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH, #40286, Life Technologies) to normalize input cDNA.

2.4. Immunohistochemistry

Immunohistochemical analysis was performed for 90 NSCLC specimens. All tumor tissues as well as the surrounding lung tissues were removed and embedded in paraffin and cut into 4-μm-thick sections. These sections were deparaffinized, rehydrated, and incubated in 0.03% H_2O_2 in 95% methanol at room temperature for 20 min to block endogenous peroxidase activity. Antigen retrieval was performed using water bath pretreatment (Immunosaver; Nisshin EM, Tokyo, Japan) at 98 °C for 45 min. All sections were incubated for 20 min with normal horse serum to eliminate nonspecific staining and were then incubated with anti-human FGF9 antibody (#ab71395, Abcam Cambridge, UK) overnight at 4 °C. This was followed by incubation with the secondary antibody (ImmPRESS Reagent Kit; Vector Laboratories, Burlingame, CA) for 30 min. Slides were then incubated in diaminobenzidine (DAB)/Tris solution (3 DAB/Tris tablets diluted in 150 ml of distilled water; Muto Pure Chemicals, Tokyo, Japan) supplemented with 15 μl of 30% H_2O_2. Finally, the slides were counterstained with hematoxylin. The proportion of cells stained and the staining intensity were assessed by the pathologist as follows: intensity score 0, absence of staining; 1, weakly stained; 2, moderately stained; and 3, strongly stained. The total score was calculated by multiplying the proportion score with the intensity score [11].

2.5. Statistical analysis

Associations between FGF9 expression and clinicopathological factors were assessed using the χ^2 test and the Mann–Whitney *U*-test. Survival time was calculated from the date of surgery to the time of death (or to the date of final follow-up), and the corresponding Kaplan–Meier curve was plotted. The log-rank test was applied for survival analysis. Multivariate analyses of the influence of variables on overall survival were performed using the Cox proportional hazards model. All statistical analyses were performed using SPSS 19.0 software (SPSS, IBM, Chicago, IL). All *p*-values were two sided and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Overexpression of FGF9 mRNA in NSCLC patients

Of the 95 patients who underwent resection for a pretreatment diagnosis of NSCLC, 90 were evaluated in this study. Five patients were excluded from the study because they were found to have small cell lung cancer (2 patients) or their clinical data could not be retrieved (3 patients) (Fig. 1A). Although most tumor samples from NSCLC patients did not show high expression levels of *FGF9* compared to the average level in normal lung tissues according to cDNA microarray data, we found that in 9 out of 90 tumor tissues (10%), the level of *FGF9* expression was higher than the normal average level (Fig. 1A). We classified these 9 cases as the *FGF9*-high group and the rest of the cases as the *FGF9*-low group. We also performed qRT-PCR for *FGF9* to confirm the microarray data for both normal and tumor tissues of 58 out of 90 NSCLC patients whose samples were still available. Finally, we performed qRT-PCR for 6 of 9 patients in the *FGF9*-high group and in 52 out of 81 patients in the *FGF9*-low group. *FGF9* mRNA expression determined by qRT-PCR and that determined by microarray analysis was well correlated ($r = 0.83$, $p < 0.0001$, Fig. 1B).

3.2. Clinical and pathological characteristics of NSCLC patients in the FGF9-high group

The clinicopathological profile of 90 patients classified according to *FGF9* expression level is summarized in Table 1. The TNM classification was performed according to the UICC-6 staging system for NSCLC in this study as all the patients were diagnosed and operated before 2006. Distribution of age, sex, smoking status, epidermal growth factor receptor (*EGFR*) mutation status, and *K-ras* mutation status did not differ between the 2 groups. Although the clinical TNM stage did not differ, the pathological stage differed significantly between the groups. Of the 9 patients in the *FGF9*-high group, 5 had adenocarcinoma, 3 had large cell carcinoma, and 1 had