SPANDIDOS PUBLICATIONS

Fibroblast growth factor receptor 4 mutation and polymorphism in Japanese lung cancer

HIDEFUMI SASAKI, KATSUHIRO OKUDA, OSAMU KAWANO, HARUHIRO YUKIUE, MOTOKI YANO and YOSHITAKA FUJII

Department of Surgery II, Nagoya City University Medical School, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan

Received March 28, 2008; Accepted August 9, 2008

DOI: 10.3892/or_00000119

Abstract. We investigated the *FGFR4* mutation status at the kinase domain and *FGFR4* single nucleotide polymorphism (SNP) at codon 388 in surgically treated non-small cell lung cancer (NSCLC) cases. The presence or absence of *FGFR4* mutations of kinase domains was analyzed by direct sequences (n=147), and the presence of *FGFR4* Arg388 allele was analyzed by genotyping assay using LightCycler hybridization probes (n=387). *FGFR4* mutations were not present in our lung cancer patients. In 61.8% of patients, homo- or heterozygous Arg388 allele was present. No correlation existed between the *FGFR4* genotype and clinicopathological features such as gender, smoking status and pathological subtypes. *EGFR* mutation status was not correlated with the *FGFR4* genotype of lung cancers. In node-negative patients, the *FGFR4* genotype was not correlated with disease outcome, while in the node-positive patients *FGFR4* Arg388 was significantly associated with worse survival. This association was not attributed to patient response to adjuvant chemotherapy. Therefore, the role of *FGFR4* polymorphism is a prognostic marker for advanced NSCLC in Japanese patients.

Introduction

Lung cancer is a leading cause of death from malignant diseases due to its high incidence, malignant behavior and lack of significant advancements in treatment strategy (1). Efforts are under way to identify the single nucleotide polymorphisms (SNPs) or mutations in lung cancers and, in clinical data, to establish correlations with disease susceptibility, progression and response to therapeutic regimens (2). Besides the imbalance in regulatory pathways due to changes in gene expression, functional alterations in key proteins caused by gene mutations contribute to the manifestation of pathological phenotypes. Thus, a distinction can be made between sequence variants with functional consequences for the encoded protein that are predisposed to pathological syndromes and common sequence variants which represent functional consequence variations and functionally neutral genetic markers.

Alterations in tyrosine kinase (TK) genes such as specific point mutations or gene amplification were found to induce neoplastic disorders. The fibroblast growth factor receptor (FGFR) family with its four closely related members is also one of the TK receptors. This family is critically involved in cell growth, differentiation, migration, angiogenesis and tumorigenesis (3). The FGF signaling system is functionally conserved in the respiratory organogenesis of several organisms (4) and the four FGF receptors are expressed in mouse lung during postnatal development. Moreover, there is evidence of altered FGFR4 expression in lung cancer cell lines (5,6).

Bange *et al* discovered a germ line polymorphism in the gene encoding for FGFR4 (6). This SNP at codon 388, from G to A, results in a change of the amino acid sequence of FGFR4 from glycine to arginine (7). The result is a charged amino acid in the highly conserved and normally hydrophobic transmembrane region. This SNP was present at a significantly higher frequency in breast and colon cancer patients with aggressive disease progression and represented a gene alteration that predisposed the carrier to poor clinical outcome (6). Streit *et al* demonstrated that a high expression of the *FGFR4* Arg388 allele was associated with poor clinical outcome in head and neck squamous cell carcinoma (8). Spinola *et al* demonstrated that the *FGFR4* Arg388 allele predicted prognosis in European lung adenocarcinoma patients (9). A recent study showed a novel somatic kinase domain mutation in FGFR4 (Pro712Thr) (10).

To determine the *FGFR4* mutation and polymorphism status, and the correlation with clinicopathological features in Japanese lung carcinoma, we performed direct sequencing and genotyping analysis using LightCycler hybridization probes. The findings were compared to the clinicopathological features of lung cancer.

Patients and methods

Patients. This was a retrospective study which included 387 lung cancer patients who had undergone surgery at the

Correspondence to: Dr Hidefumi Sasaki, Department of Surgery II, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan
E-mail: hisasaki @med.nagoya-cu.ac.jp

Key words: FGFR4, lung cancer, mutations, polymorphism, Arg388

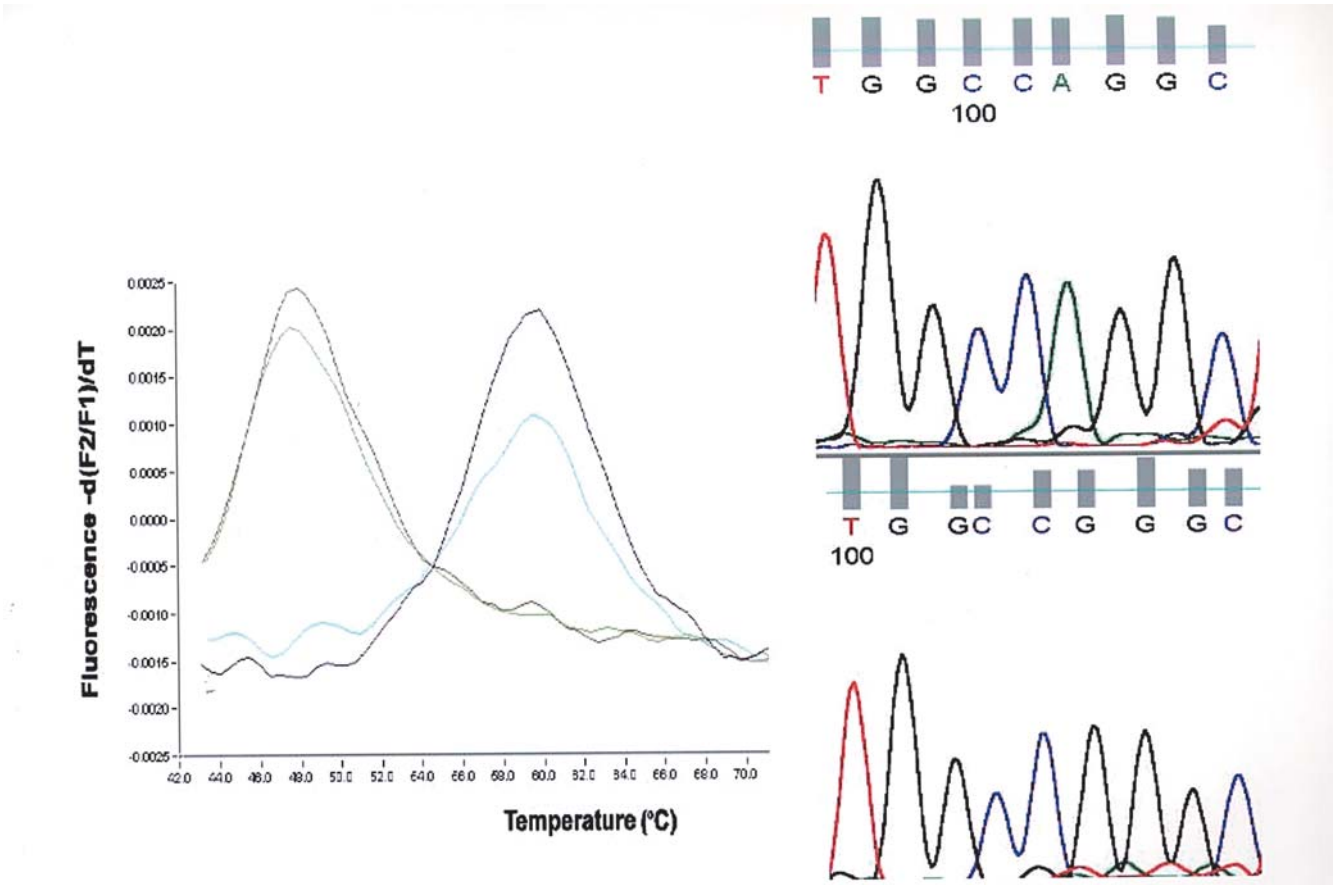

Figure 1. Left, detection of *FGFR4* polymorphism at codon 388 in DNA extracted from lung cancer tissues. The negative derivative of the fluorescence (-dF/dT) versus temperature graph shows peaks with different Tm. The wild-type product showed a single peak at 60˚C, whereas the mutant showed an additional peak at 48˚C. Right upper, sequence analysis for 388Arg allele. Right lower, sequence analysis for 388Gly.

Department of Surgery II, Nagoya City University Medical School. Written informed consent was obtained and the institutional ethics committee of the Nagoya City University Medical School approved the study. We also investigated *FGFR* mutation status at the kinase domain for 147 NSCLC patients. Lung tumors were classified according to the general rule for clinical and pathological record of lung cancer in Japan (11). Tumor samples were immediately frozen and stored at -80˚C until assayed. The clinical and pathological characteristics of the 387 lung cancer patients show that 256 (66.1%) patients were male and 131 were female. Two-hundred and twenty-three (63%) were diagnosed as adenocarcinoma, 124 (32%) were smokers and 263 were non-smokers.

PCR assays for FGFR polymorphisms. Genomic DNA was extracted using Wizard SV genomic DNA purification systems (Promega) according to the manufacturers' instructions. The primers and LightCycler Hybridization probes were designed with Primer Express 2.0 software (Applied Biosystems). The genotyping PCR reactions were performed using the Light-Cycler DNA Master Hybridization probes kit (Roche Molecular Biochemicals, Mannheim, Germany) in a 20-μl reaction volume. The primer sequences for *FGFR4* gene (codon 388) were: forward, 5-GTGTGTGTCCATGTGCGA-3 and reverse, 5-GGAGAGCTTCTGCACAGT-3. For genotyping, sensor (TCGATACAGCCCGGC-fluorescein) and anchor (LC Red 640 AGCAGCAGGAGCACAGCCAAG-phosphate) probes were used. Some of the samples were confirmed by direct sequencing, using the same primers (forward and reverse). Conditions for the real-time PCR reaction were: one cycle of 95˚C for 2 min, 50 cycles of 95˚C for 1 sec, 58˚C for 10 sec and 72˚C for 8 sec. At the end of the PCR reaction, samples were subjected to a melting analysis to confirm specificity of the amplicon. Total RNA was extracted from lung cancer tissues using an Isogen kit (Nippon Gene, Tokyo, Japan) according to the manufacturers' instructions. RNA (1 μg) was reverse transcribed by Superscript II enzyme (Gibco BRL, Gaithersburg, MD) with 0.5 μg oligo $(dT)_{12\text{-}16}$ (Amersham Pharmacia Biotech Inc., Piscataway, NJ). The *FGFR4* mutation status at exon 16 was investigated by direct sequencing using the primers: forward, TATCTGGAGTCCC GGAAGTG and reverse, AAGACAGAATCGCTGGAGGA. Conditions for the real-time PCR reaction were: one cycle of 94˚C for 5 min, 40 cycles of 95˚C for 30 sec, 60˚C for 30 sec, 72˚C for 45 sec and one cycle of 72˚C for 5 min. PCR products (542 bp) were purified using the Qiagen PCR purification kit, and then the direct sequencing for the *FGFR4* gene was performed. *EGFR* gene mutation statuses at the kinase domain for the above samples were already reported (12-14).

Statistical analysis. A statistical analysis was performed using the Mann-Whitney U test for unpaired samples and Wilcoxon's signed-rank test for paired samples. Linear

'linicopathological data of 387 lung cancer patients.

Factors	FGFR4 gene status[a]		
	388Gly patients	388Arg patients	P-value
Mean age (years): 64.9±9.0	148 (38.2%)	239 (61.8%)	
Pathological subtypes			
Adeno	83 (37.2%)	140 (62.8%)	0.6724
Non-adeno	65 (39.6%)	99 (60.4%)	
Gender			
Male	92 (35.9%)	164 (64.1%)	0.2241
Female	56 (42.7%)	75 (57.3%)	
Smoking status			
Non-smoker	52 (41.9%)	72 (58.1%)	0.3150
Smoker	96 (36.5%)	167 (63.5%)	
Pathological stages			
I	75 (38.5%)	147 (61.5%)	0.0561
II-IV	71 (48.6%)	92 (51.4%)	
Age			
≤60	47 (40.5%)	69 (59.5%)	0.5693
>60	101 (37.3%)	170 (62.7%)	
EGFR mutations			
Wild-type	111 (38.0%)	181 (62.0%)	0.9035
Mutant	37 (40.2%)	58 (39.8%)	
Lymph node metastasis			
Positive	53 (45.3%)	64 (54.7%)	0.0534
Negative	93 (34.8%)	174 (65.2%)	

[a]FGFR, fibroblast growth factor receptor; Adeno, adenocarcinoma and EGFR, epidermal growth factor receptor.

relationships between variables were determined by means of simple linear regression. Correlation coefficients were determined by rank correlation using Spearman's and the χ^2 tests. Survival of lung cancer patients was examined by the Kaplan-Meier methods, and differences were examined by the log-rank test. The analysis was performed using the Stat-View software package (Abacus Concepts Inc., Berkeley, CA), and was considered significant at $P<0.05$.

Results

FGFR4 gene mutation at kinase domain. We assessed *FGFR4* mutation statuses by direct sequencing. Of 147 lung cancer samples, no mutation was found in the kinase domain of *FGFR4*.

FGFR4 Arg388 polymorphism and clinical outcome. For genotyping, the anchor probe was matched for the *FGFR4* Gly388 allele. As shown in Fig. 1, for *FGFR4* polymorphism, Arg388 showed a single peak at 60˚C, whereas the wild type showed a single peak at 48˚C. From the 387 lung cancer patients, 239 (61.8%) had *FGFR4* polymorphism 388Arg allele (Table II). One-hundred and sixty four (164/256, 64.1%) were male, 167 (167/263, 63.5%) smokers and 140 (140/223, 62.8%) had adenocarcinomas. The correlation between *FGFR* polymorphism status and patient outcome according to *FGFR4* polymorphism is shown in Fig. 2. Consistent with Thussbas *et al* (7), no significant association between *FGFR4* genotype and patient outcome was observed for the whole cohort (p=0.4889). In node-negative patients, 93 (18 deceased) were 388Arg *FGFR4* and 174 (25 deceased) were 388Gly *FGFR4*. Thus, the *FGFR4* genotype was not correlated with disease outcome (log-rank test, p=0.4038) (Fig. 3). In node-positive patients, however, 54 (20 deceased) were 388Gly FGFR4 and 64 (39 deceased) were 388Arg. Thus, *FGFR4* Arg388 was significantly associated with survival (log-rank test, p=0.0397) (Fig. 4). We compared associations between the *FGFR4* polymorphism status and patient outcome in patients treated

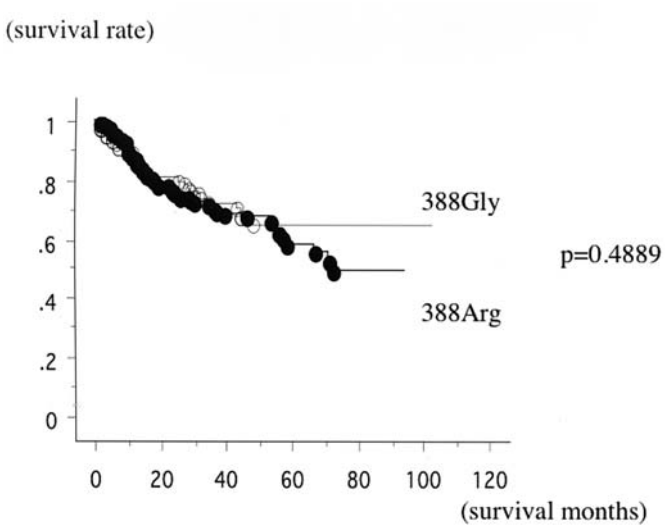

Figure 2. Survival of 387 lung cancer patients was studied as regards to the *FGFR4* polymorphism status. The prognosis from patients with 388Gly allele (n=148, 35 deceased) and those with 388Arg allele (n=239, 64 deceased) was not significantly different (log-rank test, p=0.4889).

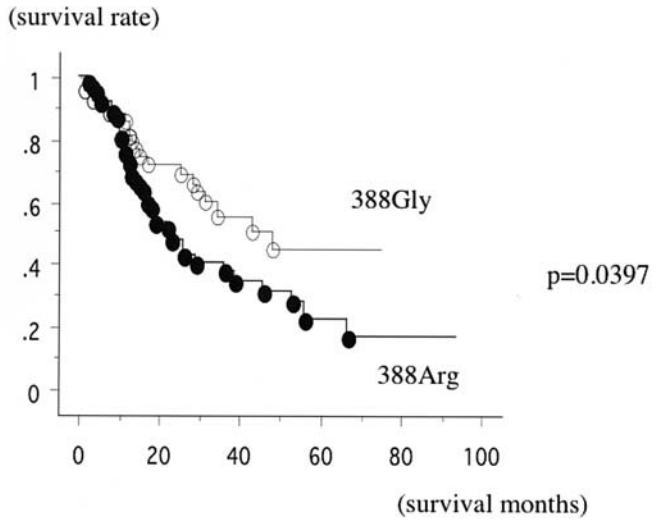

Figure 4. Survival of 118 node-positive lung cancer patients was studied as regard to the FGFR4 polymorphism status. The prognosis from patients with 388Gly allele (n=54, 20 deceased) was significantly better than those with 388Arg allele (n=64, 39 deceased) (log-rank test, p=0.0397).

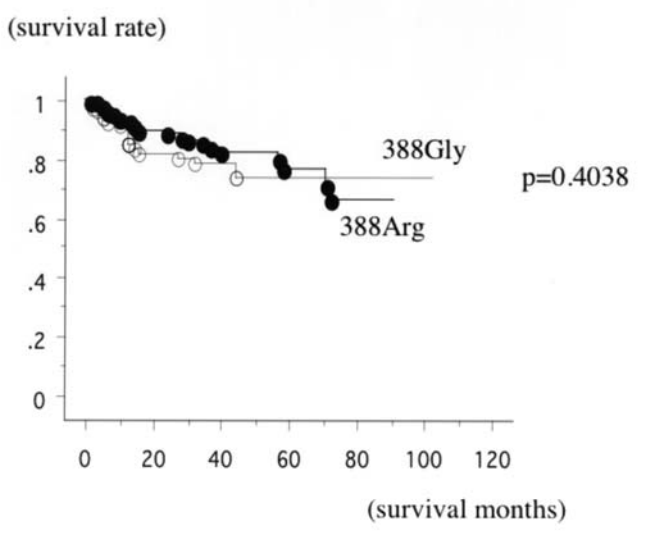

Figure 3. Survival of 267 node-negative lung cancer patients was studied as regards to the *FGFR4* polymorphism status. The prognosis from patients with 388Gly allele (n=93, 18 deceased) and those with 388Arg allele (n=174, 25 deceased) was not significantly different (log-rank test, p=0.4038).

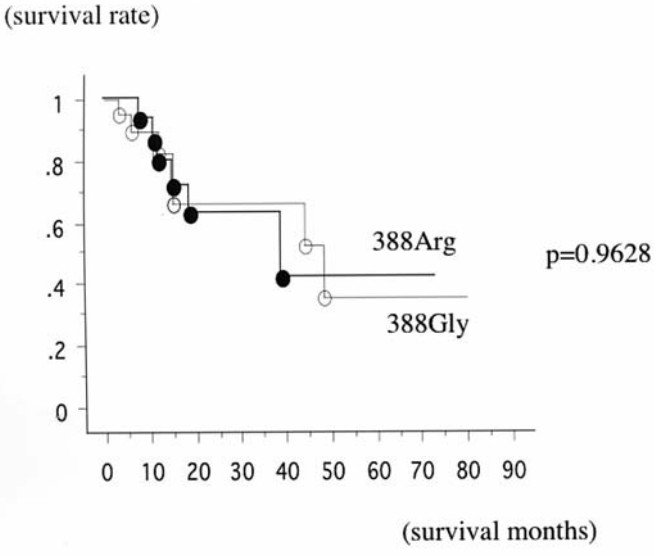

Figure 5. Survival of 36 lung cancer patients, who were treated with platinum-based adjuvant chemotherapy after they had undergone surgery, was studied as regards to the *FGFR4* polymorphism status. The prognosis from patients with 388Gly allele (n=20, 7 deceased) and those with 388Arg allele (n=16, 6 deceased) was not significantly different (log-rank test, p=0.9628).

with platinum-based adjuvant chemotherapy after they had undergone surgery. Survival of 36 lung cancer patients with follow-up through August 30, 2007 was studied concerning the *FGFR* polymorphism status. Twenty were 388Gly (wild-type, 7 deceased) and 16 were 388Arg (6 deceased). The prognosis was not significantly different according to *FGFR4* polymorphism (Fig. 5).

EGFR gene mutation status and gefitinib sensitivity in Japanese lung cancer patients. Of the 387 patients, 95 exhibited EGFR mutations, including 48 of exon 19 deletion mutations and 40 L858R mutations. Four patients had exon 20 insertion mutations and two had G719X mutation. EGFR mutation status at the kinase domain was not correlated with FGFR4 polymorphism (p=0.9035). We compared associations between the FGFR4 polymorphism status and patient outcome in patients treated with gefitinib for their recurrent diseases after they had undergone surgery. Survival of 24 gefitinib-treated lung cancer patients with follow-up through August 30, 2007 was studied concerning *FGFR* polymorphism status. Four were 388Gly (wild-type, 3 deceased) and 20 were 388Arg (13 deceased). The prognosis was not significantly different according to the *FGFR4* polymorphism (Fig. 6).

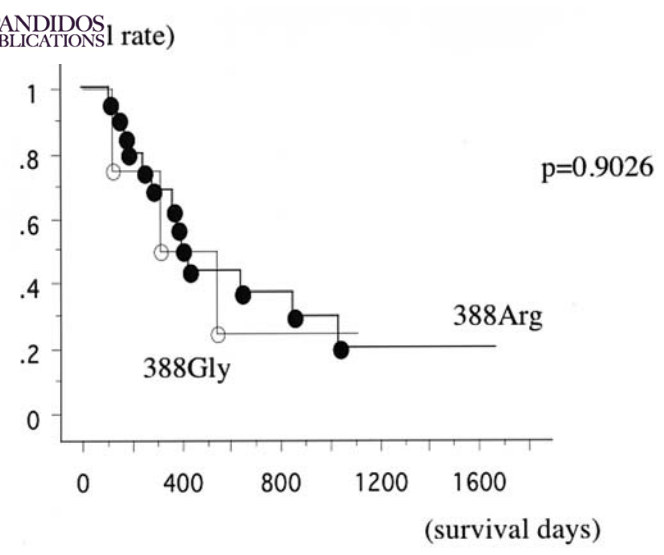

Figure 6. Survival of 24 gefitinib-treated lung cancer patients was studied regarding the *FGFR4* polymorphism status. The prognosis from patients with 388Gly allele (n=4, 3 deceased) and those with 388Arg allele (n=20, 13 deceased) was not significantly different (log-rank test, p=0.9026).

Discussion

In this study, we did not find any *FGFR4* mutation at the kinase domain. A previous report noted that *FGFR4* mutation was relatively rare in lung cancer, especially in the Japanese population. In our cohort, 61.8% of lung cancer patients presented with 388Arg allele, which was relatively higher than the Caucasian cohort. Interestingly, in node-positive but not node-negative patients, *FGFR4* polymorphism status was correlated with patient survival. This was in agreement with reports that *FGFR* gene polymorphism was a prognostic factor for node-positive breast cancer (7).

The four closely related human FGFRs and their >20 known ligands control a multitude of cellular processes, including cell growth, differentiation and migration, and it has been shown that the FGF/FGFR system plays a critical role in cancer development due to its angiogenic potential or direct enhancement of tumor growth (15). For example, autocrine growth stimulation through the coexpression of FGF and FGFR in the same cell results in the transformation of Balb/c 3T3 cells (16). Previous studies showed that a high FGFR4 expression was found in pancreatic (17), breast (18,19) and renal cell carcinoma (20). However, in lung, a previous report showed that *FGFR4* mRNA levels were not associated with the genotype of FGFR4 (9). The presence of *FGFR4* Arg388 allele was significantly overexpressed in the group of breast cancer patients with axillary lymph node involvement and early relapse (6). In patients with colorectal cancer, clinicopathological parameters such as lymph node involvement and tumor stage were closely correlated with the *FGFR4* Arg388 allele, which demonstrated that this genotype is a critical determinant in cancer progression (6). Consistent with these findings, MDA-MB-231 mammary tumor cells expressing the *FGFR4* Arg388 allele exhibited increased motility relative to cells expressing the *FGFR4* Gly388 isotype (6). An association between the *FGFR4* Arg388 allele and poor survival of patients with colon cancer was also found (6). The molecular mechanism by which *FGFR4* Arg388 polymorphism leads to a more aggressive clinical phenotype has yet to be fully understood. For *FGFR4* Arg388, no elevated tyrosine phosphorylation was observed compared with that of the *FGFR4* Gly388 allele in breast and prostate cancer cells (6). This may indicate that changes in kinase activity are too subtle to detect differences reliably. Alternatively, the two polymorphic *FGFR4* forms may use different intracellular signal transduction pathways or interact with different cell surface proteins.

When multiple members of the *FGF* family ligands were overexpressed in mouse lung epithelia, such ligands stimulated alveolar type II cell hyperplasia and adenoma formation (21-23). Davies *et al* have reported that a lung adenocarcinoma cell line harbors a non-synonymous mutation in exon 16 of *FGFR4* Pro672Thr (24). Marks *et al* investigated 158 lung cancers and found a somatic kinase domain mutation in *FGFR4* (Pro712Thr) (10). Although these data suggest a role for *FGFR4* mutations in a subset of lung adenocarcinoma, this event is very rare.

A wide range of mutation detection methodologies exist, of which sequencing has been considered the gold standard because of its ability to identify the specific DNA sequence change that has occurred. However, dideoxy sequencing is rarely sensitive below a 10% mutant allele frequency, which corresponds to a threshold of 20% of heterozygous cells for any mutation (23). For known mutations, real-time polymerase chain detection followed by melting curve analysis, using hybridization probes is highly sensitive, rapid and an efficient alternative to mutation detection (26-28). Developments in dye technology have played a major role in the emergence of this methodology.

Different base substitutions produce the differences in melting behavior. An advantage of performing melting curve analysis on a real-time PCR machine is that the PCR amplification and analysis are performed in a single run lasting 1 h with the results being available for analysis at the end of the run. A previous report indicated that melting curve analysis was able to detect as little as 5-6% of the mutant cell line DNA which was diluted in normal DNA (29).

The frequency of FGFR4 polymorphisms in our sample set was higher than previous reports on Caucasians (9). This may due to the melting curve analysis, which is a sensitive in-tube methodology used to screen for mutations in clinical samples. Another possible explanation for the discrepancies between findings from the previous studies and ours is the difference in *FGFR* polymorphism according to ethnicity.

In summary, the role of *FGFR4* polymorphism is a prognostic marker for advanced NSCLC in Japanese patients. Our results may lead to improved prediction of clinical NSCLC prognosis as well as novel therapeutic strategies that target the FGFR4 signaling pathway.

Acknowledgements

The study was supported by AstraZeneca Research Grant 2004, Grant-in-Aid for Research in Nagoya City University (2006) and Grants-in-Aid for Scientific Research, Japan Society for the Promotion of Science (JSPS) (No. 19390367, 18390381 and 18659407).

References

1. Ginsberg RJ, Kris K and Armstrong G: Cancer of the lung. In: Principles and Practice of Oncology. 4th edition, Lippincott, Philadelphia, pp673-682, 1993.
2. Corder EH, Saunders AM, Risch NJ, *et al*: Protective effect of apolipoprotein E type 2 allele for late onset Alzheimer disease. Nat Genet 7: 180-184, 1994.
3. Burke D, Wilkes D, Blundell TL and Malcolm S: Fibroblast growth factor receptors: lessons from the genes. Trends Biochem Sci 23: 59-62, 1998.
4. Warburton D, Tefft D, Mailleux A, *et al*: Do lung remodeling, repair, and regeneration recapitulate respiratory ontogeny? Am J Respir Crit Care Med 164: S59-S62, 2001.
5. Nakamura N, Iijima T, Mase K, *et al*: Phenotypic differences of proliferating fibroblasts in the stroma of lung adenocarcinoma and normal bronchus tissue. Cancer Sci 95: 226-232, 2004.
6. Bange J, Prechtl D, Cheburkin Y, *et al*: Cancer progression and tumor cell motility are associated with the FGFR4 Arg388 allele. Cancer Res 62: 840-847, 2002.
7. Thussbas C, Nahrig J, Streit S, *et al*: FGFR4 Arg388 allele is associated with resistance to adjuvant therapy in primary breast cancer. J Clin Oncol 24: 3747-3755, 2006.
8. Streit S, Bange J, Fichtner A, Ihrler S, Issing W and Ullrich A: Involvement of the FGFR4 Arg388 allele in head and neck squamous cell carcinoma. Int J Cancer 111: 213-217, 2004.
9. Spinola M, Leoni V, Pignatiello C, *et al*: Functional FGFR4 Gly 388Arg polymorphism predicts prognosis in lung adenocarcinoma patients. J Clin Oncol 23: 7307-7311, 2005.
10. Marks JL, McLellan MD, Zakowski MF, *et al*: Mutatioal analysis of EGFR and related signaling pathway genes in lung adenocarcinomas identifies a novel somatic kinase domain mutation in FGFR4. Plos One 5: e426, 2007.
11. Japan Lung Cancer Society, General Rule for Clinical and Pathological Record of Lung Cancer. 5th edition, Kanehara Press, pp1-177, 1999.
12. Endo K, Konishi A, Sasaki H, *et al*: Epidermal growth factor receptor gene mutation in non-small cell lung cancer using highly sensitive and fast TaqMan PCR assay. Lung Cancer 50: 375-384, 2005.
13. Sasaki H, Shimizu S, Endo K, *et al*: EGFR and erbB2 mutation status in Japanese lung cancer patients. Int J Cancer 118: 180-184, 2006.
14. Sasaki H, Endo K, Konishi A, *et al*: EGFR mutation status in Japanese lung cancer patients: genotyping analysis using LightCycler. Clin Cancer Res 11: 2924-2929, 2005.
15. Eswarakumar VP, Lax I and Schlessinger J: Cellular signaling by fibroblast growth factor receptors. Cytokine Growth Factor Rev 16: 139-149, 2005.
16. Matsumoto-Yoshitomi S, Habashita J, Nomura C, Kuroshima K and Kurokawa T: Autocrine transformation by fibroblast growth factor 9 (FGF-9) and its possible participation in human oncogene. Int J Cancer 71: 442-450, 1997.
17. Leung HY, Gullick WJ and Lemoine NR: Expression and functional activity of fibroblast growth factors and their receptors in human pancreatic cancer. Int J Cancer 59: 667-675, 1994.
18. Jaakkola S, Salmikangas P, Nylund S, *et al*: Amplification of fgfr4 gene in human breast and gynecological cancers. Int J Cancer 54: 378-382, 1993.
19. Lehtola L, Partanen J, Sistonen L, *et al*: Analysis of tyrosine kinase mRNAs including four FGF receptor mRNAs expressed in MCF-7 breast-cancer cells. Int J Cancer 50: 598-603, 1992.
20. Takahashi A, Sasaki H, Kim SJ, *et al*: Identification of receptor genes in renal cell carcinoma associated with angiogenesis by differential hybridization technique. Biochem Biophys Res Commun 257: 855-859, 1999.
21. Clark JC, Tichelaar JW, Wert SE, *et al*: FGF-10 disrupts lung morphogenesis and causes pulmonary adenomas in vivo. Am J Physiol Cell Mol Physiol 280: L705-L715, 2001.
22. Tichelaar J, Lu W and Whitsett J: Conditional expression of fibroblast growth factor-7 in the developing and mature lung. J Biol Chem 275: 11858-11864, 2000.
23. Zhao B, Chua SS, Burcin MM, *et al*: Phenotypic consequences of lung-specific inducible expression of FGF-3. Proc Natl Acad Sci USA 98: 5898-5903, 2001.
24. Davies H, Hunter C, Smith R, *et al*: Somatic mutations of the protein kinase gene family in human lung cancer. Cancer Res 65: 7591-7595, 2005.
25. Ogino S, Kawasaki T, Brahmandam M, *et al*: Sensitive sequencing method for KRAS mutation detection by Pyrosequencing. J Mol Diagn 7: 413-421, 2005.
26. Wittwer CT, Herrmann MG, Moss AA and Rasmussen RP: Continuous fluorescence monitoring of rapid cycle DNA amplification. Biotechniques 22: 130-138, 1997.
27. Wittwer T, Ririe M, Andrew V, David A, Gungry A and Bali J: The LightCycler: a microvolume multisample fluorimeter with rapid temperature control. Biotechniques 22: 176-181, 1997.
28. Sasaki H, Okuda K, Kawano O, *et al*: *Nras* and *Kras* mutation in Japanese lung cancer patients: Genotyping analysis using LightCycler. Oncol Rep 18: 623-628, 2007.
29. Krypuy M, Newnham GM, Thomas DM, Conron M and Dobrovic A: High resolution melting analysis for the rapid and sensitive detection of mutations in clinical samples: KRAS codon 12 and 13 mutations in non-small cell lung cancer. BMC Cancer 6: 295, 2006.