Clinical Significance of *BRAF* Gene Mutations in Patients with Non-small Cell Lung Cancer

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Abstract. *Background: V-raf murine sarcoma viral oncogene homolog B1 (BRAF) mutations are attractive molecular targets for cancer treatment. Detection of BRAF gene mutation and analyses in non-small cell lung cancer (NSCLC) are of great scientific interest. Patients and Methods: The study included 581 NSCLC patients (377 males, 204 female) undergoing pulmonary resection. BRAF gene mutations were screened using the PCR-SSCP method and were confirmed by direct DNA sequencing. Mutations of epidermal growth factor receptor (EGFR), v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (ERBB2), and v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) gene were also analyzed. Results: Five patients (0.8%) had BRAF mutations within exon 15. In 581 NSCLC patients, EGFR gene mutations within exons 18 to 21 were detected in 191 (32.8%) patients, KRAS codon 12 mutations in 56 (9.6%) patients, and ERBB2 codon 20 mutations in 11 (1.8%) patients. All mutations were mutually exclusive. The NSCLC patients with BRAF mutations were proved to be men who were heavy smokers. Conclusions: PCR-SSCP analysis of BRAF exon 15 in NSCLC patients without other gene mutations may be sufficient to identify candidates for treatment.*

Lung cancer is the leading cause of cancer-related mortality worldwide. More than 26,000 patients with lung cancer underwent curative surgical operation in Japan during 2007 (1). However, over 60,000 patients per year have advanced-stage lung cancer without indication for surgery, and conventional cisplatin-based chemotherapy for these patients, has only a limited survival benefit (2). Recent molecular biology analysis has clarified the biological behaviors of malignant tumors and facilitated the development of targeted molecular therapy. For example, epidermal growth factor receptor (EGFR)-specific tyrosine kinase inhibitors, such as gefitinib and erlotinib, are effective against lung adenocarcinomas with *EGFR* gene mutations (3, 4). Thus, the identification of abnormality in tumor-associated molecules can result in the development of new treatment strategies for cancer patients (5).

Many clinical and experimental studies demonstrated that the extracellular signal – regulated kinase (ERK) signaling pathway is one of the major pathways associated with tumorigenesis. Among them, mutations in the *BRAF* gene have been identified in a variety of human cancer types (6). Previous studies revealed that *BRAF* mutations were clustered within the P-loop (exon 11) and activation segment (exon 15) of the kinase domain (7). Among the various types of mutations, a single substitution of glutamic acid for valine at residue 600 (V600E, initially designated as V599E), which lies within the activation segment of the kinase domain, was observed in approximately 90% of case with *BRAF* mutation (6, 7). Furthermore, a previous experimental study revealed that most *BRAF* mutations result in increased kinase activity (8). The activating *BRAF* mutations, including V600E, can induce cell transformation and promote cell viability, cell proliferation, and tumorigenesis (9). Furthermore, experimental studies using RNA interference demonstrated that BRAF suppression inhibits tumor growth and induces apoptosis (10, 11). Therefore, these activating *BRAF* mutations are considered to be oncogenic. In fact, previous clinical studies have reported that *BRAF* mutations also frequently occur in melanoma, thyroid cancer, and colorectal cancer (6, 12-14).

Various specific inhibitors of *BRAF* mutations, such as sorafenib, PLX4720, and AZ628, have recently been developed for cancer treatment (15-18). Among them, sorafenib has been reported to be clinically effective for hepatocellular carcinoma (15) and renal cell cancer (16).

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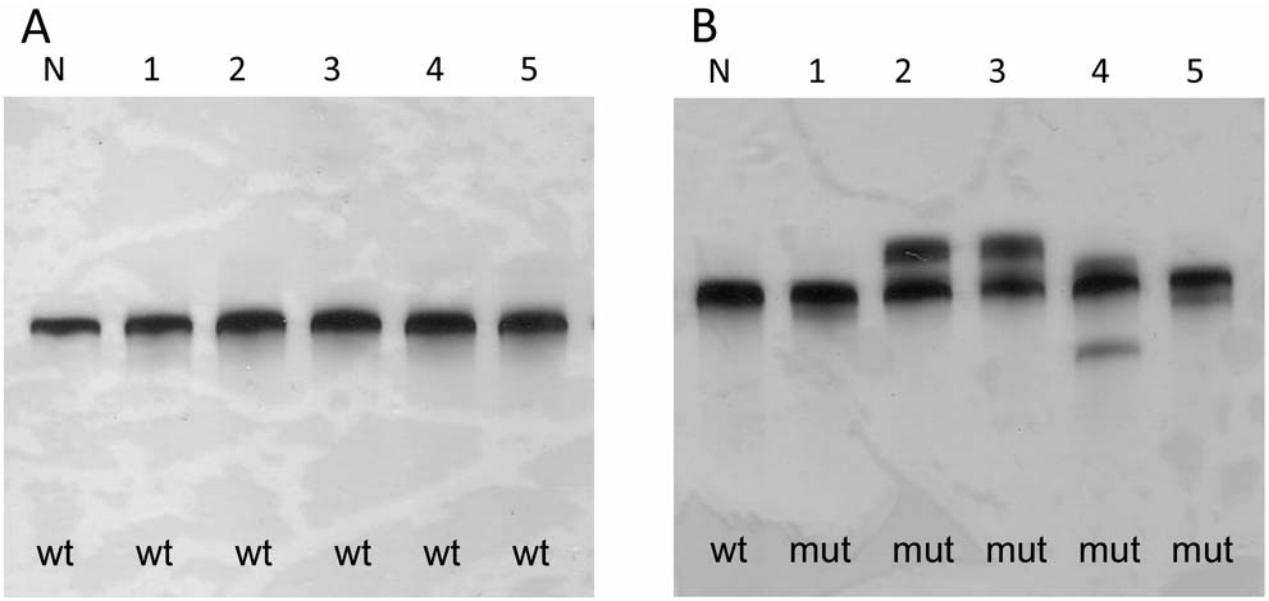

Figure 1. *A: Single strand conformation polymorphism (SSCP) analysis for v-raf murine sarcoma viral oncogene homolog B1 (BRAF) mutations in exon 11. Lane N, normal tissue; lanes patient 1 to 5, wild-type (wt) tumors. B: SSCP analysis for BRAF mutations in exon 15. Lane N, normal tissue; lanes patient 1 to 5, tumors with mutant (mut) exon 15 of the BRAF gene.*

These results suggest that these specific inhibitors could be effective in patients with *BRAF* mutant tumors (19). However, only a few clinical studies reported the clinical significance of *BRAF* mutations in non-small cell lung cancer (NSCLC) (20, 21). Therefore, we performed a clinical study to investigate the clinical significance of *BRAF* mutations in NSCLCs to apply BRAF-targeted treatment to lung cancer.

Patients and Methods

Clinical characteristics of patients. We studied consecutive NSCLC patients who underwent resection at Kyoto University Hospital from January 2001 to December 2007. This study was approved by the Ethics Committee of Kyoto University, and informed consent was obtained from each patient. All tumors were staged according to the current international tumor/node/metastasis (TNM) staging system, and histological classification was based on the WHO classification. In total, we investigated 581 tumors, which included 381 adenocarcinomas, 143 squamous cell carcinomas, and 57 tumors of other cancer types. Clinicopathological characteristics were obtained from inpatient and outpatient medical records, including the follow-up data up to April 2010.

Polymerase chain reaction (PCR) for BRAF. Genomic DNA was extracted from frozen tumor specimens by using QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA). Then, PCR was performed for exons 11 and 15 of the *BRAF* gene. Primers used were as follows: exon 11 of *BRAF*, forward: 5'-TCCCTCTCAGGCATAAGGTAA-3', reverse: 5'-CGAACAGTGAATATTTCCTTTGAT-3'; and exon 15 of *BRAF*, forward: 5'-CCTAAACTCTTCATAATGCTTGCTC-3', reverse: 5'-TTAATCAGTGGAAAAATAGCCTCAA-3', as described previously. PCR reaction was initiated by preincubation for 15 min at 95˚C, followed by 35 cycles at 72˚C for 30 s, 58˚C for 30 s, and 72˚C for 60 s by using HotStar Taq Master Mix Kit (Qiagen).

Single strand conformation polymorphism (SSCP) for BRAF mutations. After denaturing PCR products of exons 11 and 15 of the BRAF gene by incubation at 95˚C for 6 min, PCR products were immediately placed on ice. Electrophoresis was performed with a GenePhor Electrophoresis Unit with GeneGel Excel 12.5/24 (GE Healthcare UK Ltd, Amersham, UK) at 15˚C, 650 V for 80 min. The bands were visualized by silver carbonate stain (Figure 1).

Immunohistochemistry. Formalin-fixed paraffin-embedded tissue was cut into 4-μm sections. Duplicate sections were incubated overnight with primary antibodies against the Ki-67 antigen (MIB-1; DAKO, Glostrup, Denmark) diluted at 1:40. Ki-67 staining was visualized using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). The percentage of carcinoma cells with positive staining for Ki-67 in a given specimen was scored as the Ki-67 proliferation index. Samples with Ki-67 proliferation index values >25% were classified as having high Ki-67 expression (22).

Detection of mutations of epidermal growth factor receptor (EGFR), v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (ERBB2), v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS). Mutations in the EGFR (exons 18-21) and *ERBB2* (exon 20) genes were also studied by using PCR-SSCP, as reported previously (23). Mutations in *KRAS* were investigated using modified mutagenic restriction enzyme fragment-length polymorphism (PCR-RFLP), as reported previously (24).

Table I. *Clinical characteristics of subsets of patients with non-small cell lung cancer.*

	Mutation-positive				
Characteristic	*BRAF* (n=5)	*EGFR* (n=191)	*KRAS* (n=56)	*ERBB2* (n=11)	Wild-type (n=318)
Age years (median)	72	63.8	62.9	66.2	66.7
Gender					
Male	4	66	38	5	264
Female	1	125	18	6	54
Smoking					
Pack year					
<20	1	153	17	9	44
≥20	4	38	39	2	274
Histology					
Adenomatous	4	185	45	10	138
Squamous	1	2	6	1	133
Others	0	4	5	0	47
Differentiation					
Well	1	84	10	4	36
Moderate	3	99	26	7	165
Poorly	1	8	20	0	117
Pathological stage†					
I	4	141	35	7	197
II	0	18	5	2	48
III	1	25	11	2	69
IV	0	7	5	0	4

†According to the 7th edition TNM staging system for lung cancer (UICC-7).

Results

Clinical characteristics of patients with BRAF mutations. The clinical characteristics of the five patients with BRAF mutant NSCLCs are presented in Table I. Regarding tumor histology, four tumors were adenocarcinomas and one was squamous cell carcinoma. With respect to tumor differentiation, three were moderately differentiated, one was well differentiated, and one was poorly differentiated. Four patients were smokers and one patient with adenocarcinoma was a nonsmoker. The clinical outcomes were as follows: three patients had early stage-disease without postoperative recurrence; one with advanced and one with early stage-disease died at 15 and 31 months, respectively, after surgery due to distant metastases.

Mutations of the BRAF gene in NSCLCs. Among 581 carcinomas, 5 tumors (0.8%) had mutations in exon 15 of the *BRAF* gene (Figure IB). In contrast, no mutation was discovered in exon 11 of the BRAF gene. Two carcinomas had a point mutation of GTG to GAG at codon 600, which substituted glutamic acid for valine (V600E); two carcinomas had a point mutation of GAT to GGT at codon 594, which substituted glycine for aspartic acid (D594G); and one carcinoma had a point mutation without any amino acid substitution (Table II). Of the 581 carcinomas we studied, 191 tumors (32.8%) had *EGFR* mutations, 56 (9.6%) had *KRAS* mutations, and 11 (1.8%) had *ERBB2* mutations. All of these mutations were mutually exclusive (Table I).

Ki-67 proliferation index of NSCLCs with BRAF mutations. The Ki-67 proliferation index was evaluated by immunohistochemistry. Consequently, the Ki-67 proliferation index was a mean of 53%±25.4% in NSCLCs with *BRAF* mutations. In this study, high Ki-67 proliferation was identified in 4 (80%) of the 5 patients with *BRAF* gene mutations.

Histopathological analysis of tumors harboring BRAF mutations. One of the four BRAF mutation-harboring adenocarcinomas was a papillary adenocarcinoma, and two were classified as mixed subtype: one as acinar predominant subtype and one as papillary predominant subtype. The remaining tumor was not evaluated for subtype. One BRAF mutation-harboring squamous cell carcinoma was a papillary squamous cell carcinoma. The two adenocarcinomas of mixed subtype and one papillary adenocarcinoma/squamous cell carcinoma were moderately differentiated (grade 2), and one adenocarcinoma was graded as poorly differentiated (grade 3) (Table II).

Discussion

We investigated *BRAF* gene mutations in NSCLCs by using a relatively large number of patients in the present study. BRAF mutations were detected in only 0.8% (5 of 581) of

Table II. *Clinical features and details of BRAF mutation-positive patients.*

Patient no.	Gender	Age (years)	Smoker	Pack years	Histology	Differentation	p-Stage	Sequence	Ki-67 (grade)	Status
1	F	83	Never	0	Adenocarcinoma	Well	1A	GTG→GAG V600E	18% (Low)	44M Alive
2	M	61	Current	45	Squamous cell	Moderately	1A	GAG→GAT D594G	73% (High)	67M Alive
3	M	76	Ex smoker	45	Adenocarcinoma	Moderately	1A	GAG→GAT D594G	43% (High)	51M Alive
4	M	68	Ex smoker	31	Adenocarcinoma	Moderately	1A	GTG→GTA Silent	41% (High)	31M Dead
5	M	72	Ex smoker	50	Adenocarcinoma	Poorly	3B	GTG→GAG V600E	90% (High)	15M Dead

F: Female; M: male; Ki-67: Ki67-proliferation index; Status as of Prognosis; M: month. Ki-67 proliferation index values >25% were classified as high Ki-67 expression. *BRAF* mutations existed independently from other mutations of *EGFR, ERBB2,* and *KRAS*.

NSCLCs. Previous clinical studies also reported that *BRAF* mutations occurred in 1-2% of NSCLCs (20, 21). These results demonstrate that *BRAF* mutation is a rare event in NSCLC. A recent clinical study in patients with advanced NSCLC demonstrated that no clinical benefit was observed after adding sorafenib to carboplatin plus paclitaxel chemotherapy (25). This result may chiefly depend on the low frequency of *BRAF* mutations in NSCLC patients.

Although the prevalence of mutations was low, the present study also revealed that V600E is a prominent mutation in NSCLC. As previously mentioned, V600E is an oncogenic mutation and a major target of specific inhibitors. Furthermore, *BRAF* mutations existed independently from other mutations of *KRAS, EGFR*, and *ERBB2* in the present study. Intriguingly, we found *BRAF* mutation in one case of squamous cell carcinoma. All of these mutations were mutually exclusive.

Previously, we reported mutations of *EGFR* and *ERBB2* in NSCLC (23) along with its clinicopathological features. In our previous study, we were able to classify the characteristics of mutations of *EGFR* and *ERBB2* as important factors. This has contributed to the selection of chemotherapy treatment, including molecularly targeted drugs. However, in this study, we were not able to correlate specific clinicopathological findings with *BRAF* mutations in NSCLC. Most of the patient population comprised of men and smokers, although there was no significant differences in *BRAF* mutation.

Moreover, immunohistochemical staining for Ki-67 antigen, which is reported to be useful in assessing tumor proliferation (22) was higher in our study. Lower Ki-67 index values are strongly associated with histologically low-grade tumors. In a previous study of Ki-67 immunohistochemistry in NSCLC, patients with Ki-67 index values ≥25% were reported to have poor prognosis (22), with a Ki-67 proliferation index of 42.6%±30.4% (data not shown), and a high Ki-67 index in 67.6% of 173 NSCLC samples. But in the present study it was 53%±25.4%, and in those with *BRAF* mutations, 4 out of 5 tumor had a high Ki-67 index. This indicates high-grade tumors in these patients.

In addition, out of the five patients with NSCLC harboring *BRAF* gene mutations, three had early-stage NSCLC; thus, adjuvant chemotherapy was not administered.

Considering the results of the present study, it may be possible to successfully treat patients with NSCLC harboring *BRAF* gene mutations with BRAF-specific inhibitors. Furthermore, recent studies have developed new BRAF inhibitors that are more potent and more specific for BRAF, such as PLX4720 (18) and AZ628 (19). Since the frequency of *BRAF* mutations is considered to be important to evaluate the efficacy of these BRAF-specific inhibitors, further clinical studies using melanoma or thyroid cancer, in which *BRAF* mutations frequently occur, are initially required. Thereafter, we could attempt to perform BRAF-targeted therapy for NSCLC patients.

For BRAF-targeted therapy for cancer patients, it is clinically important to establish a simple method for detecting *BRAF* mutations. In the present study, PCR-SSCP analysis clearly revealed mutant bands of BRAF, including V600E. Furthermore, no mutation was found in exon 11 of *BRAF* in any of the 581 NSCLC samples. This would appear to be a very small population of patients. However, SSCP for mutations in exon 15 of *BRAF* may be sufficient to identify patient populations that would benefit from BRAF-targeted therapy for NSCLC.

Conflict of Interest

We have been no significant financial support for this work that could have influenced its outcome.

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