# *Braf* and *erbB2* mutations correlate with smoking status in lung cancer patients

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**Abstract.** The erbB pathway involves a family of tyrosine kinases and contributes to resistance or sensitivity to chemotherapy in many tumor types. Somatic mutations of the epidermal growth factor receptor (*EGFR*) gene at the kinase domain have been found in lung cancer patients. These mutations are correlated with clinical response to targeted molecular therapy. Although Caucasian lung cancer patients have been shown to harbor *Braf* and *erbB2* mutations, only a few reports exist concerning *Braf* and *erbB2* mutations in Japanese lung cancer patients. We investigated the *Braf* and *erbB2* mutation status in non-small cell lung cancer (NSCLC) patients by reverse transcription-polymerase chain reaction (RT-PCR) and direct sequencing. The study included 305 surgically removed lung cancer samples from the Nagoya City University Hospital, which were *EGFR* and *Kras* wild-type centric. Six *Braf* mutations were found in the adenocarcinoma cases. Among the adenocarcinoma cases, *Braf* mutations were more frequently noted in heavy smokers (Brinkman index >400, p=0.0476). We also detected five *erbB2* mutations all in the non-smokers. All of these mutations existed exclusively. The *erbB2* gene mutations were predominantly found in non-smokers with adenocarcinomas. However, the completely exclusive mutation status could help us design individually tailored targeted molecular therapy for lung cancer.

## Introduction

The epidermal growth factor receptor (EGFR) signaling pathway plays a crucial role in many carcinogenic processes, such as proliferation, angiogenesis, invasion and metastasis, and resistance to apoptosis (1,2). Since deregulation of EGFR pathway genes has been observed frequently in various types of tumors, including non-small cell lung cancer (NSCLC), the development of targeted agents for lung cancer therapy has focused mainly on EGFR and its downstream networks (3), such as RAS/RAF/MAP kinase and PI3K/AKT, being the two major pathways (3,4).

The pathway linking receptor tyrosine kinases to the Ras family to the Raf serine-threonine kinase to the MAP kinase cascade is critical for cell proliferation and is frequently activated in human cancers (5). MAP kinase, also known as extracellular signal-regulated protein kinase (ERK), is crucial for the transduction of growth signals from several key growth factors, such as EGF. ERK1 and ERK2, downstream effectors of the RAS-RAF-MEK-ERK-MAP kinase pathway, are constitutively active in many NSCLCs. Mutations of *Braf* were first reported in melanomas (over 60%) and colorectal cancers. The V600E (previously reported as V599E) mutant form of *Braf* activates the RAF/MEK/ERK pathway in human melanoma cells *in vitro*, and transformation of a melanocyte cell line with mutant *Braf* was found to activate the MAP kinase pathway (6). *Braf* mutations have since been reported in 3% (7) and 1.6% (8) of NSCLCs. Recently, additional studies have shown that somatic mutations of the *Braf* gene are found in approximately 2% of Caucasians (9,10) and in 1.2% of East Asians (11).

The erbB family comprises four structurally related receptors: erbB1 (EGFR) to erbB4. On ligand stimulation, the receptor forms either homodimers or heterodimers, which activate their cytoplasmic domain. Several reports have shown that somatic mutations of the *EGFR* gene are found in 25-40% of Japanese NSCLC patients (12,13), but only in 10% of NSCLC patients in the US (14,15). *EGFR* mutations were predominantly found in female, non-smokers with adenocarcinomas (12-16). Actually, *EGFR* mutations in NSCLC have been correlated with clinical response to gefitinib therapy (17-19). In addition, it has been reported that *erbB2* mutations at the kinase domain are present in 4% of European-derived NSCLC patients (20). Somatic *erbB2* mutations are more frequent in never-smoker and adenocarcinoma histology (21). Although the *erbB2* mutation was also investigated in Japanese NSCLC (13,22), the *Braf* mutation frequency in Japanese NSCLC is not well known. We previously described a single *Braf* mutation case (23), however, in this study, we investigated the *Braf* mutation status in 305 surgically treated NSCLC cases.

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## Patients and methods

*Patients*. This is a retrospective study. The study group included 305 lung cancer patients who had undergone surgery at the Department of Surgery II, Nagoya City University Medical School between 1997 and 2009. We also investigated the *EGFR*, *erbB2* (n=249) and *Kras* mutation status for the same patient group. All tumor samples were immediately frozen and stored at -80°C until assayed. The clinical and pathological characteristics of the 305 lung cancer patients are as follows: 180 cases at stage I, 51 at stage II and 74 at stage III-IV. The mean age was 66.1 years (range 39-88). Among the 305 lung cancer patients, 261 (85.6%) were diagnosed as adenocarcinoma and 38 (12.5%) were squamous cell carcinoma. The study was approved by the institutional ethics board and written

*PCR assays for Braf and erbB2*. Total RNA was extracted from lung cancer tissues and adjacent non-malignant lung tissues using the Isogen kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. RNA concentration was determined by spectrophotometer and adjusted to a concentration of 200 ng/ml. Approximately 20 cases were excluded since tumor cells were too few to sufficiently extract tumor RNA. RNA (1 µg) was reverse-transcribed by Superscript II enzyme (Gibco BRL, Gaithersburg, MD, USA) with 0.5 µg oligo(dT)12-16 (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). The reaction mixture was incubated at 42°C for 50 min and then at 72°C for 15 min. We then used 1 µl of each DNA for PCR analyses. The PCR reactions were performed using the LA-Taq kit (Takara Bio Inc., Shiga, Japan) in a 25-µl reaction volume. The primer sequences for the *Braf* gene for the kinase domain (exon 11-15) were as follows: the forward primer, 5-GACGGGACTCGAGTGATGAT-3 and the reverse primer, 5-CCACAAATGGATCCAGACA-3 (532 bp). The cycling conditions were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 40 sec, 60°C for 40 sec and 72°C for 45 sec. The primer sequences for *erbB2* gene for kinase domain (exon 19-22) were as follows: the forward primer, 5-CGCTTTTGGCACAGTCTACA-3 and the reverse primer, 5-GGGATCCCATCGTAAGGTTT-3 (594 bp). The cycling conditions were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 40 sec, 60°C for 40 sec and 72°C for 45 sec. The products were purified by the Qiagen PCR purification kit (Qiagen, Valencia, CA, USA). Amplified cDNAs were separated on 1% agarose gels, and the bands were visualized by ethidium bromide and photographed under ultraviolet transillumination. These samples were sequenced using the ABI Prism 3100 analyzer (Applied Biosystems Japan Ltd., Tokyo, Japan) and analyzed by BLAST and chromatograms by manual review from forward and reverse, both side. *EGFR* and *Kras* sequencing methods were previously submitted elsewhere (12,13,16,24).

## Results

*Braf gene mutation status in Japanese lung cancer patients*. Of the 305 patients, 93 had *EGFR* mutations within the kinase domain, including 45 exon 19 deletions, 41 L858R, 5 exon 20 insertion, 3 G719S and 2 L861Q. Twenty-two had



Figure 1. *Braf* mutations in lung cancer patients. One mutation consisted of N581I (1742 adenine to thymine; asparagine to isoleucine) (upper) and five were V600E (1799 thymine to adenine; valine to glutamic acid) (lower). Reverse sequence reading was also confirmed (right).
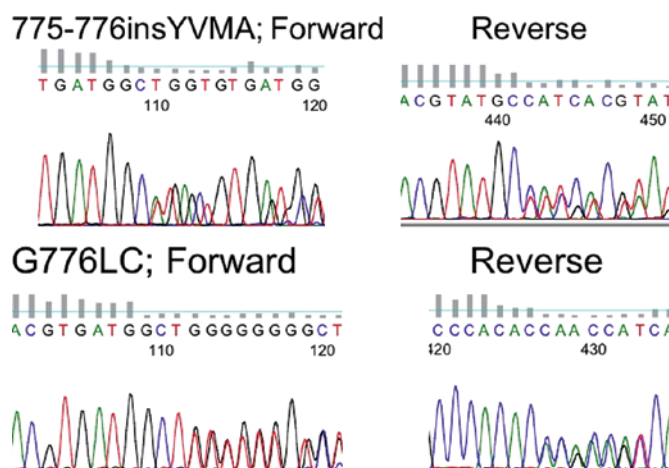


Figure 2. *erbB2* mutations in lung cancer patients. Four cases consisted of 12 amino acid insertion mutation (2324-2325 ins ATACGTGATGGC), located in exon 20 at the kinase domain (775-776 ins YVMA) (upper). Reverse sequence for the *erbB2* gene was also confirmed (right) One had an amino acid insertion mutation (2326 G to TTGT), located in exon 20 at the kinase domain (776 glycine to leucine plus cysteine) (lower). Reverse sequence reading was also confirmed (right).

*Kras* mutations at codon 12 or 13. *Braf* mutation was found in 6 (1.96%) of the 305 NSCLC patients. Reverse sequence reading was also confirmed (Fig. 1). Five consisted of V600E (1799 thymine to adenine; valine to glutamic acid) and one was N581I (1742 adenine to thymine; asparagine to isoleucine). Four were male and 2 were female. Five were smokers and 1 was a never smoker. All were adenocarcinoma cases. Within the total cohort, there was no significant difference in the *Braf* mutation rate according to gender (p=0.8234), age (p=0.5050), pathological stage (p=0.7004) or smoking status (never smoker vs. smoker; p=0.6195). However, in the adenocarcinoma cases, heavy-smoker group (Brinkman index >400) had a significantly higher *Braf* mutation rate than in the light-smoker or never-smoker group (p<0.05). There was no significant difference in the *Braf* mutation rate according to gender (p=0.6449), age (p=0.4665) and

Table I. Clinicopathological data of the 261 lung adenocarcinoma patients.

| Factors | Braf gene status | | p-value |
| | Mutant patients (n=6) | Wild-type patients (n=255) | |
|---|---|---|---|
| Mean age (years; 65.8±9.0) | 69.0±8.7 | 65.7±9.0 | 0.4665 |
| Stage | | | 0.7704 |
| I | 4 (2.5%) | 155 (97.5%) | |
| II-IV | 2 (2.0%) | 100 (98.0%) | |
| Lymph node metastasis | | | 0.8182 |
| N0 | 4 (2.2%) | 181 (97.8%) | |
| N+ | 2 (2.6%) | 74 (97.4%) | |
| Smoking status | | | 0.0476 |
| BI ≤400 | 1 (0.7%) | 146 (99.3%) | |
| BI >400 | 5 (4.4%) | 109 (95.3%) | |
| EGFR mutation | | | 0.0630 |
| Mutation | 0 (0%) | 94 (100%) | |
| Wild-type | 6 (3.6%) | 161 (96.7%) | |
| Kras mutation | | | 0.4521 |
| Mutation | 0 (0%) | 22 (100%) | |
| Wild-type | 6 (2.5%) | 233 (97.5%) | |
| Age (years) | | | 0.5054 |
| ≤65 | 2 (1.6%) | 120 (98.4%) | |
| >65 | 4 (2.9%) | 135 (97.1%) | |
| Gender | | | 0.6449 |
| Male | 4 (2.7%) | 109 (97.3%) | |
| Female | 2 (1.8%) | 146 (98.2%) | |

N0, lymph node metastasis-negative; N+, lymph node metastasis-positive; BI, Brinkman index.

Table II. Clinicopathological data of 248 NSCLC patients.

| Factors | erbB2 gene status | | p-value |
| | Mutant patients (n=5) | Wild-type patients (n=243) | |
|---|---|---|---|
| Mean age (years; 65.7±8.7) | 68.0±5.0 | 65.7±8.8 | 0.5142 |
| Stage | | | 0.3863 |
| I | 2 (1.4%) | 144 (98.6%) | |
| II-IV | 3 (2.9%) | 99 (97.1%) | |
| Lymph node metastasis | | | 0.7083 |
| N0 | 3 (1.8%) | 165 (98.2%) | |
| N+ | 2 (2.5%) | 78 (97.5%) | |
| Smoking status | | | 0.0052 |
| Never smoker | 5 (5.1%) | 93 (94.9%) | |
| Smoker | 0 (0%) | 150 (100%) | |
| EGFR mutation | | | 0.1828 |
| Mutation | 0 (0%) | 64 (100%) | |
| Wild-type | 5 (2.7%) | 179 (97.3%) | |
| Pathology | | | 0.3846 |
| Adenocarcinoma | 5 (2.3%) | 211 (97.7%) | |
| Non-adenocarcinoma | 0 (0%) | 32 (100%) | |
| Age (years) | | | 0.7046 |
| ≤65 | 2 (1.7%) | 118 (98.3%) | |
| >65 | 3 (2.3%) | 125 (97.7%) | |
| Gender | | | 0.2471 |
| Male | 2 (1.3%) | 158 (98.7%) | |
| Female | 3 (3.4%) | 85 (96.6%) | |

pathological stage (p=0.7704) among the adenocarcinoma cases (Table I).

*erbB2 gene mutation status in Japanese lung cancer patients.* We also sequenced the kinase domain of *erbB2* for 248 NSCLC patients. Among the 248 patients, 5 (2%) had *erbB2* mutations. All mutations were at exon 20. Three were female. All were non-smokers. Four had a 12 amino acid insertion mutation (2324-2325 ins ATACGTGATGGC), located in exon 20 at the kinase domain (775-776 ins YVMA). One had an amino acid insertion mutation (2326 G to TTGT) located in the exon 20 at kinase domain (776 glycine to leucine plus cysteine). Reverse sequence for the *erbB2* gene was also confirmed. Never smokers had a significantly higher *erbB2* mutation rate than the smokers (p=0.0052). There was no significant difference in the *erbB2* mutation rate according to gender (p=0.2471), age (p=0.5142) and pathological stage (p=0.3863) (Table II).

Within these NSCLCs, all four gene (*EGFR*, *erbB2*, *Kras* and *Braf*) mutations existed exclusively. All patients with *Braf* or *erbB2* mutations were alive at this point and we did not perform survival analysis (data not shown).

## Discussion

In the present study, we found six *Braf* mutations in 305 Japanese NSCLC cases. The *Braf* mutation was exclusively found without *EGFR* or *erbB2* mutations. *Braf* mutations were predominantly found in heavy smokers among the adenocarcinoma cases. This population was also thought to have a higher incidence of *Kras* gene mutations (10,24). On the other hand, in our analysis, *erbB2* gene mutations were predominantly found in non-smokers with adenocarcinomas.

The v-raf murine sarcoma viral oncogene homolog B1 (*Braf*) encodes a serine/threonine kinase that acts in the MAP kinase pathway, through both receptor tyrosine kinases and G-protein coupled receptors (5). Mutations in *Braf* were first reported in melanomas and colorectal cancers, but have since been reported in a variety of solid tumors (6,7), including stage I lung adenocarcinomas (7). Activating *Braf* mutations, especially common mutant V600E, induce constitutive activation of the signal transduction pathway, providing a potent promitogenic force that drives malignant transformation (6). The *Braf* V600E mutant showed greatly increased activity in the Raf/MEK/Erk pathway both *in vitro* and *in vivo* (6,25). An inducible transgenic mouse model of *Braf* V600E developed by Ji *et al* (26) demonstrated that mutant *Braf* was sufficient for the development of lung adenocarcinomas. Since the incidence of *Braf* mutations is highest in melanomas, the bulk of the clinical trials to date have focused on this disease, targeting either BRAF itself or MEK 1/2, the latter of which is associated with growth-dependency in *Braf* mutant cell lines (27,28). The most promising of specific agents has been PLX4032, which was associated with an 80% response rate in the extension phase of a recent multicenter phase I study that included 32 patients with advanced stage melanoma with *Braf* mutations (29).

Mutations in the *erbB2* gene were found in approximately 2% of primary NSCLCs, predominantly in never smoker-like *EGFR* mutations (12-16). It has been shown that the common *erbB2* mutation, A775 ins YVMA, led to oncogenic transformation in a cellular assay (30). The mutations target residues that are highly conserved in the erbB family, and are homologous to the exon 20 insertion mutations of *EGFR*. Murine cells transformed with the mutant were relatively resistant to the reversible EGFR inhibitor, resembling the resistant phenotype found in cells carrying the homologous mutations in exon 20 of *EGFR* (31,32). However, the mutant cells exhibited high sensitivity to the irreversible dual-specificity EGFR/ERBB2 kinase inhibitor HKI-272 (30).

In our analysis, *Braf* or *erbB2* mutations were only found in the adenocarcinomas, but not in the squamous cell carcinoma cases. It was also suggested that *Braf* mutations in Japanese NSCLC patients are not common and have an even lower frequency than that in US patients (6,7), or in *in vitro* analysis from lung cancer cell lines (11%) (5). Our data showed that mutations of the *Braf* or *erbB2* gene as a mechanism of tumorigenesis are unlikely to be associated with many cases of Japanese NSCLCs. Despite the promise of anti-BRAF therapy or irreversible erbB inhibitors, our findings indicate that a small percentage of Japanese NSCLC patients actually harbor the *Braf* or *erbB2* mutation and, in turn, a few patients with these tumors may likely benefit from these anticancer agents. However, completely exclusive *EGFR*, *erbB2* and *Braf* mutation status would help us to choose individualized molecular targeting therapy for NSCLC. Further studies are required to confirm the mechanisms of *Braf* and *erbB2* mutations involved in the sensitivity or resistance of targeted therapy for lung cancer.

## Acknowledgements

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