The Effect of Gefitinib on B-RAF Mutant Non-small Cell Lung Cancer and Transfectants

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Abstract: We previously reported one patient with squamous cell carcinoma of the lung that showed the long-term effect to gefitinib with complete response. In the present report, we examine the epidermal growth factor receptor (EGFR) and K-RAS, HER2, and B-RAF mutations in this patient to find a B-RAF exon11 mutation, resulting in a substitution of valine by phenylalanine at codon 470 (V470F) as a novel type of B-RAF mutation in human cancers. In addition, the fluorescence in situ hybridization analysis for EGFR showed the high polysomy status. B-RAF is a nonreceptor serine/threonine kinase whose kinase domain has a structure similar to other protein kinases, including EGFR members. Of interest, the B-RAF V470F mutation corresponds to a position similar to the EGFR G719X mutation located on the phosphate binding (P)-loop of EGFR that clamps ATP into the catalytic cleft. This observation suggests that gefitinib may have an anti-cancer effect on B-RAF mutant tumors. Indeed, previous reports demonstrated that H1666 cells harboring B-RAF G465V mutations showed sensitivity to gefitinib, inhibiting phosphorylation of ERK1/2. We examined the effect of gefitinib on transient transfectants of the B-RAF mutant, but no drastic inhibition of ERK1/2 phosphorylation that was one of the downstream molecules of B-RAF was induced by gefitinib.

In summary, we found a novel B-RAF V470F mutation in lung squamous cell carcinoma that showed response to gefitinib. However, our in vitro investigation did not explain the response observed in this particular patient. Further investigation is necessary to elucidate the mechanism of tumor sensitivity to EGFR tyrosine kinase inhibitors.

Key Words: Non-small cell lung cancer, B-RAF, Epidermal growth factor receptor, Gefitinib, Epidermal growth factor receptor-tyrosine kinase inhibitors.


A molecular targeting approach would enable therapeutic benefits to be maximized while minimizing toxicity to non-malignant cells. Epidermal growth factor receptor (EGFR) is highly expressed in epithelial tumors including lung cancers1 and EGFR tyrosine kinase inhibitors (TKIs), 4-anilinoquinazoline compounds, like gefitinib or erlotinib, were introduced as EGFR-targeting anti-tumor reagents.

Rapid progress has been made in identifying factors that determine responsiveness to EGFR-TKIs in non-small cell lung cancer. The EGFR mutation has been reported to be a biological predictor of responsiveness to gefitinib. These mutations occur in the ATP binding cleft of the TK domain in which EGFR-TKIs compete with ATP for binding.2,3 The EGFR-associated signaling cascade consists of several molecules with known abnormalities, including EGFR, HER2, K-RAS, and B-RAF. Interestingly, there is no coincidence among these aberrations, suggesting that each alteration plays a causative role in lung carcinogenesis.4

Previous studies indicate that EGFR-TKIs revealed responsiveness in approximately 80% of EGFR mutant tumors. However, no drug-sensitizing EGFR mutation was found in the remaining 20%, which suggests that other factors, such as increased copy number of EGFR, may cause tumor response to the drug, but this mechanism is not fully understood.5,6 We previously reported the case of one patient with squamous cell carcinoma (SCC) of the lung that showed complete response to gefitinib.7 In this study, we found a B-RAF mutation in the patient that is assumed to be an oncogenic mutationa and investigated the effect of gefitinib on HEK293T cells transfected with various types of B-RAF mutants.

MATERIALS AND METHODS

Patients and EGFR, HER2, K-RAS, and B-RAF Analysis

The mutation status of EGFR exon18–24, K-RAS exon2, HER2 exon20, and B-RAF exon11 and exon15 was
determined in three tumors (one primary tumor and two recurrent tumors) resected from the same patient by direct sequencing. The primers and polymerase chain reaction (PCR) condition will be provided on request. The EGFR copy number status was examined using fluorescence in situ hybridization (FISH) in the resected specimens of tumors and estimated using the published criteria. In addition, eight patients showing responsiveness to gefitinib with EGFR wild-type tumors in our previous studies and our recent cases were examined for K-RAS, HER2, and B-RAF mutation status. The DNA sequencing was confirmed in repeating PCR reaction by analyzing in both sense and antisense directions. We obtained the permission of our institutional review board and informed consent from each patient.

**Cell Culture, Expression Constructs, and Transfections**

HEK293T cells were cultured at 37.0°C with 5% CO2 by using Dulbecco’s modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum, 1% modified Eagle medium non-essential amino acid solution, and 2 mM L-glutamine. B-RAF expression vectors were constructed by inserting full-length human B-RAF cDNA (kindly provided by Dr. Kun-Liang Guan, University of Michigan Medical School, MI) into pcDNA3.1-Zeo vector (Invitrogen, Carlsbad, CA) containing C-terminal Flag epitope in frame. The G468E, V470F, and V599E mutations were generated by site-directed mutagenesis using a QuikChange kit (Stratagene, La Jolla, CA), and mutations were confirmed by sequencing. The EGFR L858R expression vector has been previously described. Transient transfection was performed using Lipofectamine 2000 (Invitrogen). Each transfectant was treated with 2 μM gefitinib for 6 hours. Gefitinib was provided by AstraZeneca (Waltham, MA).

**Antibodies and Western Blotting**

Cells were lysed with RIPA lysis solution as previously described. Whole cell lysates using same amount of each cell were separated with sodium dodecyl sulfate-PAGE, transferred to a membrane, and detected by antibodies using ECL plus Western blotting detection reagents (GE Healthcare Biosciences, Piscataway, NJ). The antibodies against p44/42 Erk1/2 and phospho-p44/42 Erk1/2 (Thr202/Tyr204) were purchased from Cell Signaling (Beverly, MA). The anti-B-RAF antibody was obtained from BD Biosciences (San Jose, CA).

**RESULTS**

**Patient and Molecular Analysis**

We previously reported a patient with SCC of the lung who had experienced a long-term effect with gefitinib treatment. In brief, a 62-year-old man with a 52.5 pack-year smoking history was diagnosed as having clinical stage IIIB lung SCC. He received cisplatin-based chemotherapy and subsequently underwent surgery. After the initial surgery, the patient developed two recurrences in the lung; additional surgical resections were performed for each recurrence within a 2-year period. After the patient’s third relapse in the mediastinal lymph node, gefitinib was administered, and he achieved complete response. Although the patient developed acute promyelocytic leukemia after 25 months of gefitinib administration and was subsequently treated with all-trans retinoic acid, he was eventually cured; no evidence of the recurrence of either disease has been found.

We examined the EGFR, K-RAS, HER2, and B-RAF mutation status using direct sequencing and determined the EGFR copy number status using FISH assay in the three resected metachronous tumors diagnosed as recurrent disease. Mutational analysis revealed the presence of the B-RAF codon 470 mutation at exon11, resulting in the substitution of phenylalanine for valine (V470F) in all three tumors resected, but not in nonmalignant lung tissue (Figure 1). EGFR exon18–24, K-RAS exon2, and HER2 exon20 mutations were not detected. Regarding the copy number of EGFR, the FISH analysis showed high polysomy status. We also examined the B-RAF mutation status in our additional eight patients with responsiveness to gefitinib with EGFR wild-type status, but no B-RAF exon11 or exon15 mutation was detected.

**Effect of Gefitinib on B-RAF or EGFR Mutant Transfectants**

The effect of gefitinib on phosphorylation of ERK1/2 was examined in HEK293T cells transiently transfected with EGFR L858R, B-RAF wild-type, G468E, V470F, or V599E mutant expression vector. B-RAF V470F transfectant exhibited an increased level of ERK1/2 phosphorylation, suggesting its oncogenic role (not stronger than V599E transfectant), but it did not show inhibition of ERK1/2 phosphorylation at 2 μM gefitinib. By contrast, phosphorylation of ERK1/2 in EGFR L858R transient transfectant was inhibited at the same drug concentration, suggesting that gefitinib might not inhibit the B-RAF kinase activity and its downstream pathway in B-RAF wild or mutant cells (Figure 2). We repeated this experiment twice independent from transfection, and the results were the same.

**DISCUSSION**

B-RAF is a nonreceptor serine/threonine kinase located downstream of EGFR-RAS, which activates MEK-ERK. Its mutation has been found in various human cancers. Most mutant B-RAF proteins in cancer have increased kinase activity, and the B-RAF V599E mutation is one of the most...
of B-RAF is located near the GXGXXG motif. Phosphate-binding (P) loop of B-RAF and EGFR. Codon 470 shown for B-RAF and phospho- and total ERK1/2.

FIGURE 2. The effects of gefitinib on phosphorylation of ERK1/2 in transfected HEK293T cells. Western blots are shown for B-RAF and phospho- and total ERK1/2.

Active mutants, producing a 500-fold activated protein versus the B-RAF wild-type. The kinase domain of B-RAF has a structure similar to that of other kinases, including EGFR, and most of its mutation in human cancers is also located in the phosphate-binding loop or activation loop, as seen in some of the EGFR mutations. However, the rate of B-RAF mutation is less than 3% in lung cancers. The B-RAF V470F mutation, which is a novel mutation in all kinds of human cancers, is located in the phosphate-binding loop of B-RAF. Of interest, the EGFR G719X mutation sensitizing to EGFR-TKIs is also in the phosphate-binding loop, especially in the glycine-rich (GXGXXG) motif that clamps ATP into catalytic cleft (Figure 3). Whereas the B-RAF V470F mutation was not really analogous to the EGFR G719X mutation, which occurs directly in the GXGXXG motif, these facts suggest that gefitinib may have an inhibitory effect on the kinase activity of B-RAF, causing an anti-tumor effect, if the affinity of B-RAF kinase portion to ATP and to its competitive inhibitor gefitinib may be altered because of the B-RAF mutation, although gefitinib is originally designed to selectively inhibit wild-type EGFR-TK activity. Indeed, the H1666 cell line harboring B-RAF G465V (located in the phosphate-binding loop) mutation and EGFR wild-type showed moderate response to gefitinib with inhibition of ERK1/2 phosphorylation at 1 μM and IC50 at 2 μM, despite a high IC50 value compared with EGFR drug-sensitizing mutant cell lines. However, our in vitro investigation using transient B-RAF G468E, V470F, or V599E transfectant demonstrates that gefitinib did not inhibit the phosphorylation of ERK1/2 at a concentration of 2 μM. By contrast, phosphorylation of ERK1/2 in the EGFR L858R transient transfectant was inhibited at the same drug concentration. Our study suggests that there is no strong relationship between B-RAF mutation status and gefitinib response in an in vitro system. Whereas the long-term effect of gefitinib in our patient may be the result of other mechanisms, including the increased EGFR copy number, the fact that the mutant B-RAF caused activation of the MEK-ERK cascade for proliferation, regardless of the blockage of EGFR activation by gefitinib, may explain how the B-RAF mutant protein could play a critical role in tumor response to gefitinib.

In conclusion, we had one patient with B-RAF V470F-mutant SCC of the lung that showed complete response to gefitinib. However, our investigation using transient B-RAF mutant transfection of HEK293T cells did not support our finding in this particular patient. Further investigation is necessary to determine tumor sensitivity to EGFR-TKIs and to establish individual therapy for patients with non-small cell lung cancer.

Note
The original B-RAF sequence was missing a codon (three nucleotides) in the region encoding for the amino acids 31 and 32. To avoid confusion with original published data, we use the original, if incorrect, numbering.

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REFERENCES
6. Cappuzzo F, Hirsch FR, Rossi E, et al. Epidermal growth factor receptor copy number, the fact that the mutant B-RAF caused activation of the MEK-ERK cascade for proliferation, regardless of the blockage of EGFR activation by gefitinib, may explain how the B-RAF mutant protein could play a critical role in tumor response to gefitinib.


