Featured Article

High Frequency of Epidermal Growth Factor Receptor Mutations with Complex Patterns in Non–Small Cell Lung Cancers Related to Gefitinib Responsiveness in Taiwan

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ABSTRACT

Purpose: Epidermal growth factor receptor (EGFR) mutations related to gefitinib responsiveness in non–small cell lung cancer have been found recently. Detection of EGFR mutations has become an important issue for therapeutic decision-making in non–small cell lung cancer.

Experimental Design: Mutational analysis of the kinase domain of EGFR coding sequence was done on 101 fresh frozen tumor tissues from patients without prior gefitinib treatment and 16 paraffin-embedded tumor tissues from patients treated with gefitinib. Detection of phosphorylated EGFR by immunoblot was also done on frozen tumor tissues.

Results: The 101 non–small cell lung cancer tumor specimens include 69 adenocarcinomas, 24 squamous cell carcinomas, and 8 other types of non–small cell lung cancers. Mutation(s) in the kinase domain (exon 18 to exon 21) of the EGFR gene were identified in 39 patients. All of the mutations occurred in adenocarcinoma, except one that was in an adenosquamous carcinoma. The mutation rate in adenocarcinoma was 55% (38 of 69). For the 16 patients treated with gefitinib, 7 of the 9 responders had EGFR mutations, and only 1 of the 7 nonresponders had mutations, which included a nonsense mutation. The mutations seem to be complex in that altogether 23 different mutations were observed, and 9 tumors carried 2 mutations.

Conclusions: Data from our study would predict a higher gefitinib response rate in lung adenocarcinoma patients in Chinese and, possibly, other East Asian populations. The tight association with adenocarcinoma and the high frequency of mutations raise the possibility that EGFR mutations play an important role in the tumorigenesis of adenocarcinoma of lung, especially in East Asians.

INTRODUCTION

Epidermal growth factor receptor (EGFR) is a M, 170,000 transmembrane glycoprotein. Binding of ligands to the extracellular domain results in activation of the tyrosine kinase activity of the receptor. Activation of the downstream pathways of EGFR leads to cell proliferation, differentiation, migration/motility, adhesion, protection from apoptosis, enhanced survival, and gene transcription (1, 2). Overactivity of these signaling pathways can lead to abnormal growth control and cellular transformation. Thus, deregulated expression of EGFR through mutation, amplification, or deregulation is linked with malignancy (1, 2). In recent years, inhibition of activated protein kinases with small molecule drugs or antibodies has become an effective approach to cancer therapy (3–5). Because EGFR is highly expressed in 88 to 99% of non–small cell lung cancers (6–9), and most chemotherapy regimens seem to have limited efficacy with disappointing survival results for non–small cell lung cancer (10–12), EGFR-tyrosine kinase has become a particularly promising drug target for treating non–small cell lung cancer. Among these, gefitinib is an orally active, targeted, small-molecule drug with evidence of antitumor activity in a range of cancers including non–small cell lung cancer, as shown by Phase I clinical trials (13, 14). One interesting observation about this new drug is the significant variability in the response rate. Although only 10 to 19% of patients with chemotherapy-refractory advanced non–small cell lung cancer showed a response to this new drug in large multi-institutional Phase II trials, a subgroup of patients had a dramatically rapid and profound response with only mild side effects (15, 16). Additionally, a significantly higher response rate was noted in Japanese patients than in a predominantly European-derived population (27.5% versus 10.4%; refs. 15, 16). In Taiwan, a high response rate for gefitinib for pretreated, advanced non–small-cell lung cancers has also been noted (26 to 36%; refs. 17, 18), which was similar to that of the Japanese patients. The response
rate in chemonaive advanced non–small cell lung cancer patients was even higher (56.5%; ref. 19). In addition to the difference between ethnic backgrounds, several clinical characteristics were also noted to be associated with higher response rate, i.e., adenocarcinoma, female gender, and nonsmoking history (16, 20, 21). EGFR expression detected by immunohistochemistry, however, is not correlated with the clinical response to gefitinib (22). Thus, the search for an effective predictor of the response to gefitinib and its molecular mechanism has become an important issue.

Recently, two research groups have discovered that EGFR mutations correlated with the clinical responsiveness to gefitinib in non–small cell lung cancer (23, 24). All of these mutations were within exons 18 through 21 of the kinase domain of EGFR. These mutations led to increased tyrosine kinase activity and conferred susceptibility to gefitinib. All of these mutations, except one, occurred in adenocarcinomas. The study by Paez et al. (24) also reported a much higher mutation rate in Japanese patients than in those from the United States (32% versus 3% for adenocarcinoma) and highest of all in Japanese women with adenocarcinoma (57%). These findings are highly correlated with the clinical characteristics known to be associated with a higher response rate to gefitinib (15, 16).

We have conducted mutational analysis of the EGFR gene from exons 18 to 21 in a series of 101 surgically resected non–small cell lung cancers with no prior chemotherapy or gefitinib treatment. We observed a high mutation rate of EGFR in the regions that have been reported to be associated with responsiveness to gefitinib. We also identified a range of new mutations that have not been reported previously. Additional mutational analysis of 16 patients with adenocarcinoma of lung treated with gefitinib also revealed mutations of EGFR in most of the responders.

MATERIALS AND METHODS

Fresh Frozen Tumor Tissues from Patients without Prior Gefitinib Treatment

One hundred and one cases of fresh frozen tumor tissues from surgically resected lung specimens together with their adjacent non-neoplastic lung tissues and the peripheral blood samples of the patients were collected from Chang-Gung Memorial Hospital between 2002 and 2004. Informed consent from each patient was obtained before the operation. All of the tumor and non-neoplastic lung tissues were snapped frozen and stored at −80°C within 30 minutes after resection. None of these patients had received gefitinib treatment before the surgery.

For each specimen, frozen sections were done and stained with H&E first to examine the tumor proportion of the tissue. Only tumors with >50% of tumor component were sent forward for DNA extraction and sequence analysis. Immunoblot analysis was also carried out in most of the fresh tumor specimens to check whether there was autophosphorylation of EGFR. Complete clinical data including smoking history, clinical stage, pathological diagnosis, and follow-up status were obtained from medical records and reviewed by the physicians.

Formalin-Fixed Paraffin-Embedded Tumor Tissues from Patients Treated with Gefitinib

Tumor tissues obtained before the patients received any systemic treatment were collected retrospectively from patients treated with gefitinib. All of these tumors were formalin-fixed paraffin-embedded tissues from the Department of Pathology (Chang-Gung Memorial Hospital, Taipei, Taiwan). To minimize non-neoplastic tissue contamination, the tumor portion was selected and marked on the H&E stained tissue section slide first by a pathologist (Shiu-Feng Huang). Only the tumor portion was dissected from the unstained tissue section slides and sent for DNA extraction. The latter was done with DEXPAT (Takara Biomedical, Shiga, Japan) according to the manufacturer’s instructions.

Nucleotide Sequence Analysis

For mutational analysis of the kinase domain of EGFR coding sequence, exon 18, 19, 20, and 21 were amplified with four pairs of primers, specific to the flanking sequences of individual exon, and PCR amplicons were subjected to direct sequencing (the PCR primers and amplification procedures are shown in supplemental text and supplemental Tables 1 and 2 for frozen and paraffin-embedded tissue, respectively). Forward and reverse sequencing reactions were done with the same primers for PCR amplification and ABI BigDye Terminator kit v3.1 (Applied Biosystems, Foster City, CA) according to manufacturer’s instructions. Sequencing reactions were electrophoresed on an ABI3700 genetic analyzer. Sequence variations were determined by using Seqscape software (Applied Biosystems) with the EGFR reference sequence (NM_005228.3, National Center for Biotechnology Information). All of the sequence variations were confirmed by multiple, independent PCR amplifications and repeated sequencing reactions.

Immunoblot Analysis

Tumor and adjacent non-neoplastic tissues were homogenized in lysis buffer [50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% (w/v) SDS, 1 mmol/L dithiothreitol, 5 μg/mL leupeptine, 3 μg/mL aprotinin, 0.5 mmol/L phenylmethylsulfonylfluoride, and 1 mmol/L Na3VO4] with the MagNA Lyser Green Beads kit (Roche Applied Science, Mannheim, Germany). The extracts were centrifuged at 14,000 g to remove the tissue debris. One hundred micrograms of extracted protein were resolved by 8% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and then subjected to immunoblot analyses with an antiphosphorylated EGFR monoclonal antibody (Chemicon, Temecula, CA) according to the manufacturer’s instructions. The expression level of EGFR was determined by an anti-EGFR antibody (Cell Signaling Technology, Inc., Beverly, MA), and an equal loading of extract was confirmed by immunoblotting with an anti-β–actin antibody (AC-15, Sigma, St. Louis, MO).

RESULTS

Fresh Frozen Tumor Tissues from Patients without Prior Gefitinib Treatment

The 101 non–small cell lung cancer tumor specimens included 69 adenocarcinomas, 24 squamous cell carcinomas, and 8 other types of non–small cell lung cancers. Fifty-six were male...
EGFR Mutation Spectrum

The EGFR gene encompasses 118 kb of sequence on the short arm of human chromosome 7 and consists of 28 exons (reference sequence: NM_005228.3, National Center for Biotechnology Information). We have sequenced the exon 18 to exon 21 that correspond to the catalytic kinase domain in all of the 101 tumors. Mutations were found in all four exons but mainly clustered in exons 19 and 21. In the exon 19, 13 tumors contained a small deletion starting from nucleotides 2481 to 2484, and the 15- or 18-nucleotide deletion resulted in small in-frame deletion of the kinase domain (Fig. 1, A and B). The most common form was the 15-nucleotide deletion from nucleotide 2481 to 2495 or 2482 to 2496, which resulted in elimination of codons 746 to 750. This type of deletion was found in 9 tumors. All of the exon 19 deletions occurred in tumor tissue only, and all were heterozygous. In addition, the substitution of G for T at nucleotide 2458 (2458G—T), resulting in amino acid substitution of phenylalanine for valine at codon 738 (V738F), was found in the tumor, adjacent nontumor lung tissue, and the blood sample of 1 patient (Case 27), suggesting it is not an acquired somatic mutation.

In exon 21, five types of missense mutations were observed. A 2819T—G mutation resulted in L858R change in 20 tumors. Notably, four tumors were homozygous for this mutation (Fig. 1C), whereas the others were all heterozygous mutations. The other four missense mutations were L861Q, L833V, H835L, and L838V. Each was found once only, and all were heterozygous mutations. There were 2 tumors (Case 28 and 34) with double mutations in exon 21 (Fig. 1D).

In exon 18, four missense mutations (E709A, E709G, G719S, and G719C) were found, and each occurred in 1 tumor (Fig. 1, E and F). All four patients (Case 29, 30, 38, and 39) with a mutation in exon 18 had another mutation in exon 20 (S768I) or exon 21 (L858R).

In exon 20, 2 missense mutations and two in-frame duplications were found. The S768I mutation was found in 2 tumors (Case 38 and 39). The V769M mutation occurred in 1 tumor (Case 10). This tumor also had an in-frame deletion in exon 19. The two types of duplication patterns are shown in Fig. 2A. Case 36 had a duplication of 12 bp from nucleotide 2530–5 to 2536 (Fig. 2B). The duplicated sequence spanned an intron-exon junction. Because this duplication involved the splice acceptor site of intron 19, reverse-transcriptase-PCR of the tumor RNA followed by sequencing was done. The result showed that the first splice acceptor site was used for the mutant form. The consequence was insertion of 4 amino acids (EAFQ) between codon 761 and 762 (Fig. 2C). Case 37 had a duplication of 9 bp at nucleotide 2549 to 2557, which resulted in insertion of 3 amino acids (SVD).

Although most tumors only have a single mutation in the kinase domain of EGFR gene, combination of two types of mutations were seen in 7 patients (not including Case 27). The details of the mutations are shown in Table 1.

We have additionally analyzed the entire exonic sequences of the EGFR gene in all 39 tumors with mutations and 38 other non–small cell lung cancers (31 adenocarcinomas, 4 squamous cell carcinoma, and 3 adenosquamous carcinoma) without mutations in the tyrosine kinase domain among our 101 patients. We found no additional mutation.

Formalin-Fixed Paraffin-Embedded Tumor Tissue from Patients Treated with Gefitinib

To ascertain the EGFR mutations within the tyrosine kinase domain are associated with responsiveness to gefitinib in Taiwanese lung cancer patients, we also did mutational study in tumors from 16 patients who had received gefitinib treatment as a single agent at Chang-Gung Memorial Hospital. A significant clinical response was defined according to the response evaluation criteria for global trial in solid tumors (25). Among them, 6 patients showed partial response to gefitinib, 2 patients had clinical benefit, 1 patient had serologic response, and 7 patients had no response. In total, 9 patients were considered as responders, and 7 patients were nonresponders. Mutations of EGFR were found in 7 of the 9 responders. The 2 responders with no mutations were the 2 patients with clinical benefit. Only 1 of the 7 nonresponders had mutations. The difference of the mutation rates between the responders and nonresponders is statistically significant (P = 0.041, by χ²/Fisher’s exact tests). The clinical features and the results of mutational study are shown in Table 2.

Totally 10 mutations were found in these 8 patients. Three were in-frame deletions in exon 19 (delE746_A750insA, delE746_A750, and DelL747_A750insP), which were also the most commonly found mutation region in exon 19 in our 101 fresh frozen tumor series. Five mutations were in exon 21. Two were the most common mutation 2819T—G, which resulted in L858R change. Two new mutations in exon 21 (A839T and K846R) were found, which were not seen in our 101 tumor series. Notably, both Patient 2 and Patient 4 had homozygous mutation. Patient 8 was found to have 2 mutations, including deletion in exon 19 (delE746_A750) and missense mutation in...
EGFR Mutations in Non–Small Cell Lung Cancer

Table 1 Clinical and molecular features of the 39 lung cancer patients with EGFR mutations

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Abbreviations: M, male; F, female; y, years; Pathol, pathological diagnosis; ADC, adenocarcinoma; ADSC, adenosquamous carcinoma; A.A., amino acid; del, deletion; ins, insertion; hetero, heterozygous; homo, homozygous; dup, duplication.

* Packs of cigarette per day and duration in years.
† According to the American Joint Committee on Cancer staging system.
‡ (†) represent increased phosphorylated EGFR in tumor compared with adjacent non-neoplastic lung tissue.
§ Case 29 received wedge resection of lung, all other patients received lobectomy.

exon 21(L861Q). This patient had no measurable lesion but had a serologic response with decrease of serum tumor marker (carcinoembryonic antigen) for 50% after gefitinib treatment. Patient 10 was the only nonresponder that had mutations. He had 2 mutations; one was 2564A>G (H773R) in exon 20, and the other was 2439G>A (W731Stop) in exon 19. Both were homozygous mutations.

A diagram showing all of the mutations and the amino acid sequence of the sequenced regions of EGFR discovered from the current study are shown in Fig. 3.

EGFR Phosphorylation Analyses

Immunoblot analyses were done on all of the 39 tumors with EGFR mutations and 38 other non–small cell lung cancers (31 adenocarcinomas, 4 squamous cell carcinoma, and 3 adenosquamous carcinoma) without mutations. The

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results of representative cases are shown in Fig. 4, A and B, for tumors with and without EGFR mutation, respectively. The activation of EGFR is reflected by its tyrosine-phosphorylation status. Twenty-six of the 39 tumors (66.6%) with EGFR mutation showed increased level of phosphorylated EGFR, compared with adjacent non-neoplastic lung tissues. Twenty-two of the 26 tumors also had higher expression of EGFR than adjacent non-neoplastic lung tissue. All of the new mutations identified in this study showed increased level of phosphorylated EGFR in at least one case, except Case 34. For the 38 tumors (31 adenocarcinomas, 4 squamous cell carcinomas, and 3 adenosquamous carcinomas) without EGFR mutation, only 14 (36.8%) revealed increased level of phosphorylated EGFR (12 of the 31 adenocarcinomas, 2 of the 4 squamous cell carcinomas, and none of the 3 adenosquamous carcinomas). Except one, all of these 14 tumors also had higher expression of EGFR in tumor. Furthermore, the expression level of phosphorylated EGFR of these 38 tumors without mutation was lower than the adjacent non-neoplastic tissue in 7 cases. By contrast, only 1 (Case 34) of the 39 tumors with mutation showed a lower phosphorylated-EGFR level than the adjacent non-neoplastic tissue.

**DISCUSSION**

In the current study, we report the results from a molecular analysis of the EGFR gene in 101 lung cancer patients without prior treatment of gefitinib and 16 patients received gefitinib treatment. Among the 101 patients, there were 39 patients (38.6%) carrying EGFR mutations in the tumor. In a manner consistent with the previous reports, the EGFR mutations are tightly associated with the pathology type. Among 69 patients
(33 males and 36 females) with the pathological diagnosis of adenocarcinoma, we detected 38 cases (55.5%) bearing at least one mutation in exons 18 to 21 in the **EGFR** gene. All of the adenocarcinomas carrying **EGFR** mutations were well to moderately differentiated. None of the poorly differentiated adenocarcinoma had the **EGFR** mutation. In the remaining 32 non-adenocarcinoma cases, only one had an **EGFR** mutation, and it occurred in a patient with adenosquamous carcinoma. The positive rate of **EGFR** mutation is much higher than previously reported for patients with adenocarcinoma of lung from the United States (3 to 8%) but comparable with that observed in Japanese patients (57% in female adenocarcinomas; refs. 23, 24).

We have taken a resequencing approach to detect directly any sequence variation in the exons and exon-intron junctions of the genomic region that encodes the kinase domain of **EGFR**. In total, 18 different mutations were observed in the 39 patients, and 12 of them have not been reported previously. The mutation spectrum covers deletion, duplication, and single-nucleotide substitution. Most of the mutations were within exons 19 or 21, which is a finding similar to the two previous reports (23, 24). All of the deletions were observed in exon 19, but we also identified known and new single-nucleotide substitutions in exon 18 and 21. In addition, we discovered new mutations (missense mutations and duplication) in exon 20. In nearly all of the cases, the mutation was acquired in the tumor tissue, as we did not detect mutation in the leukocyte of the same blood sample of the individual. Moreover, the mutations seem to be complex in that 7 tumors carried 2 mutations and that a homozygous mutational status was detected in 4 tumors. Among them, one (Case 30) had homozygous point mutations in both

![Fig. 2 A. Two types of duplication mutation in exon 20. Case 36 had a heterozygous in-frame duplication of 12 bp from nucleotide 2530–5 to 2536. Case 37 had a duplication of 9 bp at nucleotide 2549 to 2557. The duplicated sequence of Case 36 spanned an intron-exon junction. Intronic sequence is in lowercase letters, and exonic sequence in capital letters. B. Sequence tracing of genomic DNA of Case 36. The duplication was confirmed in both strands. C. Sequence tracing of cDNA of Case 36. The nucleotide sequence showed splicing at the first acceptor site and an insertion of 4 amino acids (shown in red) between codon 761 and 762.](image-url)
the cancer cells might be accumulating multiple molecular events in exon 18 and exon 21. These findings would suggest that the gene in these tumor specimens.

Recently, activating mutations in the EGFR gene have been reported to underlie the response to chemotherapy with a kinase inhibitor, gefitinib. A much higher mutation rate was found in Japanese patients with adenocarcinoma. Our study revealed a high mutation rate in the EGFR gene similar to that seen in Japanese patients. This result is highly consistent with an ethnical difference in the responsive rate to gefitinib as shown in Japanese patients. This result is highly consistent with an ethnical difference in the responsive rate to gefitinib as shown in Japanese patients.

We also found no evidence suggesting that the gender might play a role in determining gefitinib treatment response, especially in Japanese patients (16, 21). However, in our analysis of the 101 patients, we did not see significant difference in the EGFR mutation rate between male (18 of 33, 54.5%) and female patients (20 of 36, 55.5%) with adenocarcinoma, and the mutation patterns are also similar between the two groups. Of note, the report by Miller et al. (20) also found no significant difference in the response rate between male and female patients in their study of 139 patients. It remains to be determined whether gender might play a role in determining gefitinib treatment response in patients with EGFR mutation. We also found no evidence suggesting that the gender might play a role in determining gefitinib treatment response, especially in Japanese patients (16, 21).

There were 3 new mutations (delT747_A750InsP, A839T, and K846R) found in the responders. Interestingly, we also found double mutations in 1 responder (both mutations have been reported to be associated with gefitinib responsiveness) and in 1 nonresponder. In the latter, a nonsense mutation (W731Stop) in exon 20 and 1 missense mutation (H773R) in exon 20 were found. Because the stop codon could have resulted in protein truncation and loss of EGFR function, it is understandable why this patient did not respond to gefitinib treatment.

Female patients with adenocarcinoma have been reported to be associated with a higher gefitinib response rate, especially in Japanese patients (16, 21). However, in our analysis of the 101 patients, we did not see significant difference in the EGFR mutation rate between male (18 of 33, 54.5%) and female patients (20 of 36, 55.5%) with adenocarcinoma, and the mutation patterns are also similar between the two groups. Of note, the report by Miller et al. (20) also found no significant difference in the response rate between male and female patients in their study of 139 patients. It remains to be determined whether gender might play a role in determining gefitinib treatment response in patients with EGFR mutation. We also found no evidence suggesting that the gender might play a role in determining gefitinib treatment response, especially in Japanese patients (16, 21).
significant difference in the smoking rate between adenocarcinoma patients with or without mutation.

By immunoblot analysis, we observed EGFR activation, as evidenced by increased EGFR phosphorylation in 26 of the 39 tumors (66.6%) with EGFR mutations. This result suggests that the EGFR mutations in these tumors may enhance their kinase activity. All of the new mutations identified in this study showed EGFR phosphorylation in at least one case, except Case 34, which was unique and had two missense mutations close to each other in exon 21. The functional change caused by these 2 mutations remains to be determined. For the 38 tumors without EGFR mutations, there were only 36.8% of tumors that had increased level of phosphorylated EGFR. As a whole, the EGFR kinase domain mutations seem to have a different effect on kinase activation. The functional significance of this finding requires additional investigation.

We have analyzed the entire exonic sequences of the EGFR gene in all of the 39 tumors with mutations and 38 other non–small cell lung cancers (31 adenocarcinomas, 4 squamous cell carcinoma, and 3 adenosquamous carcinoma) without mutations in the tyrosine kinase domain among our 101 patients. We found no additional mutation. Thus, the EGFR mutations in lung cancer seem to cluster in exons 18 to 21 in a predictable pattern. In the report of Lynch et al. (23), they searched for the EGFR mutations in exon 19 and 21 in primary tumors from breast, colon, kidney, pancreas, brain, and a panel of 108 cancer-derived cell lines, and the result was totally negative. We also did not detect any mutation in exons 18 to 21 of EGFR in 30 pairs of tumor and nontumor liver tissues of hepatocellular carcinomas (data not shown). The tight association with adenocarcinoma and the recurrent pattern of EGFR mutations raise the possibility that these mutations play an important role in the tumorigenesis of adenocarcinoma of lung, especially in East Asians.

Finally, although the previous EGFR studies have revealed
that the clinical response to gefitinib is associated with specific mutations in the EGFR kinase domain, and the finding is supportive of treatment selection based on molecular classification, it is still possible that not all of the patients with EGFR mutation will respond to gefitinib. The nonsense mutation found in 1 of the nonresponders in our series is a good example. Given the wide spectrum and complexity of EGFR mutations shown by this study, it will require additional analysis to investigate whether the new EGRF mutations discovered by this study also confer a similar beneficial effect with the gefitinib treatment. Nevertheless, data from our study would suggest that there should be a higher gefitinib response rate in lung adenocarcinoma patients in Chinese and, possibly, other East Asian populations.

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