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Int. J. Cancer: **118**, 180–184 (2006)
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EGFR and ErbB2 mutation status in Japanese lung cancer patients

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Much evidence has accumulated that the epidermal growth factor receptor *(EGFR)* and its family members are strongly implicated in the development and progression of lung cancers. Somatic mutations of the *EGFR* gene were found in about 25–40% of Japanese lung cancer patients. More recently, *erbB2* mutations are found in about 4% of European-derived lung cancer patients. We have investigated *EGFR* and *erbB2* mutation status in 95 surgically treated nonsmall cell lung cancer (NSCLC) cases from Nagoya City University Hospital. Seventy-five adenocarcinoma cases were included. The presence or absence of *EGFR* and *ernB2* mutations of kinase domains were analyzed by reverse transcription polymerase chain reaction (RT-PCR) amplifications and direct sequences. We have also investigated *erbB2* mutation status in 27 surgically treated NSCLC cases followed by treatment with gefitinib from Kinki-chuo Chest Medical Center. *EGFR* mutations (CTG→CGG; L858R) were found from 14 of 95 lung cancer patients. We also detected the deletion 1a-type mutations from 9 patients and deletion 4-type mutations from 6 patients in exon 19. In exon 20, 4 mutations including 2 novel mutations were found. Total *EGFR* mutations were present in 35 patients (36.8%). These mutation statuses were significantly correlated with gender (women 73.3% *vs.* men 20%, $p < 0.0001$), smoking status (never smoker 69.4% *vs.* smoker 16.9%, $p < 0.0001$), pathologic subtypes (adenocarcinoma 45.1% *vs.* nonadenocarcinoma 12.5%, $p = 0.0089$) and differentiation status of the lung cancers (well 51% *vs.* moderately or poorly 18.4%, $p = 0.0021$). On the other hand, *erbB2* mutation was only found from 1 of 95 patients, at exon 20. This patient was female and a never smoker with adenocarcinoma. This 12 nucleotide insertion mutation (2324–2325 ins ATACGTGATGGC) was located in the exon 20 at kinase domain (775–776 ins YVMA). There was no *erbB2* mutation in 27 gefitinib-treated NSCLC patients. In total, we have found only 1 *erbB2* mutation from 122 (0.8%) Japanese NSCLC patients. There was a significantly higher erbB2 positive (2+/3+) ratio in *EGFR* mutant patients (13/25, 52.0%) compared to *EGFR* wild-type patients (10/62, 16.1%; $p = 0.0247$). The NSCLC specimen with *erbB2* mutation showed 1+ immunoreactivity. The *EGFR* mutation status might correlate with the clinicopathologic features related to good response to gefitinib, such as gender, smoking history and pathologic subtypes of lung cancers. However, *erbB2* mutation is rare from Japanese lung cancer and is of limited value for molecular target therapy.
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Key words: EGFR; lung cancer; mutations; erbB2; Japanese

Lung cancer is a major cause of death from malignant diseases, due to its high incidence, malignant behavior and lack of major advancements in treatment strategy.[1] Lung cancer was the leading indication for respiratory surgery (42.2%) in 1998 in Japan.[2] More than 15,000 patients underwent surgical operations at Japanese institutions in 1998.[2] The clinical behavior of the lung cancer is largely associated with its stage. The cure of the disease by surgery is only achieved in cases representing an early stage of lung cancer.[3]

There is much accumulated evidence that epidermal growth factor receptor *(EGFR)* and its family members are strongly implicated in the development and progression of numerous human tumors, including lung cancer.[4,5] The erbB family comprises 4 structurally related receptors: ErbB1 (EGFR), ErbB2 (HER2-neu), ErbB3 and ErbB4. On ligand stimulation, the receptor forms either homodimers or heterodimers, which activate their cytoplasmic domain. This tyrosine-auto-phosphorylated region functions as a docking site for messenger proteins, which initiate cascades of cytoplasmic and nuclear mitogenic pathways.[6] Inhibition of this pathway is facilitated by several newly developed compounds that have shown promising results in preclinical and clinical trials.[7] The *EGFR* tyrosine kinase inhibitor, gefitinib, was approved in Japan for the treatment of nonsmall cell lung cancer (NSCLC) since 2002. Trastuzumab is a recombinant DNA-derived monoclonal antibody that selectively binds to p185 HER2, the protein product of *erbB2*. Trastuzumab was approved for breast cancer[8] and clinical trials for NSCLC is underway.[9,10]

Recently, we have found that novel *EGFR* mutations' status at ATP binding pockets in Japanese NSCLC patients were correlated with the clinicopathologic features related to good response to gefitinib.[11] These *EGFR* mutations are predominantly found in Japanese lung cancer patients (about 25%) when compared to USA patients (about 8%[12–14] to 10%[15]). Kasaoka *et al.* have reported that the *EGFR* mutation ratio is 40% of Japanese lung cancer patients.[16] Actually, *EGFR* mutations in lung cancer have been correlated with clinical response to gefitinib therapy *in vivo* and *in vitro*.[11–13] More recently, it has been reported that novel *erbB2* mutations at kinase domain were found in 4% of European-derived NSCLC patients.[17]

To determine the *EGFR* and *erbB2* mutation status in Japanese lung carcinoma for screening purposes, we investigated *EGFR* and *erbB2* mutation status by the RT-PCR amplifications and direct sequences. The findings were compared to the clinicopathologic features of lung cancer.

Material and methods

Study subjects

The study group included 95 lung cancer patients who had undergone surgery (but did not receive gefitinib) at the Department of Surgery II, Nagoya City University Medical School between 1997 and 2002. We have also investigated erbB2 mutation status for 27 NSCLC patients who had undergone surgery followed by treatment with gefitinib at the National Hospital Organization, Kinki-chuo Chest Medical Center. The lung tumors were classified according to the general rule for clinical and pathologic record of lung cancer in Japan.[18] All tumor samples were immediately frozen and stored at −80°C until assayed.

The clinical and pathologic characteristics of the 95 lung cancer patients are as follows: 52 cases at stage I, 9 at stage II and 34 at stage III–IV. The mean age was 64.9 years (range, 42–82). Among the 95 lung cancer patients, 71 (74.7%) were diagnosed as adeno-

Grant sponsor: Grant-in-Aid for Cancer Research (16-1) from the Ministry of Health, Labour and Welfare; Grant sponsor: AstraZeneca Research Grant 2004.
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Received 11 March 2005; Accepted after revision 18 April 2005
DOI 10.1002/ijc.21301
Published online 7 July 2005 in Wiley InterScience (www.interscience.wiley.com).

carcinoma, 17 (17.9%) were squamous cell carcinoma and 4 (4.2%) were adenosquamous cell carcinoma. The samples from these patients had never been sequenced for *EGFR* before.

PCR assays for EGFR and erbB2

Total RNA was extracted from lung cancer tissues and adjacent nonmalignant lung tissues using 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. About 10 cases were excluded because tumor cells were too few to sufficiently extract tumor RNA. 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, Piscataway, NJ). 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 LA-Taq kit (Takara Bio, Shiga, Japan) in a 25 μl reaction volume. The primer sequences for *EGFR* gene for kinase domain (exons 18–21) were as follows: the forward primer, 5-CTCTTACACCCAGTGGAGAA-3 and the reverse primer, 5-CATCCACTTGATAGGCACTT-3 (572 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, 72°C for 45 sec The primer sequences for *erbB2* gene for kinase domain (exons 19–22) were as follows: the forward primer, 5-CGCTTTTGGCACAGTCTACA-3 and the reverse primer, 5-GGGATCCCATCGTAAGGTTT-3 (594bp). 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, 72°C for 45 sec. The products were purified by Qiagen PCR purification kit (Qiagen, Valencia, CA). 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 by ABI prism 3100 analyzer (Applied Biosystems Japan, Tokyo, Japan) and analyzed by BLAST and chromatograms by manual review.

Immunohistochemistry

Tissue blocks were cut into 4 mm sections and mounted on silane-coated slides. The slides were then deparaffinized in xylene, dehydrated in a grade alcohol series and blocked for endogenous peroxidase with 3% H_2O_2 in absolute methanol. After microwave pretreatment in Blockace solution, immunostaining was done at 4°C overnight with a rabbit polyclonal c-erbB2 oncoprotein antibody (A04085, DakoCytomation, Glostrup, Denmark) at a 1:200 dilution. The expression of erbB2 was scored as follows: −, no discernible staining or <10% of cell stained; 1+, >10% of cytoplasmic staining or plasma membrane staining with weak intensity; 2+, >10% of plasma membrane staining with moderate intensity; and 3+, >10% of plasma membrane staining with strong intensity.

Statistical methods

Statistical analyses were done using the Mann-Whitney U-test for unpaired samples and Wilcoxon's signed rank test for paired samples. Linear relationships between variables were determined by means of simple linear regression. Correlation coefficients were determined by rank correlation using Spearman's test and χ^2test. The overall survival of lung cancer patients was examined by the Kaplan-Meier methods, and differences were examined by the log-rank test. All analysis was done using the Stat-View software package (Abacus Concepts, Berkeley, CA) and was considered significant when the p-value was less than 0.05.

Results

EGFR gene mutation status in Japanese lung cancer patients

Using the primer sets for *EGFR* kinase domain, a PCR product of 572 bp was obtained. When we visualized the PCR products

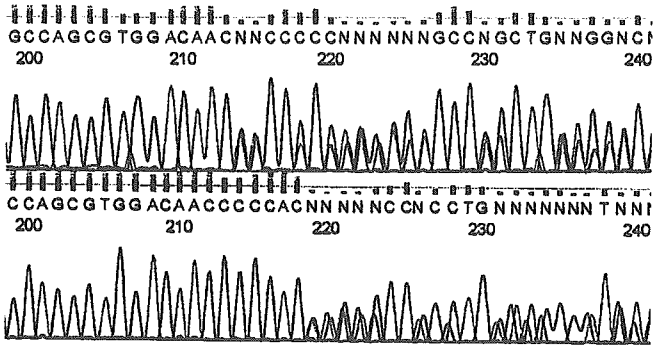

FIGURE 1 – Novel EGFR mutation at exon 20. Top: a male well-differentiated adenocarcinoma patient had the novel 2312–2313 insertion CAA. Bottom: a female, well-differentiated adenocarcinoma patient had the novel 2319–2320 insertion AACCCCCAC.

with 1% agarose gel, these samples were further studied. In exon 18, there was no G719S mutation found from this study. In exon 19, 9 patients had the del 1a type mutation, 6 patients had the deletion 4 type mutation and 1 patient had the del 1b type mutation. Seven were male and 10 were female. Thirteen were nonsmokers and 4 were smokers. Fifteen patients had adenocarcinoma, 1 had squamous cell carcinoma and 1 had adenosquamous cell carcinoma. Three of the tumors were moderately differentiated, 2 were poorly differentiated and 11 were well differentiated. Five of 15 adenocarcinomas showed bronchioloalveolar carcinoma (BAC) pattern at the edge of tumor. Thus *EGFR* mutation status at exon 19 was significantly correlated with gender ($p = 0.0172$) and tobacco-smoking ($p = 0.0008$) but not with pathologic stages (stage I *vs.* II–IV, $p = 0.9144$), subtypes (adenocarcinoma *vs.* nonadenocarcinoma, $p = 0.2675$) and differentiation of lung cancer (well *vs.* moderately or poorly differentiated, $p = 0.3812$).

In exon 20, 3 patients had theheterozygous in-frame insertion mutations. Two were male and 1 was female. All 3 were smokers. A female, well-differentiated adenocarcinoma patient had the novel 2319-2320 insertion AACCCCCAC. A male well-differentiated adenocarcinoma patient had the novel 2312-2313 insertion CAA (Fig. 1). We have found one point mutation, C2369T (T790M). This patient also has the predominant L858R mutation (Fig. 2).

For exon 21, 14 patients had the L858R mutation and 1 patient had the L861Q mutation. Four were male and 11 were female. Twelve were nonsmokers and 3 were smokers. All 15 patients had adenocarcinoma, 1 was moderately differentiated and 14 were well differentiated. Six of 15 adenocarcinomas exhibited the BAC pattern at the edge of the tumor. Thus, exon 21 mutation status was significantly correlated with gender ($p = 0.0005$), smoking status ($p = 0.0007$), pathologic stages ($p = 0.0152$), the pathologic subtypes ($p = 0.0329$) and differentiation of lung cancer ($p = 0.0033$).

The mutations detected in lung cancer specimens from 95 lung cancer patients are summarized in Table I. Taken together, 36 mutations were found from 35 lung cancer samples in our analysis. Total *EGFR* mutations were present in 35 patients (36.8%). These mutation statuses were significantly correlated with gender (women 73.3% *vs.* men 20%, $p < 0.0001$), smoking status (never smoker 69.4% *vs.* smoker 16.9%, $p < 0.0001$), pathologic subtypes (adenocarcinoma 45.1% *vs.* nonadenocarcinoma 12.5%, $p = 0.0089$) and differentiation status of the lung cancers (well 51% *vs.* moderately or poorly 18.4%, $p = 0.0021$).

The overall survival of 95 lung cancer patients from Nagoya City University, with follow-up through December 30, 2003, was studied in reference to the *EGFR* mutation status. The patient with the mutation in the *EGFR* gene ($n = 35$, 4 were dead) had a significantly better prognosis than the patient with wild-type *EGFR* ($n = 60$, 20 were dead; log-rank test $p = 0.0143$, Breslow-Gehan-Wilcoxon test $p = 0.0220$), although the observation period was short (Fig. 3). But a multivariate analysis revealed that pathologic

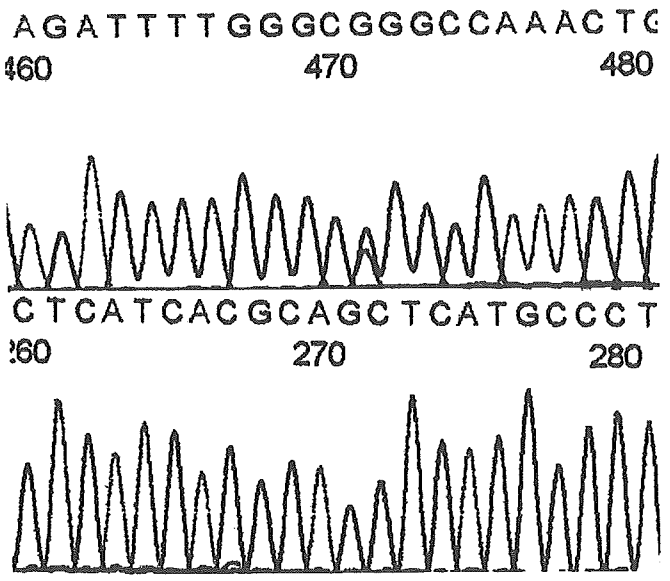

FIGURE 2 – The premoninant L858R (2573 T to G) mutation in exon 21 (top) and T790M (2369 C to T) mutation at exon 20 (bottom) within the EGFR kinase domain.

TABLE I – CLINICOPATHOLOGIC DATA OF 95 LUNG CANCER PATIENTS

Factors	*EGFR* gene status		
	Mutation patients	Wild-type patients	*p*-value
Mean age (years)			
64.9 ± 9.0	35	60	
Stage			
I	25 (72.4%)	27 (45.8%)	0.0274
II–IV	10 (28.6%)	32 (54.2%)	
Lymph node metastasis			
N0	8 (22.9%)	21 (35.0%)	0.3119
N+	27 (77.1%)	39 (65.0%)	
BI			
Never smoker	25 (71.4%)	11 (34.0%)	0.001
Smoker	10 (28.6%)	49 (66.0%)	
Differentiation			
Well	26 (78.8%)	23 (42.6%)	0.0021
Moderately or poorly	7 (21.2%)	31 (57.4%)	
Pathologic subtypes			
Adeno	32 (91.4%)	39 (74.7%)	0.0089
Nonadeno	3 (8.6%)	21 (25.3%)	
Age			
≤ 65	19 (54.3%)	29 (48.3%)	0.7269
> 65	16 (45.7%)	31 (51.7%)	
Gender			
Male	13 (37.1%)	52 (86.7%)	< 0.0001
Female	22 (62.9%)	8 (13.3%)	

N+, lymph node metastasis positive; Adeno, adenocarcinoma.

stage (p = 0.0006) was the only significant factor but not *EGFR* mutation (p = 0.1824).

ErbB2 *gene mutation status in Japanese lung cancer patients*

We identified only one *erbB2* mutation from 95 NSCLC patients. This 12-nucleotide insertion mutation (2324-2325 ins ATACGTGATGGC) was located in the exon 20 at kinase domain (775-776 ins YVMA) (Fig. 4). This patient was a female non-smoker with well-differentiated adenocarcinoma, without *EGFR* mutation. Adjacent normal lung tissue exhibited a wild-type sequence for the *erbB2* gene, suggesting that this mutation was somatic. We have also done sequencing for 27 gefitinib-treated NSCLC patients. Among 27 patients, 9 patients had *EGFR* mutations (data not shown). However, no *erbB2* mutation was found

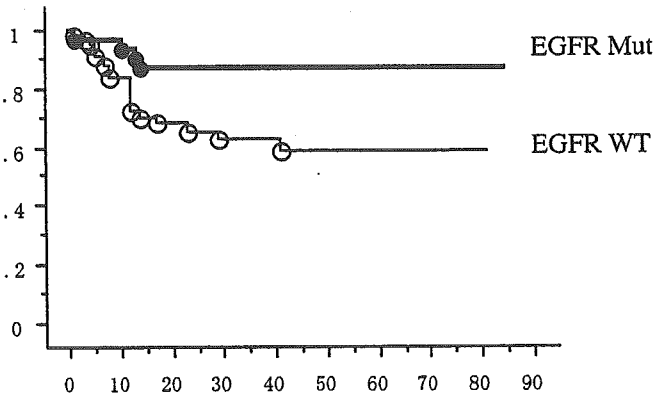

FIGURE 3 – The patient with a mutation in the *EGFR* gene (n = 35, 4 were dead) had a significantly better prognosis than the patient with wild-type *EGFR* (n = 60, 20 were dead) (log-rank test, p = 0.0143; Breslow-Gehan-Wilcoxon test, p = 0.0220), although the observation period was short.

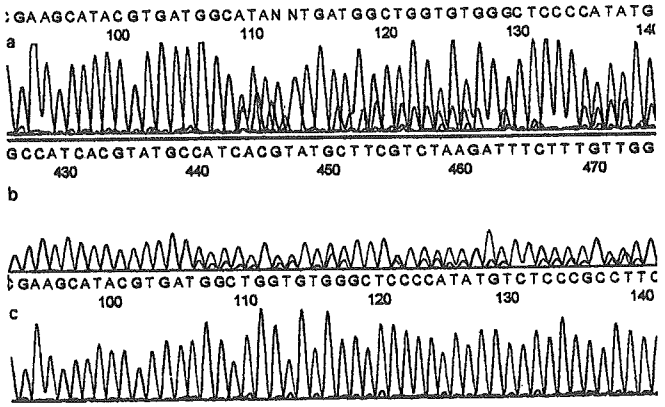

FIGURE 4 – Detection of the insertion mutation in the *erbB2* gene in genomic DNA extracted from lung cancer. *(a)* The 12 nucleotide insertion mutation (2324–2325 ins ATACGTGATGGC) was located in the exon 20 at kinase domain (775–776 ins YVMA). *(b)* Reverse sequence was performed and confirmed. *(c)* Adjacent normal lung tissue showed a wild-type sequence for the *erbB2* gene.

within the kinase domain. Totally, we have found only 1 *erbB2* mutation from 122 (0.8%) Japanese NSCLC patients.

Immunohistochemistry

The immunohistochemical evaluation was done according to the scoring system described in Material and Methods. Immunohistochemistry was done only for 87 patients because the tissue blocks were not available for other patients. The erbB2-positive (2+/3+) ratio was 26.4% (23/87). There was a significantly higher erbB2-positive ratio in *EGFR*-mutant patients (13/25, 52.0%) compared to *EGFR* wild-type patients (10/62, 16.1%) (p = 0.0247). The patient with *erbB2* mutation exhibited 1+ immunoreactivity (Fig. 5).

Discussion

We obtained findings that *EGFR* mutation status was significantly correlated with gender and smoking history of lung cancers. This was in agreement with the recent reports that *EGFR* gene mutations are common in lung cancers from never smokers[13,14] and females with adenocarcinoma.[11,14] However, our analysis also suggested that *erbB2* mutation might be less common in Japanese NSCLC patients.

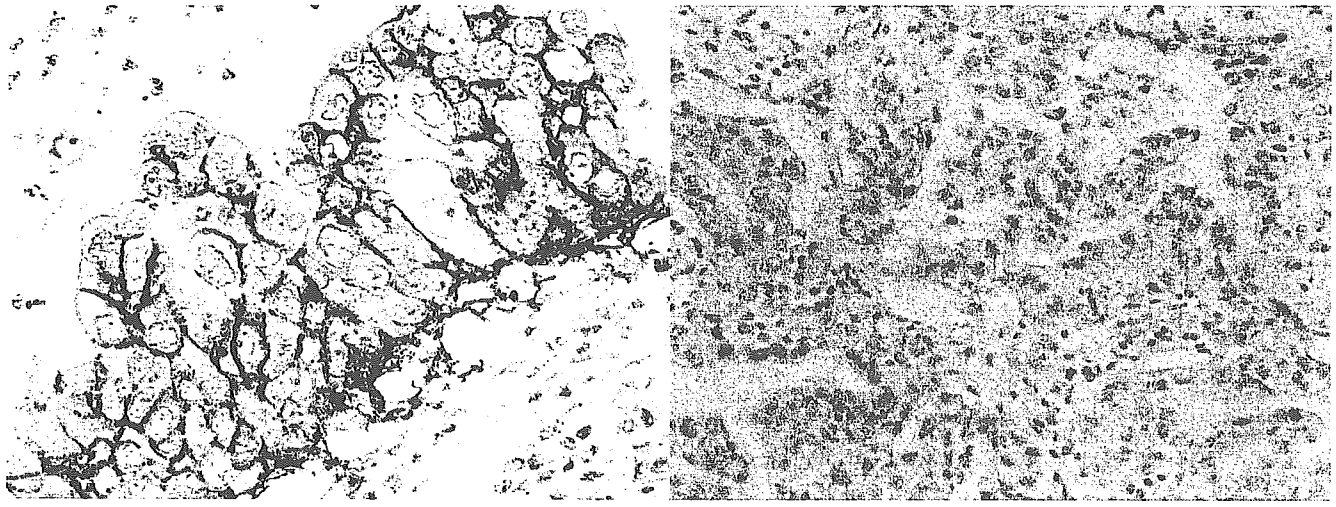

FIGURE 5 – Immunohistochemistry for ErbB2. Left: erbB2-positive (3+) section. Right: the NSCLC specimen with *erbB2* mutation exhibited 1+ immunoreactivity.

Overexpression of EGFR/ErbB2 and ErbB ligands is correlated with advanced diseases and poor patient prognosis.[19] Although *EGFR* is more abundantly expressed in lung carcinoma,[20,21] *erbB2* overexpression is less common; it is found in <35% of patients with nonsmall cell lung cancers, mainly in those with adenocarcinoma.[21] Amplification of *EGFR* and *erbB2* mRNA[22] or overexpression of their proteins[23] has been found to relate to survival in patients with NSCLC, although contradictory results have also been reported.[24,25] The drug trastuzumab, a humanized antibody against the extracellular domain of erbB2, has been approved for treatment of metastatic breast cancer and is most effective in breast cancer with *erbB2* amplification. Preliminary results suggested that the combination of chemotherapy and trastuzumab is well tolerated for NSCLC.[21] However, results from phase II trials of trastuzumab as a treatment for NSCLC have not shown any advantage for most patients[22] and have provided insufficient evidence to proceed to phase III trials.[23] Because the presence of a mutation appears to be a determination of response to therapy, as is the case with gefitinib and *EGFR* mutations, we therefore investigated the *erbB2* and *EGFR* gene mutation status. However, we have found only 1 *erbB2* mutation from 122 Japanese lung cancer patients. More recently, Shigematsu *et al.* reported that *erbB2* mutations were found in 3% (8/269) of Japanese NSCLC.[26] The single *erbB2* mutation we have found was the same as the one repeatedly found by Shigematsu *et al.*[26] Because very few NSCLC patients have gene amplification of *erbB2*, trastuzumab in the treatment of NSCLC might have a limited role.[9] Lung cancers that coexpress both EGFR and erbB2 appear to have more virulent behavior.[27] In addition, EGFR-erbB2 heterodimers are associated with a stronger and more sustained proliferative signal than EGFR homodimers.[22,28] Blockade of a signaling pathway may in theory be overcome by compensatory activation of a separate pathway in the same tumor cell. Because there was a significantly higher erbB2-positive ratio in *EGFR*-mutant patients, blockade of both may ultimately yield superior results.

Because so many *EGFR* mutation phenotypes were discovered, it would be of interest to determine whether resistance to *EGFR* inhibition emerges through secondary mutation as is the case in imatinib-treated chronic myelogenous leukemia.[29] In our analysis, a female never smoker adenocarcinoma patient had the predominant L858R mutation as well as T790M mutation. Actually, this case was untreated with EGFR kinase inhibitors. Threonine 315 to isoleucine substitution in the Abl kinase domain was a critical structural determinant controlling inhibitor sensitivity of STI571.[29] Introduction of bulkier hydrophobic side chains at the Thr-790 position fully preserved the cellular kinase activity of the *EGFR* in the presence of selective kinase inhibitors, indicating potential mechanisms of molecular resistance formation as previously found for BCR-Abl at T315I. Previous *in vitro* study showed that mutation of T790M in the *EGFR* revealed a hotspot for resistance formation against gefitinib,[30] also *in vivo*.[31]

Over the decades, the incidence of lung adenocarcinoma has increased worldwide. Most individuals with lung adenocarcinoma (especially women) are nonsmokers,[32] who are corresponding with the sensitive population to gefitinib. In Taiwan, *EGFR* mutation ratio from adenocarcinoma was also high (55%, 38 of 69), and all of the adenocarcinomas with *EGFR* mutation were well to moderately differentiated.[33] These data were compatible for our results. Because well-differentiated adenocarcinoma patients had a better prognosis,[34] *EGFR* mutant patients showed better prognosis in our univariant analysis. The reason why many mutations were especially found in Asian, female nonsmoker adenocarcinoma remains unknown. Human papilloma virus type 16/18 infections,[35] cooking oil fume,[36] nutritional status, genetic susceptibility, immunologic infection, tuberculosis and asthma[32] have been investigated as causes of lung cancer occurring in nonsmoking women.

The findings of the breakdown of *EGFR* mutations among the 3 exons were interesting. The exon 21 mutations correlated with pathologic stage and subtype, unlike mutations in exon19. Since exon 21 mutations are more closed to the activation loop of *EGFR*, these may be more correlated with gefitinib sensitivity. Especially since 3 patients with exon 20 mutations were smokers, all of the mutations might not be equally correlated with sensitivity for gefitinib.

Acknowledgements

The authors thank Mr. N. Hosono, Mr. R. DeBiasi and Dr. J. Lee for their excellent technical assistance.

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