EGFR T790M Mutation

A Double Role in Lung Cancer Cell Survival?

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Abstract: Even though lung cancer patients harboring a mutation in the epidermal growth factor receptor (*EGFR*) gene exhibit an initial dramatic response to EGFR tyrosine kinase inhibitors (EGFR-TKIs), acquired resistance is almost inevitable after a progression-free period of approximately 10 months. A secondary point mutation that substitutes methionine for threonine at amino acid position 790 (T790M) is a molecular mechanism that produces a drug-resistant variant of the targeted kinase. The T790M mutation is present in about half of the lung cancer patients with acquired resistance, and reported to act by increasing the affinity of the receptor to adenosine triphosphate, relative to its affinity to TKIs. Nevertheless, several lines of evidence indicate that the T790M mutation confers growth advantage to cancer cells, and it was shown that mice expressing tetracycline-inducible EGFR transgenes harboring the T790M mutation develop lung tumors. Thus, T790M mutation seems to play a double role in the survival of lung cancer cells. Several second-generation EGFR-TKIs are currently being developed to overcome the acquired resistance caused by the T790M mutation. *MET* (met proto-oncogene) amplification or activation of IGF1R are reported as alternative mechanisms for acquired resistance to EGFR-TKIs. Clarification of the pathways leading to acquired resistance is essential to maximize the efficacy of EGFR-TKI therapy for patients with lung cancer.

Key Words: Molecular target therapy, *EGFR* mutation, Acquired resistance, EGFR tyrosine kinase inhibitor.

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Gefitinib and erlotinib are low molecular-weight epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKI) that mimic adenosine triphosphate (ATP). Both drugs reversibly and specifically inhibit EGFR in a competitive fashion. About 70 to 80% of non-small cell lung cancers harboring a somatic mutation in the tyrosine kinase domain of the *EGFR* gene respond to gefitinib/erlotinib, whereas only 10% of tumors without this mutation are responsive to these drugs;[1–3] however, acquired resistance to EGFR-TKI therapy almost always develops after a median of approximately 10 months from the onset of treatment, even in patients who exhibited initial dramatic responses.[4]

A secondary point mutation in the *EGFR* tyrosine kinase domain that substitutes methionine for threonine at amino acid position 790 (T790M) has been described as a mechanism to explain the acquired resistance to TKIs.[5,6] In this review, we discuss the role of the T790M mutation in the mechanism of resistance to EGFR-TKI therapy and in oncogenesis.

EGFR and Downstream Signaling Cascades

Upon binding to its ligands, EGFR forms homo- or heterodimers with other ERBB (erythroblastic leukemia viral oncogene homolog avian) receptors; in addition, tyrosine residues within the cytoplasmic domain are phosphorylated and downstream signaling cascades are activated. These include the phosphatidylinositol-3-kinase (PI3K)-Akt pathway (Figure 1 *A*) or the STAT pathway, which are mainly associated with cell survival, and the RAS-RAF-MAPK pathway, which is mainly associated with cell cycle progression.[7,8]

Somatic activating mutations in the *EGFR* genes were described in 2004, mainly in patients with lung adenocarcinoma who were female, never-smokers, and of Asian ethnicity.[1,2] *EGFR* mutation usually occurs in the first 4 exons of the tyrosine kinase domain, and a deletion involving 5 amino acids (codons 746–750) together with a point mutation at codon 858 (L858R) account for 90% of all *EGFR* mutations.[3] EGFRs harboring these mutations are constitutively activated without ligand binding and cancer cells harboring this mutation become highly dependent on the EGFR pathway, a state often referred to as "oncogene addiction."[9,10] It is reported that the PI3K-Akt signaling pathway is mainly activated in EGFR-mutant cells where ERBB3 acts as a dimer partner of EGFR, and the down-regulation of this pathway is required for the process of gefitinib-induced apoptosis in these cells.[11] In addition, mutant EGFR kinases have a higher affinity to EGFR-TKIs (Figure 1*B*)[12]; therefore, patients with lung cancer harboring *EGFR* mutations often exhibit a dramatic response to EGFR-TKIs.

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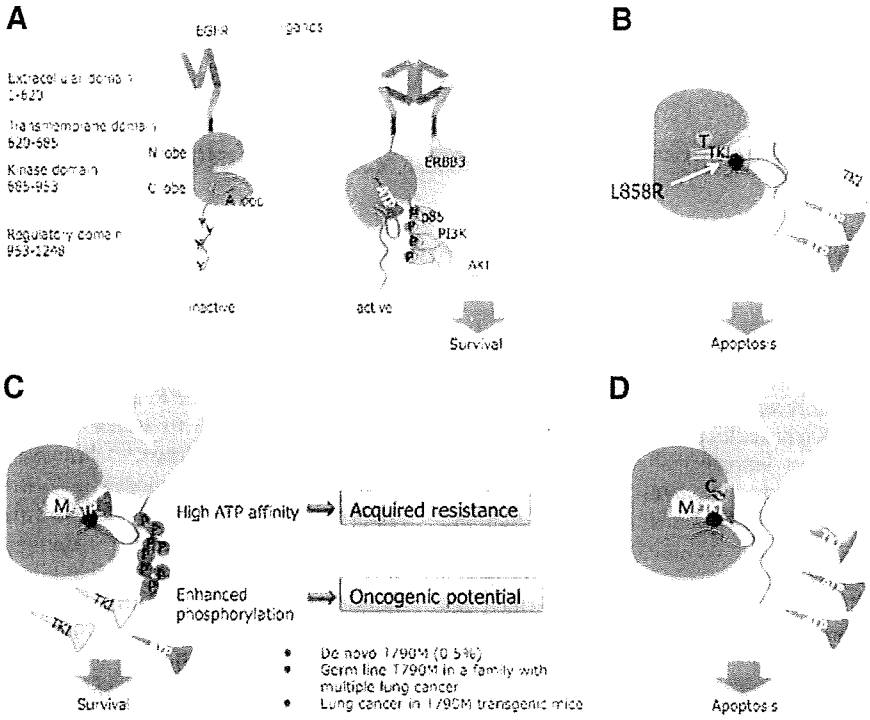

FIGURE 1. The T790M mutation plays a double role in lung cancer cell survival. *A*, In the inactive conformation of the epidermal growth factor receptor (EGFR), the activation loop (A-loop) precludes the binding of peptide substrate. When the specific ligands bind the extracellular domain, EGFR dimerizes with other members of the ERBB family (ERBB3 in this scheme) in a tail-to-head fashion.[35] The C lobe of the kinase domain plays a role analogous to that of cyclin in activated cyclin-dependent kinase (CDK)/cyclin complexes.[35] The A-loop becomes extended to allow peptide substrate binding (active conformation), resulting in phosphorylation of tyrosine residues in regulatory domains. Phosphorylated tyrosine residues serve as docking sites for adaptor molecules that facilitate downstream signaling pathways, the most significant of which is the phosphatidylinositol-3-kinase (PI3K)-Akt pathway. *B*, *EGFR* mutation (L858R in this case) also promotes formation of active conformation. Gefitinib or erlotinib competitively inhibit binding of ATP to the EGFR kinase, resulting in inhibition of phosphorylation and downstream signaling. Gefitinib binds 20-fold more tightly to the L858R mutant than to the wild-type enzyme.[36] *C*, When threonine 790 is substituted by methionine (T790M), the ATP affinity of the oncogenic L858R mutant is increased by more than an order of magnitude, leading to resistance to gefitinib/erlotinib.[18] The T790M mutation also possesses enhanced phosphorylating activity, especially in combination with the L858R mutation. Several lines of evidence indicate that the T790M mutant is actually an oncogene. *D*, An irreversible EGFR-tyrosine kinase inhibitor (TKI) forms a covalent bond at cysteine 797 (C) even when the T790M mutation is present, and thus is able to inhibit the T790M mutant kinase.

T790M Mutation as a cause of Acquired Resistance to TKI Treatment

Patients with lung cancer who show an initial dramatic response to EGFR-TKI almost always acquire resistance to the drug after a progression-free period of approximately 10 months.[4] A 2002 report described a secondary mutation in chronic myeloid leukemia patients that substitutes isoleucine for threonine at codon 315 of the *ABL* gene and causes acquired resistance to imatinib.[13] Since the T315 of ABL corresponds to the T790 of EGFR, based on amino acid homology, researchers investigated whether an artificial T790M mutation conferred resistance to gefitinib and showed that this was the case.[14] Based on this report, 2 groups of investigators confirmed that the T790M mutation is present in patients who develop acquired resistance to EGFR-TKI treatment in 2005.[5,6] We and others showed that the *EGFR* T790M mutation, in *cis* with the primary activating mutation, occurs in approximately 50% of patients with acquired resistance to EGFR-TKI treatment.[15,16] Nomura et al.[17] showed that the *EGFR* mutations were found to favor the shorter allele of polymorphic CA dinucleotide repeat in intron one of the *EGFR* gene, and this or another genetic factor can be the reason that the acquired T790M mutation occurs in *cis*, in addition, secondary somatic activating mutation occurs in *cis* with the inherited T790M mutation discussed below.

The T790 in EGFR is located at a key position in the ATP binding cleft, often referred to as the "gatekeeper residue." Initially, it was thought that the larger methionine residue caused steric hindrance to the binding of EGFR-TKI[5,6]; however, it is difficult to explain why structurally similar, irreversible EGFR-TKI is able to overcome the T790M mutation, as discussed later. A recent analysis showed that the T790M mutant retains affinity to gefitinib; i.e., the T790M-mutant EGFR kinase binds gefitinib with a Kd of 4.6 nM, nearly as tightly as the L858R mutant (Kd = 2.4 nM).[18] In contrast, introduction of the T790M mutation

increases the ATP affinity of the oncogenic L858R mutant by more than an order of magnitude.[18] The authors of this report claim that increased ATP affinity is the primary mechanism by which the T790M mutation confers drug resistance (Figure 1*C*).[18]

The T790M Mutation as an Oncogenic Agent

We previously reported two examples of surgically-treated patients who carry both the T790M and L858R mutations, among 397 patients with *EGFR* mutations (0.5%) who had never been exposed to EGFR-TKI treatment[19]; one of the patients showed inherent resistance to gefitinib when she was treated with this drug after tumor recurrence.[19] Others reported a family with multiple cases of lung cancer associated with germ line transmission of the T790M mutation, and four of the six tumors analyzed showed a secondary somatic activating *EGFR* mutation (either L858R, del L747-T751, or G719A) occurring in *cis* with the germ line T790M mutation[20]; however, the T790M mutation was never found among 237 lung cancer family probands.[21] These results suggest that the T790M mutation not only confers resistance to EGFR-TKIs, but also grants growth advantage to cancer cells (Figure 1*C*).

Although it was initially reported that the kinase activity of the EGFR T790M mutant was indistinguishable from wild-type EGFR,[5,6,14] Mulloy et al. showed that the T790M mutant exhibits tyrosine phosphorylation levels comparable to wild-type EGFR, whereas the T790M/L858R double mutant exhibits a substantial increase in phosphorylation, compared with the L858R mutant alone. Thus, the T790M resistance mutation, when combined with activating EGFR kinase domain mutations, confers a significant enhancement of its catalytic phosphorylating activity, which suggests that these mutations cooperate to produce a more potent kinase. This may potentially explain the additional role of the T790M mutation in predisposing to tumorigenesis.[22] Vikis et al.[21] further indicated that the T790M mutation alone leads to increased phosphorylation levels. A human bronchial epithelial cell line overexpressing EGFR carrying the T790M mutation displayed a growth advantage over wild-type EGFR.[21]

Animal models were generated to inducibly express the T790M mutation, alone or together with the L858R mutation, in type II pneumocytes that develop lung adenocarcinomas. Mice expressing the T790M mutation alone develop tumors with longer latency than those expressing both the T790M and L858R mutations.[23] In contrast to what is observed in tumors of patients carrying human germ line T790M mutations (discussed above), no additional kinase domain mutations were detected in the tumors of these mice.[23] These results indicate that the T790M mutation is not only a cause of resistance to gefitinib/erlotinib but is also an oncogenic mutation that confers growth advantage to cancer cells. Its oncogenic potential is maximized when the mutation arises in combination with other common *EGFR* activating mutations (Figure 1*C*).

Strategies to Overcome the Resistance Conferred by the T790M Mutation

Since the T790M mutation confers resistance to gefitinib/erlotinib by increasing the affinity of EGFR to ATP, relative to that of EGFR to TKIs,[18] it is possible to overcome the resistance caused by this mechanism by developing a novel class of EGFR-TKIs that have a higher affinity for the T790M kinase, when compared with the affinity of ATP for the mutant kinase. Several kinds of so-called second generation TKIs are currently in various stages of development. BIBW2992,[24] PF00299804,[25] and HKI-272[26] are examples of this new type of EGFR-TKI and belong to the class of irreversible TKIs that covalently bind the sulfhydryl group of cysteine 797 at the catalytic pocket of EGFR (Figure 1*D*); however, Yun et al.[18] indicate that irreversible binding is not required for effective inhibition of the T790M mutant: a reversible inhibitor that binds EGFR-T790M with an affinity sufficient to compete with ATP should be as effective. Accordingly, the XL647 is reported to inhibit the T790M EGFR mutant, even though this compound is a reversible TKI.[27] Nevertheless, it should be noted that one of the acquired resistance mechanisms was also the T790M mutation in the cell culture model of acquired resistance to an irreversible EGFR-TKI, HKI-272.[28] This observation seems to be somewhat puzzling, but it reflects the fact that HKI-272 can overcome T790M only at high doses (approximately 1 μM), but not at clinically achievable concentrations (approximately 0.2 μM).[28]

Inhibition of the heat shock protein 90 (HSP90) is also effective in inhibiting the T790M mutant.[29] Addition of rapamycin (an inhibitor of mammalian target of rapamycin [mTOR]) to irreversible TKI potentiates the antitumor effect in the L858R/T790M mouse model.[26]

MET Amplification and Other Mechanisms for Acquired Resistance to TKI Treatment

Over 50 secondary mutations of the *ABL* gene are reported in acquired resistance to imatinib in chronic myeloid leukemia[30]; however, the *EGFR* D761Y and L747S mutations are the only two other rare examples of secondary mutations associated with acquired resistance to gefitinib (other than T790M).[15,31]

In 2007, Engelman et al.[32] reported amplification of *MET*, a receptor tyrosine kinase for hepatocyte growth factor, as another mechanism of resistance to EGFR-TKIs. The authors isolated gefitinib-resistant clones from HCC827 lung cancer cells (*EGFR* exon 19 deletion and amplified) by exposing the cells to increasing concentration of gefitinib. The resistant cells maintained activation of the ERBB3/PI3K/Akt antiapoptotic pathway in the presence of gefitinib. The resistant cells but not parental cells harbor *MET* amplification by a factor of 5 to 10, and inhibition of MET by specific TKI restores gefitinib sensitivity. They concluded that *MET* amplification activates the PI3K/Akt pathway through ERBB3 activation.[32] *MET* amplification is present in about 20% of patients with acquired resistance but only in 3% of untreated patients.[32,33] Interestingly, *MET* amplification sometimes coexists with the T790M mutation,[32,33] and one patient is reported to have two independent resistant tumors;

one of which had the T790M mutation, whereas the other had *MET* amplification.[32] More recently, it was shown that hyperphosphorylation of the insulin-like growth factor I (IGF-I) receptor (IGF-IR) and constitutive association of IRS-1 with PI3K through loss of expression of IGF binding protein in gefitinib-treated cells is another mechanism of acquired resistance.[34]

The mechanisms underlying acquired resistance to gefitinib/erlotinib treatment are still unknown in more than 30% of cases. Further clarification of the pathways leading to acquired resistance is essential to maximize the efficacy of EGFR-TKI therapy in patients with non-small cell lung cancer.

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Activation of MET by Gene Amplification or by Splice Mutations Deleting the Juxtamembrane Domain in Primary Resected Lung Cancers

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Introduction: MET (Met proto-oncogene) activation either by gene amplification or mutation is implicated in various types of human cancers. For lung cancer, *MET* gene amplification is reported to occur in a subset of adenocarcinomas. Although somatic mutations of *MET* in lung adenocarcinomas are rare, all but one of those reported so far entail a splice mutation deleting the juxtamembrane domain for binding the c-Cbl E3-ligase; normally such binding leads to ubiquitination and receptor degradation, and loss of this domain leads to MET activation. The purpose of this study was to clarify in the role of MET activation in lung carcinogenesis.

Materials and Methods: *MET* gene copy number was determined by real-time quantitative polymerase chain reaction in 187 of the patients with lung cancer and the *MET* gene splice mutation deleting the juxtamembrane domain was examined by direct sequencing in 262. The results were correlated with various clinical and pathologic features including mutations of the epidermal growth factor receptor, *KRAS*, and *HER2* genes.

Results: All the instances of MET activation occurred in patients with adenocarcinomas. The prevalences of *MET* gene amplification and splice mutations were 1.4% (2 of 148) and 3.3% (7 of 211), respectively. We identified four different intronic mutations that disrupted a splice consensus sequence in genomic DNA. Activation of MET and mutations of the epidermal growth factor receptor, *KRAS*, and *HER2* genes had strict mutual exclusionary relationships.

Conclusions: About 5% of pulmonary adenocarcinomas in this cohort of Japanese patients were driven by activated MET by gene amplification or splice mutations. Such patients would be candidates for targeted therapy against MET.

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MET (met proto-oncogene) is a receptor tyrosine kinase that phosphorylates several tyrosine residues after binding its specific ligand, hepatocyte growth factor. Receptor phosphorylation activates downstream signals, including phosphatidyl inositol 3-kinase, mitogen-activated protein kinase, and phospholipase C-γ and leads to epithelial-mesenchymal transition, cell scattering, angiogenesis, proliferation, enhanced cell motility, invasion, and metastasis.[1,2]

Accumulating evidence suggests that MET plays an important role in the pathogenesis of human lung cancer. Amplification and overexpression of the *MET* gene (located at 7q31) is reported to occur in a subset of patients with pulmonary adenocarcinomas that do not harbor mutation of the *EGFR* (epidermal growth factor receptor) gene.[3] Lung and gastric cancer cell lines with *MET* gene amplification has been reported to lead to enhanced phosphorylation of the *MET* gene.[4,5] Furthermore, *MET* gene amplification could be detected in about 20% of patients with lung adenocarcinomas who developed acquired resistance to EGFR TKI.[6,7]

In Caucasian patients with lung adenocarcinoma, mutations of the *MET* gene have been reported to occur in 7.1% (9 of 127)[8] and 12.1% (4 of 33).[9] When confined to somatic mutations, the prevalences have been reported to occur in 1.6% (2 of 127)[8] and 6.0% (2 of 33).[9] Of these 4 somatic mutations, 3 were splice mutations that resulted in deletion of exon 14 coding for the juxtamembrane domain. Another was a point mutation in an extracellular semaphorin domain (L229F). Mutations in the kinase domain were not detected in any lung adenocarcinomas (0 of 160).[8,9] On the contrary, all the mutations found in sporadic papillary-type renal cell carcinomas (17 of 129; 13%), childhood hepatocellular carcinomas (3 of10; 30%), and head and neck squamous cell carcinomas (4 of 15; 27%) occurred within the tyrosine kinase domain.[10–13] This fact contrasts strongly with other types of cancer.

Tyrosine 1003 in the juxtamembrane domain of MET is a binding site for c-Cbl, an ubiquitin protein ligase (E3), which causes ubiquitination, receptor endocytosis, and degradation of MET.[14] Therefore, deletion of the juxtamembrane domain (exon

14) is one mechanism for MET activation, as shown by Kong-Beltran et al.[9] The mutant *MET* exhibits decreased ubiquitination and delayed down-regulation correlating with elevated, distinct MET production.[9] Therefore, phospho-MET levels and downstream mitogen-activated protein kinase activation are sustained following ligand stimulation.[9]

Because the mutational frequencies of the *EGFR* or *KRAS* genes differ markedly between Japanese and Western patients, it would be of interest to determine the prevalence of MET activation by gene amplification or mutation in a Japanese cohort. Therefore, we decided to search for *MET* amplification and for mutations deleting the juxtamembrane domain, and evaluated their clinicopathological significance including any associations with *EGFR* and *KRAS* mutations. Additionally, we performed the mutational search of the *HER2* gene.

PATIENTS AND METHODS

Patients

We studied 262 patients with lung cancer who underwent potentially curative pulmonary resection at the Department of Thoracic Surgery, Aichi Cancer Center Hospital, from May 2000 through 2002. Tumor samples were frozen rapidly in liquid nitrogen, after obtaining the appropriate approval from the Institutional Review Board and the patients' written informed consent. All the patients were Japanese; 149 were men and 113 were women, with ages at diagnosis ranging from 26 to 89 years (median 64 years). One hundred forty-nine patients had stage I disease, 33 had stage II, 75 had stage III, and 5 had stage IV. There were 211 adenocarcinomas, 33 squamous cell carcinomas, 6 adenosquamous carcinomas, 10 large cell carcinomas, and 2 small cell carcinomas. One hundred and seven patients had never smoked and 155 were current or former smokers. We had previously determined the *EGFR*, and *KRAS* mutational status in this cohort.[15–17]

Cell Lines

Twenty-two lung cancer cell lines were available for this study. These comprised 9 adenocarcinomas, A549, ACC-LC-319, NCI-H358, NCI-H838, NCI-H1666, NCI-H1993, NCI-H2009, NCI-H2882, RERF-LC-MT; 5 squamous cell carcinomas, Calu1, PC-1, PC-10, SK-MES-1 and RERF-LC-AI; 3 large cell carcinomas, Calu6, NCI-H460 and SK-LC-6; one non-small cell lung carcinoma (NSCLC), NCI-H1299; 4 small cell carcinomas, ACC-LC-48, ACC-LC-49, ACC-LC-80, and SK-LC-2. Two gastric cancer cell lines, KATOIII and MKN45 were also used. NCI-H358, H460, H838, H1299, H1666, H1993, H2009, and H2882 were gifts from Dr. Adi F. Gazdar. KATOIII and MKN45 were obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer (Tohoku University, Sendai, Japan). The derivation of other cell lines has been described previously.[18,19] All cell lines were cultured in RPMI-1640 (Sigma-Aldrich, Irvine, UK) with supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and 1×antibiotic-antimycotic (Invitrogen) at 37°C in a humidified incubator with 5% CO_2. NCI-H1993[4,7] and MKN45[5] cell lines have amplification of the *MET* gene, while KATO III does not.[5]

RNA and DNA Extraction

Frozen tumor tissue sections were grossly dissected by a surgical pathologist (Y. Y.) to enrich tumor cells as much as possible. Total RNAs and genomic DNAs contained at least 20% of tumor content in our cohort. Total RNA was isolated using the RNeasy Kit (Qiagen, Valencia, CA) in 262 cases. For extraction of genomic DNA, tissues were incubated with 1×PCR (polymerase chain reaction) buffer containing 100 μg/ml proteinase K for 1 hour at 54°C. Next, the solution was incubated for 3 minutes at 95°C. Genomic DNA was extracted in 187 unselected patients for whom tumor blocks were available.

Analysis of *MET* Gene Amplification

The copy number of the *MET* gene relative to a LINE-1 repetitive element was determined by quantitative real-time PCR using the SYBR Green Method (QuantiTect SYBR Green PCR Kit; Qiagen) using an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster city, CA) according to Engelman et al.[7] The copy number of the LINE-1 is reported to be similar between normal and cancerous cells.[20] The standard curve method was used to calculate *MET* gene copy number in the cell line or tumor DNA sample relative to the Line-1 repetitive element.[20] Quantification was based on standard curves from a serial dilution of Calu6 genomic DNA. Calu6 was selected as a standard sample, because amount of *MET* gene relation to *Line-1* was almost 1.00. Primer sequences for the MET gene were 5′-TAGAAGAGCCCAGCCAGTGT-3′ (forward), 5′-CGAATGCAATGGATGATCTG-3′ (reverse), and for LINE-1 were 5′-AAAGCCGCTCAACTACATGG-3′ (forward), 5′-TGCTTTGAATGCGTCCCAGAG-3′(reverse). All the specimens were analyzed in triplicate using 20 ng of genomic DNA. We defined that amplification was present when the copy number was two or more.

Analysis of the Splice Mutation of the *MET* Gene Around the Juxtamembrane Domain

The cDNA sequence of the *MET* gene was obtained from GenBank (accession number NM 000245.2). The exon 14 that codes for the juxtamembrane domain of the *MET* gene was amplified with primer F (5′-TGAAATTGAACAGC-GAGCTAAAT-3′) and R (5′- TTGAAATGCACAATCAG-GCTAC-3′), in an one-step reverse transcription (RT)-PCR setup with Qiagen OneStep Reverse Transcription-PCR kits (Qiagen) using 4 ng of total RNA. The conditions for RT-PCR were one cycle of 50°C for 30 minutes, 95°C for 15 minutes, 45 cycles of 94°C for 40 seconds, 62°C for 40 seconds, 72°C for 1 minute, and one cycle of 72°C for 10 minutes. After RT-PCR, free nucleotides and excess primer were removed using PCR Purification kit (QIAGEN). PCR products were diluted and cycle sequenced using the BigDye Terminator Cycle Sequencing Kit v. 3.1/1.1 (Applied Biosystems). Sequencing reaction products were separated electrophoretically on an ABI PRISM 3100 apparatus (Applied Biosystems). Both the forward and reverse sequences obtained were analyzed with BLAST and by manual review.

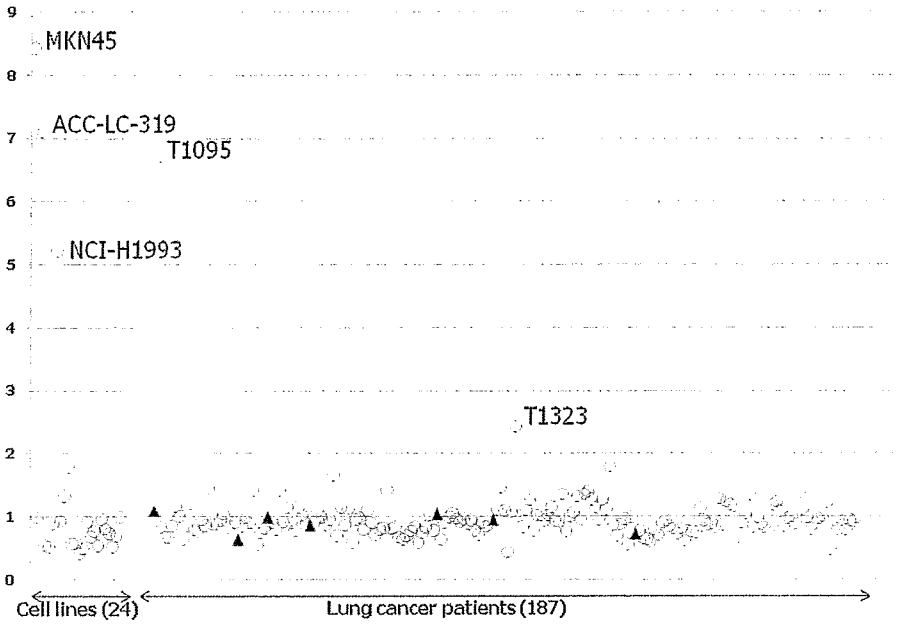

FIGURE 1. Relative copy number of the *MET* gene determined by real-time quantitative PCR (polymerase chain reaction) in 24 cell lines and 187 lung cancer specimens. MKN45 and NCI-H1993 cell lines showing *MET* amplification served as positive controls. Solid triangles indicate samples that had *MET* splice mutations in the following experiments.

For cases with *MET* mutations identified by sequencing of the RT-PCR product, we also analyzed 100 ng aliquots of genomic DNA to examine the mechanism for alternative splicing. The genomic DNA sequence of the *MET* gene was obtained from GenBank (accession number NC 000007.12). PCR of genomic DNA was carried out using AmpliTaq Gold (Applied Biosystems) for introns 13 and 14. PCR primers (encompassing from the 5′ splice site of intron 13 to the 5′ splice site of intron 14; nucleotide numbers of genomic DNA from 99615 to 99728) were as follows: 5′-GATTGCTGGTGT-TGTCTCAATATC-3′ (forward) and 5′-TGTCAAATACT-TACTTGGCAGAGG-3′ (reverse). The PCR condition was: one cycle of 95°C for 10 minutes, 40 cycles of 94°C for 30 seconds, 62°C for 30 seconds, 72°C for 40 seconds, and one cycle of 72°C for 10 minutes. Sequence analysis was carried out as for the RNA analysis.

Analysis of the *HER2* Gene Mutation

We sequenced exon 20 of the tyrosine kinase domain of the *HER2* gene where all the mutations are reported.[21–23] The cDNA sequence of the *HER2* gene was obtained from GenBank (accession number NM 004448). Primer sequences were 5′-ACAGTCTACAAGGGCATCTGGA-3′ (forward), and 5′-AACTCCACACATCACTCTGGTG-3′ (reverse). The RT-PCR conditions were one cycle of 50°C for 30 minutes, 95°C for 15 minutes, 40 cycles of 94°C for 40 seconds, 62°C for 40 seconds, 72°C for 1 minute, and one cycle of 72°C for 10 minutes.

Statistical Analysis

The χ^2 test was used to compare proportions. The two-sided significance level was set at $p < 0.05$. All analyses were carried out using StatView software (version 5; SAS Institute, Cary, NC).

RESULTS

MET Gene Amplification in Lung Cancer Specimens

First, we searched for amplification of the *MET* gene in 22 lung cancer cell lines and 2 gastric cancer cell lines (Figure 1). *MET* amplifications were detected in the ACC-LC-319 adenocarcinoma cell line. Two of the cell lines (NCI-H1993, lung cancer and MKN45, gastric cancer) have been reported to harbor *MET* gene amplifications[4,5,7] and we confirmed this findings. Relative copy numbers of the *MET* gene for the ACC-LC-319, NCI-H1993, and MKN-45 cell lines were 7.26, 5.22, and 8.43, respectively.

In 187 clinical specimens (148 adenocarcinomas, 28 squamous carcinomas, 4 adenosquamous carcinomas, 6 large cell carcinomas, and 1 small cell carcinoma), we detected only 2 *MET* gene amplifications (Figure 1). The *MET* copy numbers of these 2 patients were 6.72 (T1095) and 2.43 (T1323). The prevalence of *MET* gene amplification in adenocarcinomas was thus 1.4% (2 of 148).

MET Gene Splice Mutations Deleting the Juxtamembrane Domain

We searched for splice mutations of the *MET* gene deleting the juxtamembrane domain using 22 lung cancer cell lines, however, mutation was not detected. Next, we performed mutational search in a cohort of 262 lung cancers that included those examined for gene amplification. We identified 7 *MET* mutations in 262 lung cancer specimens (2.7%; Table 1, Figure 2*A*). All 7 mutations were confirmed by a second independent PCR. All the mutations were deletions of nucleotides 3075 to 3215 (according to NM 000245.2) corresponding with the sequence of exon 14. This would result in a 47 amino-acid

TABLE 1. Clinicopathological Features of the Patients with the *MET* Amplification or Splice Mutation in Lung Cancer Patients

	Patient Number	Age	Sex	Histology (differentiation)	Stage	Smoking Status	Prognosis (d)
Amplification	T1095	64	Male	AD(M)	IB	S	1094:DOD
	T1323	71	Male	AD (P)	IB	S	516:DOD
Mutation	T1021	62	Male	AD (P)	IA	S	2732:NED
	T1148	74	Female	AD(M)	IB	NS	2029:NED
	T1165	76	Male	AD(M)	IA	S	1611:DOD
	T1181	67	Male	AD(W)	IA	S	1953:NED
	T1241	60	Female	AD(M)	IIB	NS	2241:NED
	T1307	75	Male	AD (P)	IB	S	2101:NED
	T1384	56	Female	AD(M)	IA	NS	1872:NED

AD, adenocarcinoma; M, moderately differentiated; P, poorly differentiated; W, well differentiated; S, smoker; N, never-smoker; DOD, dead of disease; NED, no evidence of disease.

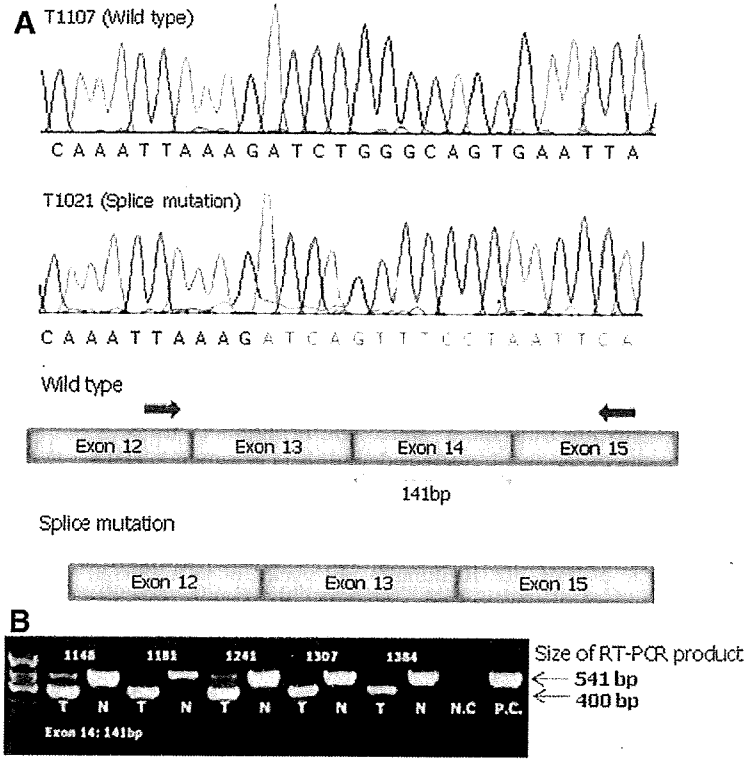

FIGURE 2. *A*, Examples of cDNA sequencing chromatograms of the *MET* gene. In patient T1021, exon 13 was spliced directly to exon 15, skipping exon 14. The chromatogram for the wild type sequence is shown in the upper panel for comparison. Red characters represent a splice mutation. Red arrows indicate the primer position for reverse transcription-polymerase chain reaction (RT-PCR). *B*, Agarose gel electrophoresis of the RT-PCR product encompassing exon 14 of the *MET* gene of the paired tumor and normal samples. T, tumor; N, normal lung tissue; N.C, negative control; P.C, positive control. The difference between the larger and smaller bands appears to correspond with length of the exon 14 (141 bp).

deletion of the exon 14 (L964 through D1010 in the juxtamembrane domain), identical with that reported.[8,9]

RNAs from matched tumor and normal lung tissues were available for 5 patients with *MET* mutations (except T1021 and T1065). Deletion of exon 14 was only present in tumor samples by RT-PCR and sequencing experiments indicated that this was a somatic event (Figure 2*B*). In addition, we noticed that bands for the mutant allele (shorter) were always stronger than bands for the wild type allele (Figure 2*B*). In 3 tumors (patients T1181, T1307, and T1384), bands for the wild type allele were almost invisible. However, none of seven tumors with *MET* mutations harbored *MET* amplification in the preceding experiments.

Mechanisms for the Deletion of Exon 14 of the *MET* Gene

To investigate the mechanism for the deletion of exon 14 of the *MET* gene in cDNA, we sequenced introns 13 and14 for any sequencing alterations, using genomic DNA. We identified four intronic mutations that would affect RNA splicing by disrupting consensus sequences.[24,25] Patient T1021 had a point mutation at the 5′ splice site of intron 14. Patients T1148, T1165, T1307 had deletions either involving the 3′ splice site, a branch site, or the polypyrimidine tract of intron 13 (Figure 3*A*, *B*), respectively.[24] Contrary to the cDNA sequencing, these

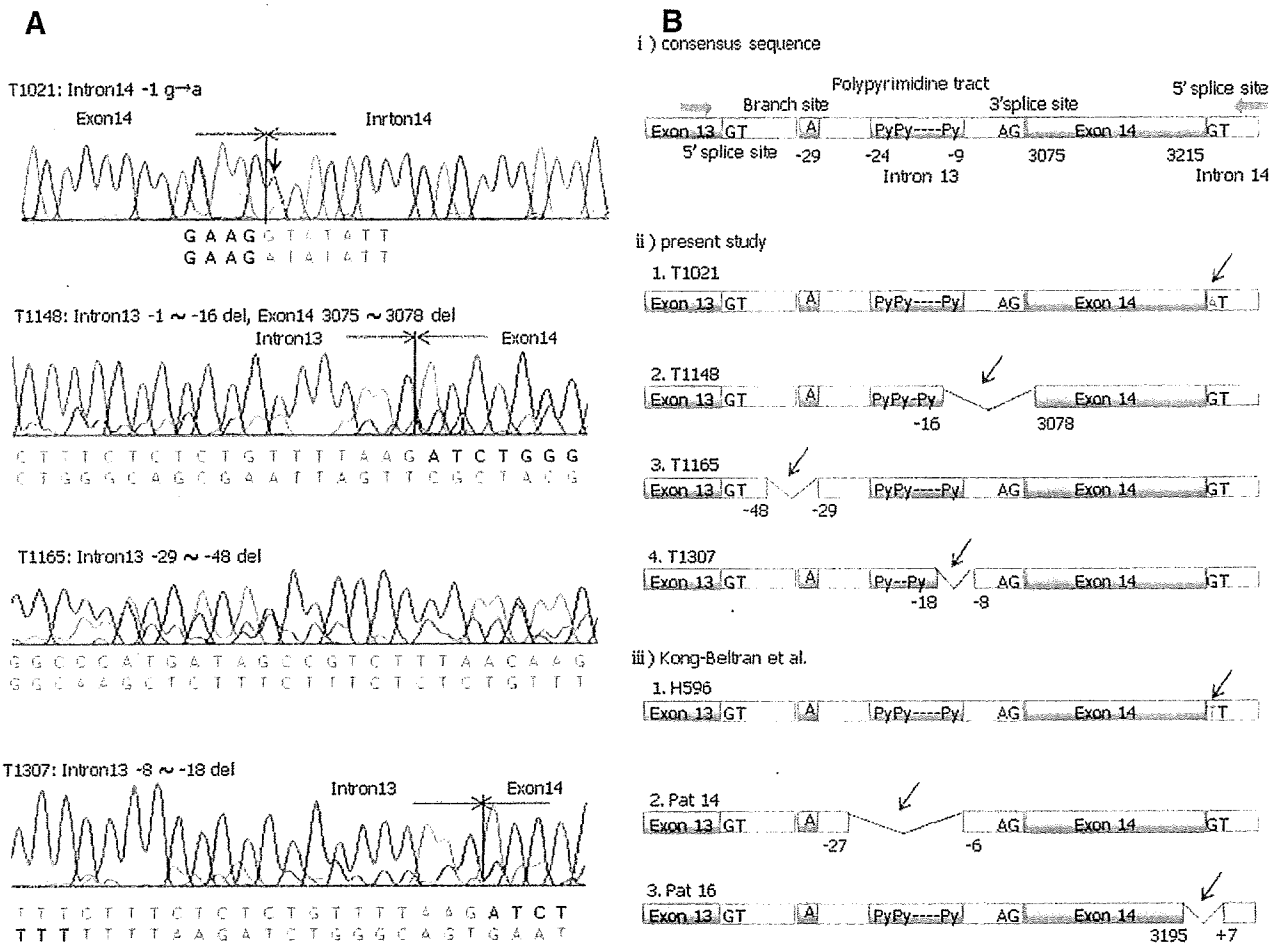

FIGURE 3. *A*, Sequencing chromatograms of genomic DNA with intronic mutation of the *MET* gene: Red characters represent deletions or point mutations in genomic DNA. Four intronic mutations were detected in this analysis. *B*, Diagrammatic representations of the mechanisms for eliminating exon 14 of the *MET* gene: (i) The 5′ splice site, 3′ splice site, branch site, and polypyrimidine tract are indicated as consensus motifs at the top of figure.[24,25,28] Blue arrows indicated primers for polymerase chain reaction (PCR). (ii) Diagrammatic representations of Figure 3A. (iii) Three intronic mutations reported previously by Kong-Beltran et al.[9] All intronic mutations detected in this analysis were different from those reported.[9] Each mutation is indicated by a red arrow. Py, pyrimidine.

mutations were heterozygous (Figure 3*A*). Matched normal lung of these four patients had wild-type sequences (data not shown).

HER2 Mutation in Lung Cancers

We identified 6 *HER2* mutations in the 262 lung cancer specimens (2.3%). Five mutations were a 12-bp duplication/insertion of the amino acids YVMA at codon 776 and one mutation was a 9-bp insertion of the amino acids VGS at codon 779. All patients with the *HER2* mutation, except for one with an adenosquamous carcinoma, had adenocarcinomas. Five patients were women and one was a man. Four patients had stage IA tumors, one had stage IB, and one had stage IIIA. Four patients were never smokers and two were smokers.

Relationships Between Patients with *MET* Gene Amplification or Splice Mutation and Clinicopathological Backgrounds Indicating Mutations of the *EGFR*, *KRAS*, and *HER2* Genes

Table 1 shows the clinicopathological backgrounds for 9 patients with *MET* gene amplification or splice mutation. All the patients had adenocarcinomas. Two patients with amplification were male smokers. Four patients with the *MET* mutation were male smokers and three were female never-smokers. All except for T1241 had stage I tumors. There were no significant differences among clinicopathological factors according to *MET* mutation status (Table 2).

EGFR, *KRAS*, and *HER2* mutations were identified in 103 (48.8%), 29 (13.7%), and 5 (2.4%) of 211 lung adenocarcinomas, respectively, and these 3 mutations were mutually exclusionary as reported.[15,26] *MET* gene activation by

TABLE 2. Characteristic Clinicopathological Factors According to *MET* Mutation Status in 211 Patients with Lung Adenocarcinomas

Characteristic	Mutated	Wild Type	P (univariate)	P (multivariate)
Sex				
Male	4 (57%)	98 (48%)	0.714	0.552
Female	3 (43%)	106 (52%)		
Age				
≤64	2 (29%)	104 (51%)	0.279	0.736
>64	5 (71%)	100 (49%)		
Stage				
I–II	7 (100%)	140 (69%)	0.104	0.981
III–IV	0 (0%)	64 (31%)		
Smoking status				
Never-smoker	3 (43%)	102 (50%)	>0.999	0.644
Smoker	4 (57%)	102 (50%)		
Differentiation				
W-M	5 (71%)	139 (68%)	>0.999	0.365
P	2 (29%)	65 (32%)		
EGFR				
Mutated	0 (0%)	103 (50%)	0.014	0.976
Wild type	7 (100%)	101 (50%)		
KRAS				
Mutated	0 (0%)	29 (14%)	0.597	0.987
Wild type	7 (100%)	175 (86%)		
HER2				
Mutated	0 (0%)	5 (2%)	>0.999	0.995
Wild type	7 (100%)	199 (98%)		

M, moderately differentiated; P, poorly differentiated; W, well differentiated; EGFR, epidermal growth factor receptor.

amplification or mutation occurred selectively in these adenocarcinomas without *EGFR*, *KRAS* or *HER2* mutations with a statistical significance ($p < 0.0001$; Figure 4).

DISCUSSION

We found that about 5% of lung adenocarcinomas had MET activation either by gene amplification (2 of 148 patients, 1.4%) or splice mutations deleting the juxtamembrane domain (7 of 211 patients, 3.3%). Unlike *EGFR* or *HER2* mutations that mainly target female, Asian and nonsmoking patients, and unlike *KRAS* mutations that mainly target male, Caucasian and smoking patients,[15–17,21,26] there was no such relationship for MET activation.

MET gene amplification was detected in 22% (4 of 18) of NSCLCs that had developed acquired resistance to EGFR tyrosine kinase inhibitors (TKIs).[7] The prevalence of *MET* amplification in primary lung cancer patients unexposed to such inhibitors had not been elucidated until recently. However, Bean et al.[6] reported that *MET* amplification was present only in 2 of 62 EGFR-TKI-untreated patients, but that it was present in 9 of 43 patients with acquired resistance. This is in agreement with our results, suggesting that there is no ethnic difference in this clinical feature and that gene amplification does not play a major role for development of pulmonary adenocarcinomas without EGFR-TKI treatment.

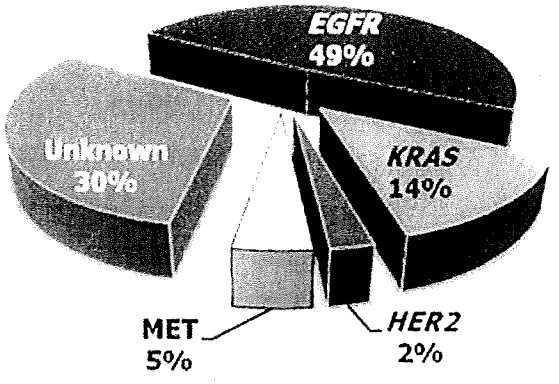

FIGURE 4. Frequencies of MET activation by gene amplification or splice mutation and the mutations of each gene in 211 Japanese patients with lung adenocarcinomas. Thirty percent of the specimens had no mutations of the epidermal growth factor receptor (*EGFR*), *KRAS* or *HER2* genes and did not show MET activation. Each instance of MET activation detected in this series was mutually exclusionary with these gene mutations. *FGFR4* mutation and *EML4-ALK* fusion gene have been reported to occur in similar exclusionary fashion and be present in 1 of 158 (0.6%) and 5 of 149 (3.4%) of lung adenocarcinomas, respectively, these may be encompassed in the part of Unknown.[29,30]

There was also no significant difference in the prevalence of *MET* somatic mutation between our study (7 of 211, 3.3%) and previous studies from Western countries (2 of 127, 1.6%[8] or 2 of 33, 6%[9]). We were able to identify cis-acting intronic mutations of the *MET* gene that disrupted splice consensus sequence in four of seven patients. However, in the remaining three patients, the mechanisms for abnormal splicing could not be determined. Other possible mechanisms included mutations and nongenetic alterations of factors required for constitutive or alternative splicing or formation of the fusion proteins involving splice factors resulting from cancer-associated chromosomal translocations.[24] Interestingly, mechanisms leading to deletion of exon 14 in 7 cases including those reported by Kong-Beltran et al. (Figure 3*B*) were all different. This also suggest the notion that deletion of exon 14 plays an important role in the development of lung adenocarcinomas, conferring various advantages to the tumor cells and that *MET* splice mutations are "driver mutations" and not just "passenger mutations."[27]

Although a *MET* splice mutation did not occur in tumors with *MET* amplification, we noticed that mutant alleles were transcribed preferentially in RT-PCR or sequencing experiments. Kong-Beltran et al.[9] reported similarly that the deleted form of the MET receptor is expressed predominantly despite their tumor samples being heterozygous for exon 14, suggesting preferential expression of the variant transcript.

Shibata et al.[3] reported that *MET* amplification occur in tumors without *EGFR* mutations. Kong-Beltran et al.[9] found that tumors harboring these intronic mutations were wild type for *KRAS*, *BRAF*, *EGFR*, and *HER2*. In the present study, we were able to confirm and extend these findings. MET activation by amplification or splice mutation was present only in tumors without any mutations of the *EGFR*, *KRAS*, or *HER2*

genes. This again suggests that MET activation plays an important role that is equivalent to mutations of the *EGFR*, *KRAS* or *HER2* genes. Altogether, lung adenocarcinomas containing the MET activation seem to form a novel and independent subclass of such tumors.

In the present study, 30% of Japanese patients with adenocarcinomas of lung did not harbor any mutations of *EGFR*, *KRAS* or *HER2* and MET activation by gene amplifications or splice mutations (Figure 4). To generate a therapeutic strategy for these patients, it will be important to discover activated genes that have complementary roles.

Tumor cell lines with activated MET either by gene amplification or splice mutations are highly sensitive to MET targeted therapy.[4,5,9] A dramatic reduction in tumor cell numbers was observed using the MET TKI, PHA665752, in gastric cancer cell lines with a *MET* amplification including the MKN45 cells used in the present study.[5] Short hairpin RNA-mediated MET knockdown induced significant growth inhibition, G1/S cell cycle arrest, and apoptosis in EBC-1 and H1993 cells showing MET amplification, whereas it had little or no effect on cell lines not exhibiting MET amplification.[4] Similarly, a lung cancer cell line (NCI-H596) harboring a splice variant of *MET* was sensitive to an anti-MET OA-5D5 antibody.[9] Altogether, these results strongly suggest that MET activation identifies a subset of NSCLCs that is likely to respond to new molecular therapies targeting MET.

In conclusion, we found that about 5% of pulmonary adenocarcinomas were driven by activated MET by gene amplifications or splice mutations. Although this prevalence seems low, a considerable number of patients with lung cancers would benefit from anti-MET strategy, considering that over one million deaths are caused by lung cancer worldwide annually.

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MINIREVIEW

Epidermal growth factor receptor in relation to tumor development: *EGFR* gene and cancer

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Keywords
cancer; epidermal growth factor receptor (EGFR); gefitinib; non-small cell lung carcinoma (NSCLC); tyrosine kinase inhibitor (TKI)

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Epidermal growth factor receptor (EGFR) and its three related proteins (the ERBB family) are receptor tyrosine kinases that play essential roles in both normal physiological conditions and cancerous conditions. Upon binding its ligands, dynamic conformational changes occur in both extracellular and intracellular domains of the receptor tyrosine kinases, resulting in the transphosphorylation of tyrosine residues in the C-terminal regulatory domain. These provide docking sites for downstream molecules and lead to the evasion of apoptosis, to proliferation, to invasion and to metastases, all of which are important for the cancer phenotype. Mutation in the tyrosine kinase domain of the *EGFR* gene was found in a subset of lung cancers in 2002. Lung cancers with an *EGFR* mutation are highly sensitive to EGFR tyrosine kinase inhibitors, such as gefitinib and erlotinib. Here, we review the discovery of EGFR, the EGFR signal transduction pathway and mutations of the *EGFR* gene in lung cancers and glioblastomas. The biological significance of such mutations and their relationship with other activated genes in lung cancers are also discussed.

Identification of epidermal growth factor, epidermal growth factor receptor and ERBB family proteins

Epidermal growth factor (EGF) was originally isolated by Stanley Cohen in 1962 as a protein extracted from the mouse submaxillary gland that accelerated incisor eruption and eyelid opening in the newborn animal [1]. Therefore, it was originally termed 'tooth-lid factor', but was later renamed EGF because it stimulated the proliferation of epithelial cells [1]. In 1972, the amino acid sequence of the EGF was determined. The presence of a specific binding site for EGF, the EGF receptor (EGFR), was confirmed in 1975 by showing that ^{125}I-labeled EGF binds specifically to the surface of fibroblasts [1].

In 1978, EGFR was identified as a 170kDa protein that showed increased phosphorylation when bound to EGF in the A431 squamous cell carcinoma cell line that had an amplified *EGFR* gene. The discovery (in 1980) that the transforming protein of Rous sarcoma virus, v-src, has tyrosine-phosphorylation activity led to the discovery that EGFR is a tyrosine kinase activated by binding EGF [1]. In 1984, the cDNA of human *EGFR* was isolated and characterized. A high degree of similarity was found between the amino acid sequence of *EGFR* and that of v-erbB, an oncogene of the avian erythroblastosis virus [1].

Abbreviations
ALK, anaplastic lymphoma kinase; BAC, bronchioloalveolar cell carcinoma; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EML4, echinoderm microtubule-associated protein-like 4; NRG, neuregulin; STAT, signal transducer and activator of transcription; TKI, tyrosine kinase inhibitor; TRU, terminal respiratory unit.

Screening of cDNA libraries using an EGFR probe identified a family of proteins closely related to EGFR. This family consists of EGFR (also known as ERBB1/HER1), ERBB2/HER2/NEU, ERBB3/HER3 and ERBB4/HER4. ERBB2, ERBB3 and ERBB4 show extracellular homologies, relative to the EGFR, of 44, 36 and 48%, respectively, while those for the tyrosine kinase domain are 82, 59 and 79%, respectively. The degrees of homology in the C-terminal regulatory domain are relatively low, being 33, 24 and 28%, respectively.

Structure of the ERBB proteins and diversity of their ligands

The EGFR gene is located on chromosome 7p12-13 and codes for a 170kDa receptor tyrosine kinase. All ERBB proteins have four functional domains: an extracellular ligand-binding domain; a transmembrane domain; an intracellular tyrosine kinase domain; and a C-terminal regulatory domain [2]. The extracellular domain is subdivided further into four domains. The tyrosine kinase domain consists of an N-lobe and a C-lobe, and ATP binds to the cleft formed between these two lobes. The C-terminal regulatory domain has several tyrosine residues that are phosphorylated specifically upon ligand binding, as described below (Fig. 1A).

Eleven ligands are known to bind to the ERBB family of receptors [3]. These can be classified into three groups (a) ligands that specifically bind to EGFR (including EGF, transforming growth factor-a, amphiregulin and epigen); (b) those that bind to EGFR and ERBB4 (including betacellulin, heparin-binding EGF and epiregulin); and (c) neuregulin (NRG) (also known as heregulin) that binds to ERBB3 and ERBB4. NRG1 and NRG2 bind to both ERBB3 and ERBB4, whereas NRG3 and NRG4 only bind to ERBB4 [3]. Although these ligands show redundancy, heparin-binding-EGF is the only ligand whose absence in knockout mice results in postnatal lethality as a result of heart and lung problems, while mice lacking other EGF ligands, or even triple null mice deficient for amphiregulin, EGF and transforming growth factor-a are viable [4]. These ligands are synthesized as transmembrane proteins, and soluble ligands (growth factors) are released into the extracellular environment via proteolytic processing. This shedding is mediated by ADAM (a disintegrin and metalloprotease) proteins that are membrane-anchored metalloproteases [4].

Signal transduction by ERBB proteins

Binding of a family of specific ligands to the extracellular domain of ERBB (except for ERBB2, see below) leads to the formation of homodimers and heterodimers. This process is mediated by rotation of domains I and II, leading to promotion from a tethered configuration to an extended configuration (Fig. 1B) [2]. This exposes the dimerization domain. ERBB2 does not have corresponding ligands but is expressed constitutively in the extended configuration. ERBB2 is a preferred dimerization partner, and heterodimers containing ERBB2 mediate stronger signals

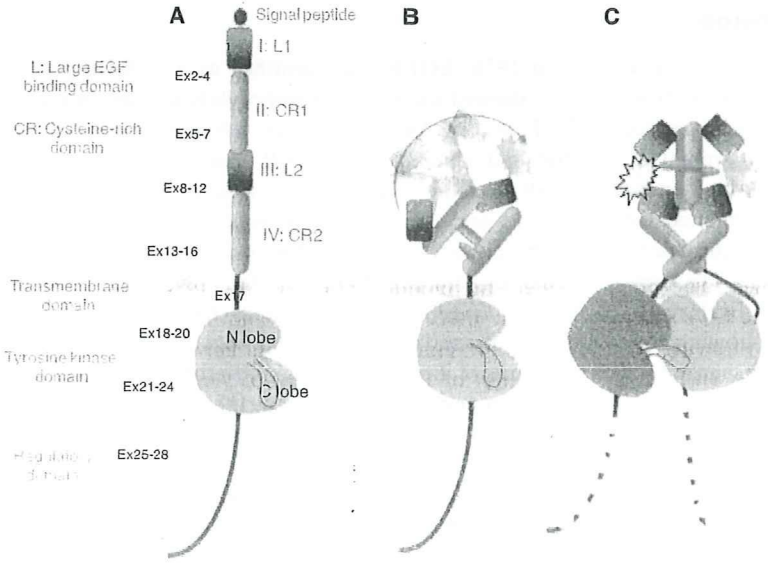

Fig. 1. Structure of the EGFR protein (A), activation (B) and dimerization by ligand binding (C).

than other dimers. In the cytoplasm, the kinase domain dimerizes asymmetrically in a tail-to-head orientation (Fig 1C) [5]. In this manner, tyrosine kinase becomes activated, as in the case of activation of cyclin-dependent kinases by cylclins. Dimerization consequently stimulates intrinsic tyrosine kinase activity of the receptors and triggers autophosphorylation of specific tyrosine residues within the cytoplasmic regulatory domain.

These phosphorylated tyrosines serve as specific binding sites for several adaptor proteins, such as phospholipase Cg, CBL, GRB2, SHC and p85. For example, tyrosine-X-X-methionine (where X is any amino acid) is a motif for the p85 binding site. Several signal transducers then bind to these adaptors to initiate multiple signalling pathways, including mitogen-activated protein kinase, phosphatidylinositol 3-kinase/AKT and the signal transducer and activator of transcription (STAT)3 and STAT5 pathways (Fig. 2) [3]. These eventually result in cell proliferation, migration and metastasis, evasion from apoptosis, or in angiogenesis, all of which are associated with cancer phenotypes. ERBB3 lacks tyrosine kinase activity because of substitutions in crucial residues in the tyrosine kinase domain. However, it has many binding sites for p85, a regulatory subunit of phosphatidylinositol 3-kinase, and thus is a preferred dimerization partner.

EGFR overexpression and cancer

EGFR is expressed in a variety of human tumors, including those in the lung, head and neck, colon, pancreas, breast, ovary, bladder and kidney, and in gliomas. EGFR expression and cancer prognosis have been investigated in many human cancers. Although there some discrepancies have been reported, patients with tumors that show high expression of EGFR tend to have a poorer prognosis in general. However, it was not possible to predict super-responder of gefitinib degree of EGFR expression, as determined by immunohistochemistry or immunoblotting.

Mutations of the extracellular domain are frequent in glioblastomas

Three different types of deletion mutations (categorized according to the extent of deletion, and termed *EGFR vI, EGFR vII* and *EGFR vIII*) have been reported in the extracellular domain of the *EGFR* gene [6]. In the *EGFR vI* mutation, the extracellular domain has been totally deleted and resembles the v-erbB oncoprotein. In the *EGFR vII* mutation, 83 amino acids in domain IV of the extracellular domain have been deleted; however, this mutation does not appear to contribute to a malignant phenotype. The most

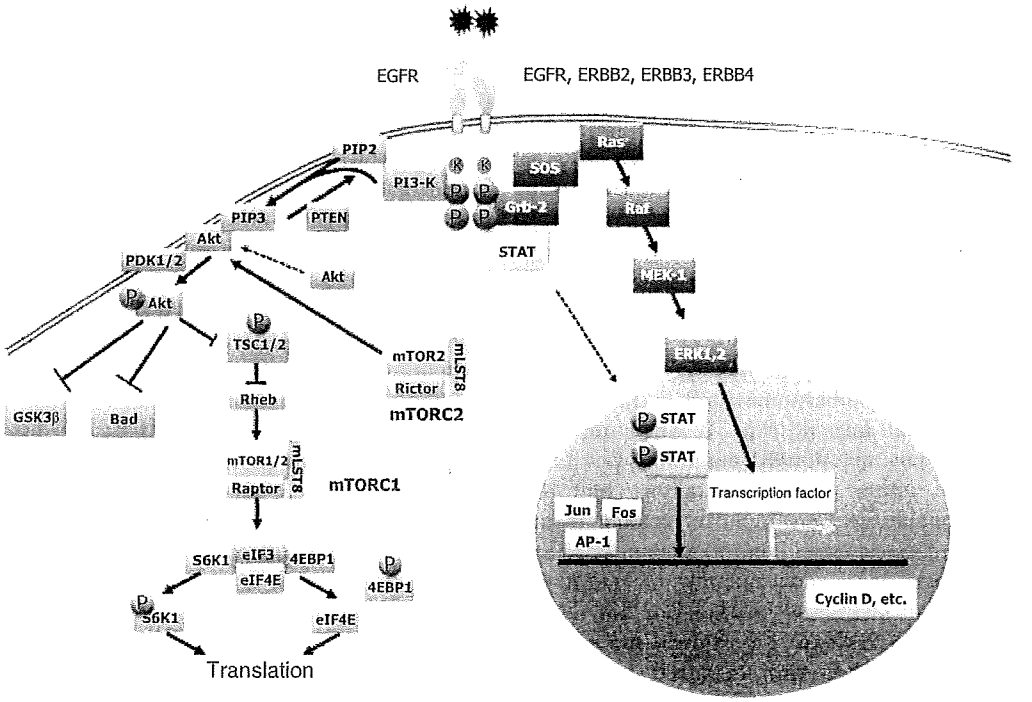

Fig. 2. EGFR and ERBB proteins and their downstream pathways.

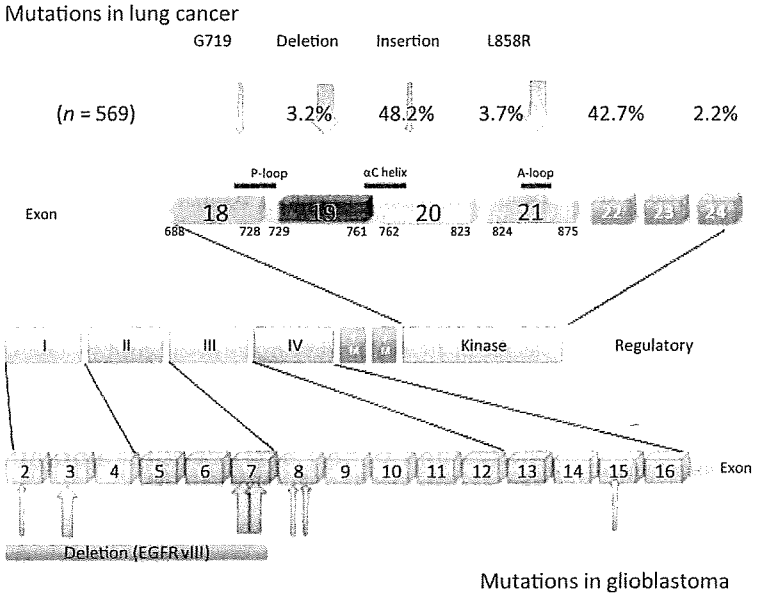

Fig. 3. Distribution and frequency of *EGFR* mutations occurring in the kinase domain in lung cancer (upper part of the figure) [12] and in the extracellular domain in glioblastoma (lower part of the figure) [8].

common of the three types of deletion mutations is *EGFR vIII*. This mutation often accompanies gene amplification, resulting in the overexpression of EGFR lacking amino acids 30–297, corresponding to domains I and II. In this case, the EGFR tyrosine kinase is activated constitutively without ligand binding, as in the case of *EGFR vI*. *EGFR vIII* is reported to occur in 30–50% of glioblastomas [6]. In lung cancers, *EGFR vIII* is found in 5% of squamous cell carcinomas, while none of 123 adenocarcinomas were found to harbor this mutation [7]. It is also known that tissue-specific expression of *EGFR vIII* leads to the development of lung cancer [7]. There is also a suggestion that lung tumors with *EGFR vIII* are sensitive to the irreversible EGFR tyrosine kinase inhibitor (TKI), HKI272, despite the fact these tumors are relatively resistant to the reversible inhibitors, gefitinib and erlotinib [7].

Recently, novel missense mutations in the extracellular domain of the *EGFR* gene have been identified in 13.6% (18/132) of glioblastomas and in 12.5% (1/8) of glioblastoma cell lines [8] (Fig. 3). There appear to be several hot spots: five R108K mutations were found in domain I, three T263P mutations and five A289V/D/T mutations were found in domain II, and two G598V mutations were found in domain IV. These *EGFR* mutations occur independently of *EGFR vIII* and provide an alternative mechanism for *EGFR* activation in glioblastomas [8]. Furthermore, these mutations are associated with increased *EGFR* gene dosage and confer anchorage-independent growth and tumorigenicity to NIH-3T3 cells. Cells transformed by expression of these *EGFR* mutants are sensitive to small-molecule EGFR kinase inhibitors [8]. In contrast, none of 119 primary lung tumors was found to harbor these ectodomain mutations [8].

EGFR mutations in the tyrosine kinase domain

In April 2004, two groups of researchers in Boston [9,10], and subsequently a group in New York [11], reported that activating mutations of the *EGFR* gene are present in a subset of non-small cell lung cancer and that tumors with *EGFR* mutations are highly sensitive to EGFR-TKIs. This discovery solved the enigma of why female, nonsmoking, adenocarcinoma patients of East Asian origin with lung cancers had a higher response to EGFR-TKIs, because patients with these characteristics have a higher incidence of *EGFR* mutations. Figure 4 shows the incidence of *EGFR* mutations found in 559 mutations in 2880 lung cancer patients in the literature [12]. It is also intriguing that *EGFR* mutations in the tyrosine kinase domain are almost exclusively seen in lung cancers and not in other types of tumor.

It is of particular interest that *EGFR* mutations are the first molecular aberrations found in lung cancer that are more frequent among patients without a smoking history than among those with one. Furthermore, the *EGFR* mutation frequency is inversely associated with the total amount of tobacco smoked [13]. However, it should be noted that *EGFR* mutations

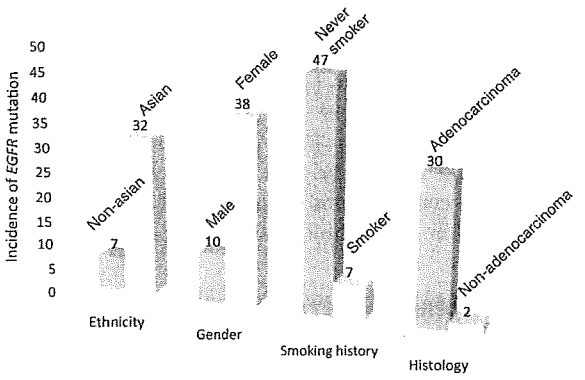

Fig. 4. Incidences of *EGFR* mutations in lung cancer in various different clinical backgrounds [12]. Hx, history; adeno, adenocarcinoma.

have been detected in more than 20% of patients with a history of heavy smoking [13]. These findings do not necessarily mean that smoking has a preventive effect on *EGFR* mutations. Rather, they suggest that *EGFR* mutations are caused by carcinogen(s) other than those contained in tobacco smoke, and indicate that the apparent negative correlation with smoking dose occurs as a result of diluting the number of tumors containing *EGFR* mutations with an increased number of tumors containing wild-type *EGFR* as the smoking dose increases. Indeed, this was shown in our case–control study [14].

Pathology of lung cancers with *EGFR* gene mutations

Bronchioloalveolar cell carcinoma (BAC) is defined as a carcinoma *in situ* without stromal, vascular or pleural invasion, showing growth of neoplastic cells along pre-existing alveolar structures (lepidic growth). Although it is relatively rare to present with pure BAC, invasive adenocarcinomas with areas exhibiting lepidic growth are frequently seen. This type of adenocarcinoma is sometimes referred to as an adenocarcinoma with BAC features. Such tumors respond more to gefitinib than do other types of adenocarcinoma [15] and thus have a higher incidence of *EGFR* mutations. As expected, adenocarcinomas with BAC features are more common in adenocarcinomas of never-smoking patients (13%) than in smokers (5%).

We proposed a terminal respiratory unit (TRU)-type of adenocarcinoma [16]. This type of cancer is characterized by distinct cellular features (expression of thyroid transcription factor 1 and surfactant proteins, and lepidic growth in the periphery), and it resembles adenocarcinomas with nonmucinous BAC features. Although, according to the World Health Organization classification, mucinous BACs form a subset of BACs, this type of BAC does not express thyroid transcription factor 1 or surfactant apoprotein, and is thus not a TRU-type adenocarcinoma. It is also known that *KRAS* mutations are more frequent in mucinous BAC than in nonmucinous BAC.

In our series of 195 adenocarcinomas, 149 were of the TRU type and 46 were of other types [17]. TRU-type adenocarcinomas are associated with a significantly higher incidence of female patients, never-smokers and *EGFR* mutations, but with fewer *KRAS* and *TP53* mutations than other types of adenocarcinoma [17]. An *EGFR* mutation was detected in 97/195 adenocarcinomas, in 91/149 TRU-type adenocarcinomas and in 6/46 tumors of other types. Conversely, 91/97 *EGFR*-mutated adenocarcinomas were categorized as TRU-type adenocarcinomas [17]. In addition, *EGFR* mutations were detected in some cases of atypical adenomatous hyperplasias known to be precursor lesions for BAC [17]. These findings further confirm that the TRU-type adenocarcinoma is a distinct adenocarcinoma subset involving a particular molecular pathway. It is of note that *EGFR* mutations can also occur in poorly differentiated adenocarcinomas, as long as the tumor belongs to the TRU cellular lineage.

Types of *EGFR* mutations

EGFR mutations are mainly present in the first four exons of the gene encoding the tyrosine kinase domain (Fig. 3) [12]. About 90% of the *EGFR* mutations are either small deletions encompassing five amino acids from codons 746–750 (ELREA) or missense mutations resulting in a substitution of leucine with arginine at codon 858 (L858R). There are more than 20 variant types of deletion, including larger deletions, deletions plus point mutations and deletions plus insertions. About 3% of the mutations occur at codon 719, resulting in the substitution of glycine with cysteine, alanine or serine (G719X). In addition, about 3% are in-frame insertion mutations in exon 20. These four types of mutations seldom occur simultaneously. There are many rare point mutations, some of which occur together with L858R [12].

Exon 19 deletional mutation and L858R result in increased and sustained phosphorylation of EGFR and other ERBB family proteins without ligand stimulation. It has been shown that mutant EGFR selectively activates the AKT and STAT signaling pathways that promote cell survival, but has no effect on the mitogen-activated protein kinase pathway that induces cell proliferation [18]. EGFR mutants in the

kinase domain are oncogenic [19]. The mutant EGFR protein can transform both fibroblasts and lung epithelial cells in the absence of exogenous EGFR, as evidenced by anchorage-independent growth, focus formation and tumor formation in immunocompromised mice [19]. Transformation is associated with constitutive autophosphorylation of EGFR, SHC phosphorylation and STAT pathway activation [19]. Whereas transformation by most EGFR mutants confers cell sensitivity to erlotinib and gefitinib, transformation by an exon 20 insertion (D770insNPG) makes cells resistant to these inhibitors but more sensitive to the irreversible inhibitor CL-387,785 [19]. In that study, the G719S mutation of exon 18 showed intermediate sensitivity *in vitro* [19]. However, the authors did not observe any difference between the exon 19 deletion and L858R in their cell-based assay. However, biochemical analysis of the kinetics of purified wild-type and mutant kinases revealed that mutant kinases have a higher K_m for ATP (wild-type, 5 $\mu mol \cdot L^{-1}$; L858R, 10.9 $\mu mol \cdot L^{-1}$; deletion, 129.0 $\mu mol \cdot L^{-1}$) and a lower K_i for erlotinib (wild-type, 17.5 $\mu mol \cdot L^{-1}$; L858R, 6.25 $\mu mol \cdot L^{-1}$; deletion, 3.3 $\mu mol \cdot L^{-1}$;) [20]. Mulloy *et al.* [21] showed that the Del747–753 kinase had a higher autophosphorylation rate and higher sensitivity to erlotinib than L858R kinase. These data reflect differences in the clinical response rate between the exon 19 deletion and L858R.

Oncogenic activity of *EGFR* mutants has also been shown *in vivo*. Two groups of researchers have developed transgenic mice that express either the exon 19 deletion mutant or the L858R mutant in type II pneumocytes under the control of doxycyclin [22,23]. Expression of either *EGFR* mutant led to the development of adenocarcinomas similar to human BACs, and the withdrawal of doxycycline to reduce expression of the transgene, or erlotinib treatment, resulted in tumor regression. These experiments show that persistent EGFR signaling is required for tumor maintenance in human lung adenocarcinomas expressing *EGFR* mutants.

EGFR gene copy numbers

EGFR amplification is detectable in 40% of human gliomas and is often associated with deletion mutations, as discussed below. When the topographical distribution of *EGFR* amplification in lung cancers with confirmed mutations was examined, gene amplification was found in 11 of 48 specimens [24]. Nine of the cancers showed heterogeneous distribution, and amplification was associated with higher histological tumor grades or invasive growth [24]. However, the amplification status of the metastatic lymph node was not always associated with gene amplification of the primary tumors [24]. Only one of 21 carcinomas *in situ*, and none of 17 precursor lesions, harbored gene amplifications [24]. These results suggest that mutations occur early in the development of lung adenocarcinomas and that amplification might be acquired in association with tumor progression.

Relationship between EGFR and mutations of the related genes

The activating mutation of the *KRAS* gene was one of the earliest discoveries of genetic alterations in lung cancer, and has been known as a poor prognostic indicator since 1990 [25]. We were the first group to report that the occurrence of *EGFR* and *KRAS* mutations are strictly mutually exclusive [13]. One explanation is that the KRAS–mitogen-activated protein kinase pathway is one of the downstream signaling pathways of EGFR. Interestingly, *KRAS* mutations predominantly occur in White people with a history of smoking. Mutations of the *ERBB2* gene are present in a very small fraction ($\sim$ 3%) of adenocarcinomas and they appear to target the same population targeted by *EGFR* mutations: never-smokers and female patients [26]. Most of the *ERBB2* mutations are insertion mutations in exon 20 [26]. As anticipated, tumors with *ERBB2* mutations are resistant to treatment with EGFR-TKIs [27] because constitutively activated ERBB2 kinase will phosphorylate other ERBB family proteins, resulting in the activation of downstream molecules even when the EGFR tyrosine kinase is blocked. Mutation of the *BRAF* gene occurs in about 1–3% of lung adenocarcinomas.

By retrieving transforming genes from mouse 3T3 fibroblasts transfected with a cDNA expression library constructed from a lung adenocarcinoma arising in a male smoker, Soda *et al.* [28] identified the gene resulting from the fusion of that for transforming echinoderm microtubule-associated protein-like 4 (*EML4*) and the gene for anaplastic lymphoma kinase (*ALK*). This *EML4–ALK* fusion gene resulted from a small inversion within chromosome 2p. The *EML4–ALK* fusion transcript is detected in about 5% of non-small cell lung cancers. *ALK* translocation was associated with patients being never-smokers of a younger age and acinar-type adenocarcinomas, in a larger study [29]. It is also noteworthy that *EGFR*, *ERBB2*, *BRAF*, *KRAS* and *ALK* mutations almost never occur simultaneously in individual patients, suggesting a complementary role of these mutations in lung carcinogenesis.

Conclusions

In this minireview, we have described how Cohen's discovery of the 'tooth-lid factor' led to the identification of the genetic causes of certain types of human cancers, and to the genetic classification of a variety of tumors of apparently the same phenotype that has significant therapeutic implications.

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Prognostic Implication of *EGFR*, *KRAS*, and *TP53* Gene Mutations in a Large Cohort of Japanese Patients with Surgically Treated Lung Adenocarcinoma

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Introduction: Although mutation of the epidermal growth factor receptor (*EGFR*) gene is predictive for the response to EGFR-tyrosine kinase inhibitor, its prognostic impact for patients without EGFR-tyrosine kinase inhibitor treatment remains controversial. We examined for *EGFR*, *KRAS* or *TP53* mutations in a consecutive large cohort of patients with lung adenocarcinoma, and evaluated their prognostic impact.
Methods: We analyzed 397 patients with lung adenocarcinoma who underwent potentially curative pulmonary resection. Total ribonucleic acid was extracted and direct sequencing of each gene was performed after reverse transcription-polymerase chain reaction.
Results: We found that 196 patients (49%) had *EGFR* mutations. Of these, 83 were exon 19 deletions (42%) and 92 were L858R (47%). Univariate analysis showed that patients with *EGFR* mutations survived for a longer period than those without mutations (p = 0.0046). However, there was no difference in overall survival between the patients with exon 19 deletion and those with L858R (p = 0.4144). Patients with *KRAS* mutations or *TP53* mutations tended to survive for a shorter period (p = 0.2183 and 0.0230, respectively). Multivariate analysis using the Cox proportional hazards model revealed that smoking status (p = 0.0310) and disease stage ($p < 0.0001$) were independent prognostic factors. However, none of the gene mutations was independent prognostic factors (*EGFR*, p = 0.3225; *KRAS*, p = 0.8500; *TP53*, p = 0.3191).
Conclusions: *EGFR*, *KRAS*, and *TP53* gene mutations were not independently associated with the prognosis for Japanese patients with surgically treated lung adenocarcinoma.

Key Words: Lung cancer, *EGFR*, *KRAS*, *TP53*, Gene mutations.

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Multiple genetic alterations result in the activation of oncogenes and the inactivation of tumor suppressor genes during the formation of lung adenocarcinoma. In particular, mutations of genes in the epidermal growth factor receptor (EGFR) signaling pathway, such as *EGFR*, *KRAS*, *HER2*, *BRAF*, and phosphatidyl inositol 3 kinase catalytic alpha (*PIK3CA*), are thought to be important for the pathogenesis of adenocarcinomas.[1–4]

Activating mutation of the *EGFR* gene was first reported in 2004.[5–7] *EGFR* mutations are more prevalent in females, never smokers, patients of Asian ethnicity, and those with histology of adenocarcinoma[8]. We previously showed that about 50% of lung adenocarcinomas from Japanese patients harbored *EGFR* mutations.[1] Tumors with *EGFR* mutations are highly sensitive to small molecule EGFR-specific tyrosine kinase inhibitors (TKIs), such as gefitinib or erlotinib. According to the published data for 1335 patients, the response rate of non-small cell lung cancers (NSCLCs) with *EGFR* mutations for EGFR-TKI was about 70%, whereas those without mutations was about 10%.[8] Furthermore, several retrospective studies showed that patients with *EGFR* mutations have a significantly longer survival than those without mutations when treated with EGFR-TKIs.[9–12] These results indicate that the *EGFR* mutations are important predictive factors for successful treatment with EGFR-TKIs. However, prognostic impact of EGFR gene mutations in lung adenocarcinoma remains controversial some investigators claim that *EGFR* mutations are prognostic rather than predictive, because reports showed that patients with NSCLCs harboring *EGFR* mutations survived for a longer period than those without mutations irrespective of therapy (chemotherapy with EGFR-TKIs or placebo).[13,14] However, we identified previously that *EGFR* mutations did not affect the prognosis for patients with adenocarcinoma who were not treated with gefitinib.[1] Similar results were reported from two independent groups.[15,16] Thus, prognostic impact of *EGFR* mutations in the patients with NSCLCs remains controversial.

Activating mutation of the *KRAS* gene was one of the earliest discoveries of genetic alteration in lung cancers,[17] and about 10% of NSCLCs of Japanese patients harbored *KRAS* mutations.[18–20] We and others reported the strictly mutually exclusionary manner of *EGFR* and *KRAS* mutations.[1,15] Several meta-analyses revealed that *KRAS* mutations may be