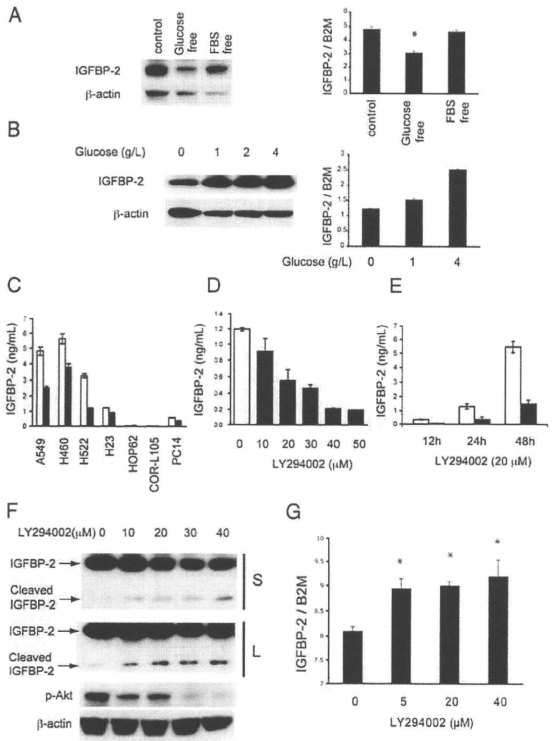

Figure 2. A: The effect of glucose and fetal bovine serum on intracellular IGFBP-2 levels. A549 cells (5×10^5) were incubated for 2 hours in fetal bovine serum (FBS) free media, followed by a 24-hour incubation in glucose free, FBS free, or regular media. The cells were then harvested and subjected to both immunoblotting and quantitative RT-PCR for IGFBP-2. IGFBP-2 mRNA was normalized to human β2 microglobulin (B2M). Values represent means ± SD. Statistical analysis was performed by Welch's *t*-test. *$P < 0.01$. **B:** A549 cells were cultured in media with the indicated concentrations of glucose for 24 hours. IGFBP-2 expression levels were measured by both immunoblotting and quantitative RT-PCR. Values represent means ± SD. Significant slope of regression line between IGFBP-2 mRNA and glucose concentration was obtained ($P = 0.012$). **C:** Effect of LY294002, a PI3K inhibitor, on extracellular IGFBP-2 levels. Seven lung adenocarcinoma cell lines were treated either with a vehicle control DMSO (white bar) or 20 μmol/L of LY294002 (black bar) for 24 hours. Secreted IGFBP-2 was measured by ELISA as described before. **D:** A549 cells were treated with the indicated concentration of LY294002 for 24 hours. A significant slope of regression line between secreted IGFBP-2 and LY294002 concentration was obtained ($P = 0.0048$). **E:** Time course of IGFBP-2 secretion in A549 cells treated with 20 μmol/L of LY294002. The 95% CI based test of slope regression was significant ($P < 0.05$): 0.134 to 0.18 vs. 0.029 to 0.045, in control DMSO and LY294002, respectively. **F:** The effect of LY294002 on intracellular levels of IGFBP-2. A549 cells were treated with the indicated concentration of LY294002, followed by immunoblotting for IGFBP-2, phosphorylated Akt (Ser 473), and β-actin. S, short exposure; L, long exposure. **G:** A549 cells were treated with the indicated concentration of LY294002, and IGFBP-2 mRNA levels were evaluated by a real-time RT-PCR. Values represent means ± SD. Statistical analysis was performed by Welch's *t*-test. *$P < 0.01$.

knockdown cells were more sensitive to camptothecin rather than vector control cells (95% CI: -2.7×10^{-4} to -1.6×10^{-4} vs. -4.5×10^{-4} to -3.0×10^{-4}, in negative control and IGFBP-2 siRNA, respectively; Figure 4B). In caspase-3 activity assay (Figure 4C), there were no significant changes in caspase-3 activity between negative control and IGFBP-2 siRNA with DMSO treatment (white bars). When cells were treated with camptothecin, IGFBP-2 siRNA significantly increased caspase-3 activity than negative control siRNA (black bars). The sensitivity to camptothecin was significantly potentiated by IGFBP-2 inhibition ($P < 0.0001$). Apoptosis was significantly increased in cells with IGFBP-2 siRNA compared with negative control siRNA ($P = 0.0009$; Figure 4D). Cleaved PARP was more substantial in IGFBP-2 siRNA treated cells compared with vector control H522 cells (Figure 4E). As a PI3K inhibitor induced IGFBP-2 degradation (Figure 2F), we examined whether a PI3K inhibitor has an additive effect on apoptosis with camptothecin. As expected, combination therapy of LY294002 and camptothecin enhanced PARP cleavage in H522 cells when compared with camptothecin or LY294002 alone. IGFBP-2 levels were inversely correlated with the increase in the levels of cleaved PARP (Figure 4F, left panels). In contrast, there were no substantial effects of LY294002 on PARP cleavage in COR-L105 cells, which have low IGFBP-2 levels (Figure 4F, right panels).

These data strongly suggest that IGFBP-2 regulates apoptosis via caspase-3. Moreover, IGFBP-2 becomes a therapeutic target as well as a biomarker for the treatment of PI3K inhibitors.

Tissue IGFBP-2 Is Overexpressed in Lung Adenocarcinoma

Next, we examined tissue expression levels of IGFBP-2 in human lung adenocarcinoma and normal tissue by using

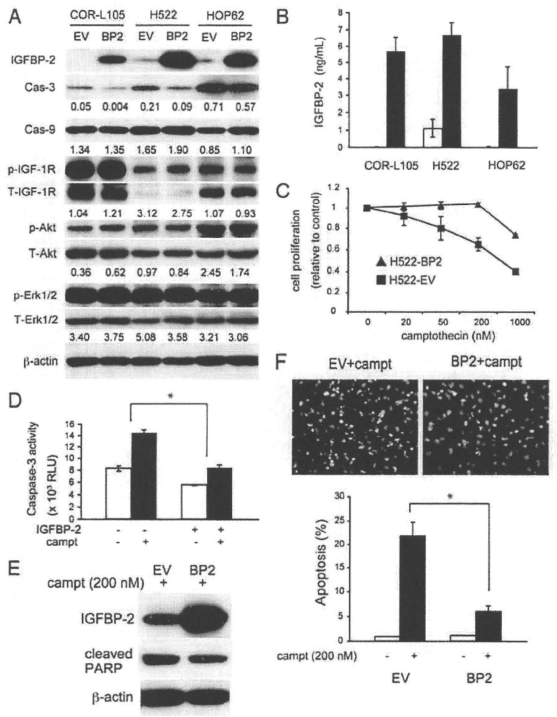

Figure 3. A: IGFBP-2 overexpression inhibits procaspase-3 expression independent of the IGF signaling pathway. Empty vector (EV) and IGFBP-2 (BP2) were transfected in COR-L105, NCI-H522, and HOP62 lung adenocarcinoma cell lines, and stably IGFBP-2 overexpressing cells were obtained. Whole cell lysates were subjected to SDS-polyacrylamide gel electrophoresis, followed by immunoblotting for IGFBP-2, procaspase-3, procaspase-9, phosphorylated and total IGF-1R, phosphorylated and total Akt, phosphorylated and total Erk1/2, and β-actin. Signal densities were quantified by ImageJ, and then procaspase-3/β-actin, procaspase-9/β-actin, p-IGF1R/T-IGF1R, p-Akt/T-Akt, and p-Erk1/2/T-Erk1/2 ratios were calculated. **B:** Secreted IGFBP-2 levels were measured by ELISA in three different stable vector- and IGFBP-2-transfected (white and black bars, respectively) cell lines. Data represent means ± SD. **C:** IGFBP-2 overexpressing and empty vector NCI-H522 cells were plated in 96 wells and treated with indicated concentration of camptothecin for 24 hours. Cell proliferation was determined by microplate reader using cell count reagent. Data represent means ± SD. The IC50 values were 686 nmol/L and more than 1000 nmol/L in empty vector and IGFBP-2 cells, respectively. **D:** Caspase-3 assay in IGFBP-2 overexpressing and empty vector NCI-H522 cells. Cells were plated in 96 wells and treated with 200 nmol/L of camptothecin for 24 hours. Caspase-3 activity was determined by a microplate reader. Data represent means ± SD. Statistical analysis of comparison between empty vector and IGFBP-2 overexpressing cells was performed by Welch's *t*-test. EV (camptothecin/DMSO) versus BP2 (camptothecin/DMSO); $^*P < 0.02$. **E:** Apoptosis was also evaluated by immunoblotting for PARP cleavage with whole cell lysate. **F:** Twenty-four hours after exposure of 200 nmol/L of camptothecin, cells were stained with Hoechst 33342. Apoptotic and nonapoptotic cells were counted by microscopy at least in three different areas, and the apoptotic rate was represented. Values represent means ± SD. Statistical analysis was performed by Welch's *t*-test. $^*P < 0.01$.

a real-time RT-PCR and Western blotting. IGFBP-2 mRNA was significantly higher in tumors than in paired normal tissue, as examined by a real-time RT-PCR ($P = 0.021$; Figure 5A). A higher amount of IGFBP-2 protein was also frequently observed in tumor tissue compared with in paired normal tissue (Figure 5B).

Inverse Relationship between IGFBP-2 and Caspase-3 Expression

Finally, immunohistochemical analysis was performed on tissue microarray including 169 cases of lung adenocarcinoma. IGFBP-2 expression was mostly confined to cancer cells, whereas normal lung epithelium revealed very low or undetectable IGFBP-2 levels (Figure 6A, arrowheads). In most cases, IGFBP-2 was localized in cytoplasm of lung adenocarcinoma cells, as shown in Figure 6A. Membraneous IGFBP-2 expression was found in only 3 of 169 cases (1.8%; Figure 6B). IGFBP-2 was expressed in early precursor lesions, and its expression levels increased gradually as the lesions progress from benign (Figure 6C, arrows) to malignant cells (Figure 6C, arrowheads). In particular, a strong IGFBP-2 expression was found in cancer cells with high nuclear grade distinct from ones with low nuclear grade even within the same gland (Figure 6D). It should be noted that the mutually exclusive expression between IGFBP-2 (Figure 6E, left panel, arrowheads and Figure 6F, left panel, upper area) and procaspase-3 (Figure 6E, right panel, arrowheads and Figure 6F, right panel, lower area) was frequently observed in lung adenocarcinomas. To summarize the immunohistochemical data, a significant inverse correlation between the groups in the numbers of patients with IGFBP-2 and procaspase-3 expression was observed in lung adenocarcinomas (Table 1).

Discussion

The IGF signaling pathway plays a pivotal role in cellular proliferation, differentiation, survival, and metabolism.

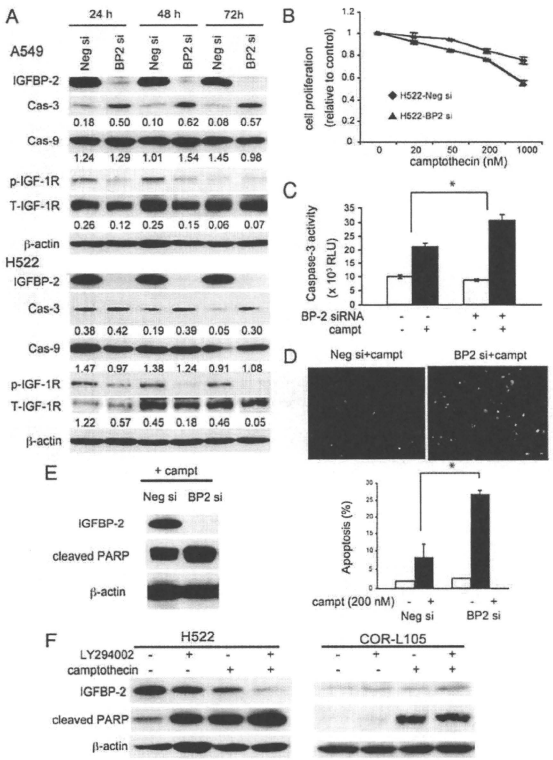

Figure 4. A: Specific IGFBP-2 inhibition resulted in the increase in procaspase-3. A549 or NCI-H522 cells were transfected with negative control or IGFBP-2 siRNA oligonucleotides, followed by immunoblot for IGFBP-2, procaspase-3, procaspase-9, phosphorylated and total IGF-1R, and β-actin at indicated times after transfection. Signal densities were quantified by ImageJ, and then procaspase-3/β-actin, Procaspase-9/β-actin, and p-IGF1R/T-IGF1R ratios were calculated. **B:** NCI-H522 cells were treated with negative control or IGFBP-2 siRNA for 24 hours and then exposed to different concentrations of camptothecin for 24 hours. Cell proliferation was determined as described before. Data represent means ± SD. A 95% CI based test of slope regression was significant ($P < 0.05$): −2.7E-04 to −1.6E-04 in negative siRNA vs. −6.5E-04 to −3.0E-04 in IGFBP-2 siRNA. **C:** Caspase-3 assay in NCI-H522 cells treated with negative control or IGFBP-2 siRNA. NCI-H522 cells were treated with negative control or IGFBP-2 siRNA for 48 hours in 96 wells and then treated with 200 nmol/L of camptothecin for 24 hours. Caspase-3 activity was determined by a microplate reader. Data represent means ± SD. Statistical analysis of comparison between negative control and IGFBP-2 siRNA was performed by Welch's *t*-test. *$P < 0.0001$. **D:** Twenty-four hours after exposure of 200 nmol/L of camptothecin, siRNA-treated NCI-H522 cells were stained with Hoechst 33342. The apoptotic rate was measured as described previously. Values represent means ± SD. Statistical analysis was performed by Welch's *t*-test. *$P < 0.001$. **E:** Apoptosis was also evaluated by immunoblot for PARP cleavage in NCI-H522 cells. **F:** NCI-H522 and COR-L105 cells were treated with 20 μmol/L of LY294002 or 200 nmol/L of camptothecin or combination of LY294002 and camptothecin for 24 hours. Immunoblot was performed with IGFBP-2, cleaved PARP, and β-actin antibodies.

IGFBPs are circulating proteins and function as modulators of IGF signaling through sequestration of IGFs in serum and the extracellular fluid. Increased levels of serum IGFBP-2 are found in certain pathophysiological conditions including fasting, diabetes mellitus, growth hormone deficiency, hepatic or renal failure, and cancer.[31] In cancer, IGFBP-2 exerts various biological functions by virtue of IGF-dependent or -independent mechanisms. Soluble IGFBP-2 binds to IGFs and consequently inhibits IGF signaling in various human cancers, including lung cancer.[19,32–34] Membrane-associated IGFBP-2 stimulates or inhibits cell proliferation and migration through a direct binding to serum and extracellular matrix molecules, such as cell surface integrin receptors, proteoglycans, and heparin.[2–5,35] Meanwhile, a number of studies demonstrate that intracellular IGFBP-2 promotes cancer cell growth in various cell types.[9,11,36] Moreover, IGFBP-2 overexpression confers resistance to apoptosis induced by chemotherapy in breast cancer cells[6] and by androgen ablation in prostate cancer.[9] Serum IGFBP-2 can be used for prediction of chemotherapy response and prognosis in ovarian cancer[37] and acute lymphoblastic leukemia.[38] Notably, IGFBP-2 is a marker for antiestrogen resistance, but not for cell growth in human breast cancer cells.[39] These observations invoke that intracellular IGFBP-2 mainly contributes to cancer cell survival independently of secreted IGFBP-2.

In the present study, we have shown that (1) intracellular IGFBP-2 regulates caspase-3 expression in an IGF independent manner; (2) IGFBP-2 overexpression prevents camptothecin-induced apoptosis, whereas IGFBP-2 inhibition promotes apoptosis; and (3) there is an inverse expression pattern between intracellular IGFBP-2 and caspase-3 in human lung adenocarcinomas.

We demonstrated a novel mechanism of antiapoptotic effect of IGFBP-2 via procaspase-3 inhibition in lung can-

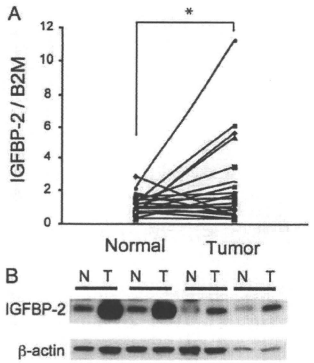

Figure 5. A: IGFBP-2 mRNA expression was measured by real-time RT-PCR in 24 pairs of human normal and corresponding tumor tissue. The mRNA levels of IGFBP-2 are presented as arbitrary units for the mRNA levels of human β2 microglobulin (B2M). A paired *t*-test was used for statistical significance (*P = 0.021). **B:** Representative picture of Western blots. IGFBP-2 protein levels were measured with four pairs of normal (N) and corresponding tumor (T) tissue from lung adenocarcinoma patients.

cer. Caspases are cysteine proteases that play essential roles in mammalian apoptosis. Procaspase-3 cleavage and consequent activation is the final step of caspase cascades in response to various apoptotic stimuli. Several authors have proved that enforced procaspase-3 potentiates sensitivity to chemotherapy and promotes apoptosis.[40–42] In lung cancer, decreased caspase-3 expression has been shown as a poorer prognostic factor in non-small-cell lung cancer.[43–45]

Our results raise the important question regarding the regulatory mechanisms involved in caspase-3 inhibition via IGFBP-2. A recent report has shown that transcriptional factor Sp1 activates the caspase-3 promoter.[46] Mammalian IGFBP-2 also has the Sp1 binding regions upstream of the transcriptional start site.[47] One possible explanation for the regulation of caspase-3 via IGFBP-2 is that IGFBP-2 overexpression in cancer cells inhibits Sp1 through negative feedback mechanism, and thereby inhibits caspase-3 gene and protein expression. Another possibility is PTEN. IGFBP-2 has been identified as the most significant molecular signature for loss of PTEN in brain and prostate cancer.[30] It has been shown that PTEN is cleaved by caspase-3 in a PTEN phosphorylation-regulated manner.[48] IGFBP-2 overexpression may induce PTEN up-regulation and protein stabilization through feedback mechanisms, and thereby negatively regulating caspase-3 activation. Future studies will help to identify the precise regulatory mechanism of caspase-3 mediated by IGFBP-2. Recent studies demonstrate caspase-3 has apoptosis-independent physiological functions, including differentiation, maturation, proliferation, and immuno response.[49,50] Thus, caspase-3 may contribute to lung cancer development and progression by multiple functions including apoptosis.

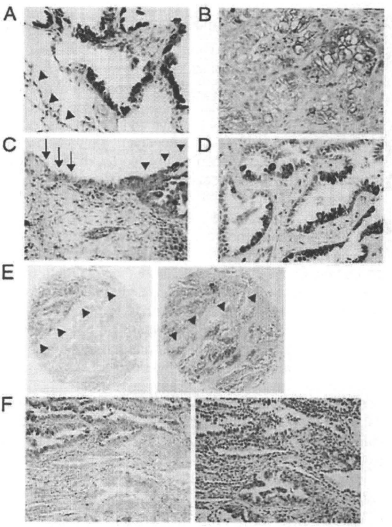

Figure 6. A: Representative pictures of immunohistochemistry for IGFBP-2 in lung adenocarcinomas. Note a strong immunoreactivity in cytoplasm of cancer cells, whereas almost negligible in normal epithelium (**arrowheads**). **B:** Typical membraneous IGFBP-2 expression. **C:** IGFBP-2 expression is gradually increased from benign cells (**arrows**) to malignant cells (**arrowheads**). **D:** Strong IGFBP-2 expression is only localized in cancer cells with high nuclear grade. **E:** Representative mutually exclusive expression between IGFBP-2 (**left, arrowheads**) and procaspase-3 (**right, arrowheads**) in serial sections on tissue microarray. **F:** Another case also demonstrates an inverse expression pattern between IGFBP-2 (**left**) and procaspase-3 (**right**) in serial sections. Original magnification: ×400 (**A–D**, and **F**); ×100 (**E**).

Because IGF signaling was not altered by the overexpression of intracellular IGFBP-2, our data suggest that intracellular and secreted IGFBP-2 are functionally independent. Interestingly, IGFBP-5 is another cancer-associated IGFBP, and it has been reported that intracellular IGFBP-5 induces growth inhibition and caspase-dependent apoptosis of breast cancer cells, whereas adding secreted-IGFBP-5 was not internalized and had no effects on growth and apoptosis.[51] Further, endogenous and exogenous IGFBP-5 is suggested to exhibit opposing actions on cell survival in osteosarcoma cells.[52] IGFBP-3, a most major IGFBP in serum, also induces

Table 1. Inverse Relationship between IGFBP-2 and Caspase-3 Expression in 169 Cases of Lung Adenocarcinomas

	Caspase-3		
IGFBP-2	Weak	Moderate	Strong
Weak	48	20	10
Moderate	40	9	2
Strong	40	0	0

Fisher's exact test was used for statistical significance (P = 0.0002). Data represent the number of patients.

growth inhibition and apoptosis in cancer cells, but it does not require the cell surface binding and nuclear translocation of IGFBP-3 in breast and prostate cancer.[53,54] These lines of evidence prompt us to propose that intracellular IGFBP-2 elicits antiapoptosis effects on cancer cells via intracrine mechanism, independent of secreted IGFBP-2. Although not yet identified in IGFBP-2, the posttranslational modification (ie, glycosylation) of secreted IGFBP-3 or -5 can be involved in the functional difference between intracellular and secreted form.[51]

There are a number of lines of evidence that IGFBP-3 is able to induce apoptosis and potentiate the apoptotic effects of UV or chemotherapy.[55,56] The inverse relationship between IGFBP-2 and IGFBP-3 expression at tissue and serum levels in a variety of cancers, including prostate,[31,55,57] ovarian,[58] and testicular cancer, has been well recognized.[59] We also found a relatively inverse relationship between secreted levels of IGFBP-2 and IGFBP-3 in lung adenocarcinoma cell lines (unpublished data). Remarkably, IGFBP-2 is predominantly expressed in cytoplasm and nucleus of lung epithelium when exposed to hyperoxia, whereas IGFBP-3 is localized in the extracellular compartment.[60] These findings suggest that IGFBP-2 and -3 may be differentially regulated and also exert a distinct action for cell proliferation and apoptosis in different compartments.

Our immunohistochemical analysis demonstrated that most adenocarcinomas revealed a cytoplasmic IGFBP-2 expression pattern, and a significant inverse association between IGFBP-2 and procaspase-3 expression. These results support the evidence that intracellular IGFBP-2 regulates procaspase-3 expression *in vitro*, thereby inhibiting apoptosis. Interestingly, IGFBP-2 expression showed a marked heterogeneity within lung adenocarcinoma tissue. At cellular levels, a strong IGFBP-2 expression was found in cancer cells having high nuclear grade. This finding suggests IGFBP-2 overexpression in cancer cells is caused by adaptive mechanisms in tumor microenvironment and confers aggressive biological nature to survive under the toxic conditions.

IGFBP-2 protein is degraded by proteases such as matrix metalloprotease-1 and -7, calpain, as well as by basic fibroblast growth factor and an androgen blockade.[61–64] We found IGFBP-2 protein was degraded by a treatment of PI3K inhibitor in A549 cells. Because a various new PI3K inhibitors have been entered clinical trials,[65] IGFBP-2 would be a useful biomarker for the treatment with PI3K inhibitors in lung cancer as well as in glioma, prostate, and breast cancers.[30,66] Further, our results suggested that IGFBP-2 is a therapeutic target in lung cancer, in line with the results in breast and ovarian cancers.[6,10] In general, lung adenocarcinomas typically showed a resistance to multiple cancer chemotherapy. Because cytoplasmic IGFBP-2 may provide cancer cells with an antiapoptotic ability, IGFBP-2 is an attractive therapeutic target especially for chemotherapy resistant tumors. The combination of chemotherapy and the IGFBP-2 or PI3K inhibitors may also potentiate drug-sensitivity.

Lung cancer is the leading cause of cancer death worldwide. Despite the availability of some cytotoxins and molecular target therapy, the efficacy of these agents is limited. It has thus become increasingly necessary to identify novel approaches to treat lung cancer. We propose that IGFBP-2 is not only a useful biomarker for predicting chemotherapy response, but also a novel therapeutic target in lung cancer.

Acknowledgments

We thank Ms. Tomoyo Kakita, Ms. Mayumi Ogawa, and Mr. Hironori Murayama for their excellent technical assistance, and Dr. Hiroaki Kataoka for IGFBP-2 constructs. We also thank Dr. Farid Gizatullin for helpful discussion.

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Lung Cancer 70 (2010) 94–102

Contents lists available at ScienceDirect

Lung Cancer

journal homepage: www.elsevier.com/locate/lungcan

Activation status of receptor tyrosine kinase downstream pathways in primary lung adenocarcinoma with reference of *KRAS* and *EGFR* mutations

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ARTICLE INFO

Article history:
Received 16 September 2009
Received in revised form 2 December 2009
Accepted 5 January 2010

Keywords:
Lung adenocarcinoma
Receptor tyrosine kinases
Survival
Akt
TTF-1
Signal pathway

ABSTRACT

The activation status of signal transduction pathways involving receptor tyrosine kinases and its association with *EGFR* or *KRAS* mutations have been widely studied using cancer cell lines, although it is still uncertain in primary tumors.

To study the activation status of main components of growth factor-induced pathways, phosphorylated Akt (pAkt), extracellular signal-regulated kinases 1 and 2 (pERK) and other downstream proteins were immunohistochemically examined using surgical samples of 193 primary lung adenocarcinomas. Also, thyroid transcription factor-1 (TTF-1) expression and mutation status of *EGFR* and *KRAS* were examined.

Advanced tumor stages ($p < 0.001$), negative TTF-1 expression ($p < 0.001$) and Akt activation ($p = 0.015$) were independent and significant poor prognostic markers. Akt activation related to advanced stage ($p = 0.021$), invasiveness ($p = 0.004$), and not to mutations. TTF-1 expression associated with never-smoker ($p = 0.013$), pre- or minimally invasiveness ($p < 0.001$) and *EGFR* mutations ($p = 0.017$) as well as with pERK ($p = 0.039$) expression. *EGFR* mutations did not correlated with pAkt and pERK expression, which was different from the results based on cultured cells, while *KRAS* mutations were solely and significantly linked to ERK activation ($p = 0.009$).

In lung adenocarcinoma, tumors with TTF-1 expression have distinct characteristics regarding mutations, signal protein activation and clinical issues. Moreover, this property was revealed to be important in outcome estimation at any tumor stage, whereas Akt activation is abnormally affected according to the tumor stage regardless of their cell origin. The signal proteins were differently related to mutation status from cultured cells.

1. Introduction

Lung cancer is one of the leading causes of cancer-related deaths worldwide [1] and adenocarcinoma is recently becoming a frequent histologic type among non-small-cell lung cancers (NSCLCs) in many countries. Gefitinib, an inhibitor of epidermal growth factor receptor (*EGFR*) tyrosine kinase, has shown remarkable efficacy for control of a subset of lung adenocarcinomas, reflecting improved understanding of the underlying biology [2]. Especially the detection of somatic mutations in *EGFR* shed light on the mechanisms of acquisition of tumor growth advantage, featuring dysregulated signal transduction in tumor cells [3,4].

Akt, a serine/threonine kinase, and extracellular signal-regulated kinases 1 and 2 (ERK) are major target proteins, downstream of EGFR and various other oncoproteins such as Ras and Raf. They are known to be activated in a wide spectrum of human cancer together with various downstream substrates such as glycogen synthase kinase 3-beta (GSK3B), mammalian target of Rapamycin (mTOR), p70 ribosomal protein S6 kinase (S6K) and forkhead proteins FKHR/FKHRL1 (FKHR) [5–8] and to play central roles in tumorigenesis or cell proliferation. The present study was performed to elucidate, by immunohistochemistry (IHC), whether there might be selective activation of downstream pathways of receptor tyrosine kinases (RTKs) in lung adenocarcinomas, depending on tumor-cell lineage or with/without *EGFR* and *KRAS* mutations. We further evaluated the clinicopathological and prognostic significance of such activation in various adenocarcinoma subtypes. Since we earlier revealed by expression profiling that

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doi:10.1016/j.lungcan.2010.01.001

adenocarcinoma cell lines might have different characteristics of gene expression from clinical adenocarcinomas [9], we here used tissue materials of surgically resected adenocarcinomas rather than cell lines.

2. Materials and methods

2.1. Patients and pathological review

A series of 193 Japanese cases with primary lung adenocarcinoma surgically resected between 1998 and 2001 at the Department of Thoracic Surgical Oncology, The Cancer Institute Hospital, Japanese Foundation for Cancer Research (JFCR), Tokyo, were selected for the present study. Informed consent was obtained from all the subjects at the time of surgery. This study was approved by the Institutional Review Board of the JFCR. All patients were staged pathologically according to the 5th edition of the UICC-TNM staging system [10]. For accuracy of survival analysis, only death of lung cancer was counted as cause-specific death. Smoking history was ascertained with all patients in detail.

Histological diagnosis was made according to the WHO classification [11], using sections through the largest cut surface of each tumor stained by hematoxylin–eosin and alcian-blue methods and PAS reaction. However, with its subdivision of lung adenocarcinomas, more than 80% tumors fell into the mixed subtype category. We therefore additionally used an noninvasive/invasive dichotomy as well as a predominance classification for invasive carcinomas, which is mostly based on the WHO classification except for the mixed subtype, such as bronchioloalveolar carcinoma (BAC) predominant, papillary predominant, acinar predominant, etc. The noninvasive carcinoma includes BAC. In the predominance classification of invasive carcinomas, we diagnosed by a component that makes up the predominant portion in the largest cut surface, or the cut surface containing a solid part shown by CT scans, of a tumor. Also, we employed a concept of "minimally invasive adenocarcinomas", which were defined to be lesions where an invasive area of less than 5 mm in diameter or less.

2.2. Tissue microarrays

Tumor tissues were fixed in 15% neutral formalin and embedded in paraffin. Three histologically representative sites were selected per tumor, considering the well-known heterogeneity of lung adenocarcinomas (including the peripheral boundary and the central part of each tumor) and tissue microarrays were constructed as follows. Selected points of the donor paraffin blocks of the largest cut surface were punched with a 2-mm-diameter coring needle, and transferred to the array in the recipient block using a manual tissue arrayer (KIN-1, Azumaya, Inc, Japan). 48 human tissue rods (16 tumors) were embedded in one tissue array block. Based on our preliminary examinations using whole sections of tumor for several cases, we adopted 2 mm needles and three points to take tissues, rather than smaller needles and only one or two points. As controls, 3 mouse xenografts were selected from the panel of 39 cell lines (termed JFCR 39) [12] and embedded together with clinical samples in each array block as detailed below.

2.3. Protein expression analysis

Phosphorylated protein levels of Akt, ERK, GSK3B, mTOR, S6K and FKHR were immunohistochemically examined using antibodies for phosphorylated proteins designated by pAkt, etc. Also, thyroid transcription factor-1 (TTF-1) was examined for cell lineage analysis. The primary antibodies and citrate buffer used in this study are listed in Suppl. Table 1 and details of our immunohistochemical technique are also available on this. Antigen–antibody complexes were detected by labeling with the Envision+/HRP system (DAKO, Carpinteria, CA, USA), using 3,3′-diaminobenzidine tetrachloride as the chromogen and hematoxylin as counterstain. As well as using some normal cells as internal controls, mouse xenografts of the three cancer cell lines (PC-3, a prostate cancer line, showing high pAkt and low pERK expression, U251, a brain tumor line, showing moderate pAkt and low pERK expression and HTB26, a breast cancer line, showing low pAkt and high pERK expression) were included as external controls (Fig. 1), because we sought better quantification of immunoreactivity of each antibody by using well-known cell lines with well-documented reactivity. Immunoreactivity of each case was evaluated for all the tumor cells (or other cells of interest) appearing in all the three portions, applying the staining results for these xenografts. Essentially, for pAkt and pFKHR cytoplasmic staining (Fig. 1 and Suppl. Fig. 1), and for pS6K and pGSK3B whole cell staining were evaluated respectively, referring to the U251 levels. For pmTOR staining, comparison was with HTB26. All these were recorded as dichotomous parameters categorized as "negative" (weaker than or equal to xenograft staining) and "positive" (stronger than xenograft staining). For pERK immunoreactivity, the percentages of cells with positive staining were recorded and a score of 10% or less was categorized as "negative" and a score of more than 10% as "positive".

2.4. Mutation analysis of EGFR and KRAS

The mutation status of four exons of the *EGFR* gene and three codons of the *KRAS* gene was evaluated in the subset ($n = 93$) of the 193 cases. The primer sequences for exons 18 and 21 of *EGFR* were as follows (forward and reverse, respectively). exon 18 (5′-TCCAAATGAGCTGGCAAGTG-3′ and 5′-TCCCAAATACTCAGTGAACAAA-3′), exon 21 (5′-GATGCAGAG-CTTCTTCCCAT-3′ and 5′-ATACAGCTAGTGGGAAG GCA-3′). For *KRAS*, codon 12 and codon 13 (5′-CCTTATGTGTGACATGTTCT-3′ and 5′-CTATTGTTGGATCATATTCG-3′), codon 61 (5′-TTCC-TACAGGAAGCAAGTA-3′ and 5′-GGCAAATACACAAAGAAAGC C-3′). All PCR assays were carried out in a 20 μL volume that contained 0.2 μL of Taq DNA polymerase (NEB Phusion TM High Fidelity DNA polymerase sets, Finnzymes Oy, Finland). DNA was amplified for 35 cycles at 98 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s, followed by a 7 min extension at 72 °C. All PCR products were incubated with exonuclease I and shrimp alkaline phosphatase (USB corporation, Exo SAP-IT, OH, USA) according to the manufacturer's instructions and then sequenced directly by a cycle sequencing method (Beckman Coulter Inc, DTLS-Quick Start Kit, CA, USA). All sequence variants were confirmed by sequencing the products of independent PCR amplifications in both directions. To detect deletion in exon 19 and insertion in exon 20 of *EGFR*, common fragment analysis was used. Sample DNA was amplified with a Cy5-labeled primer set as follows: exon 19 (5′ Cy5-GTCTTCCTTCTCTCTCTGTCAT-3′ and 5′-TGTGGAGA GTGAGCAGGGTCT-3′), exon 20 (5′ Cy5-A CCATGCGAAGCCACACTGA-3′ and 5′-TCCTTATCTCCCCTCCCCGTAT-3′) and any deletion or insertion mutation was detected as a new peak of amplified products in the electrophoregram.

2.5. Statistical analysis

Statistical analyses were accomplished with STATA software, version 9 (Stata Corp. LP, College Station, TX, USA) and statistical programming language of R [13]. We studied the relationships between the survival and other clinicopathological factors and phospho-protein expression by univariate analyses of log-rank test. Then multivariate analyses using Cox regression model together with an AIC (Akaike Information Criteria) stepwise selection were applied to those factors to evaluate their relative risks. Then, correlation coefficients between each clinicopathological or

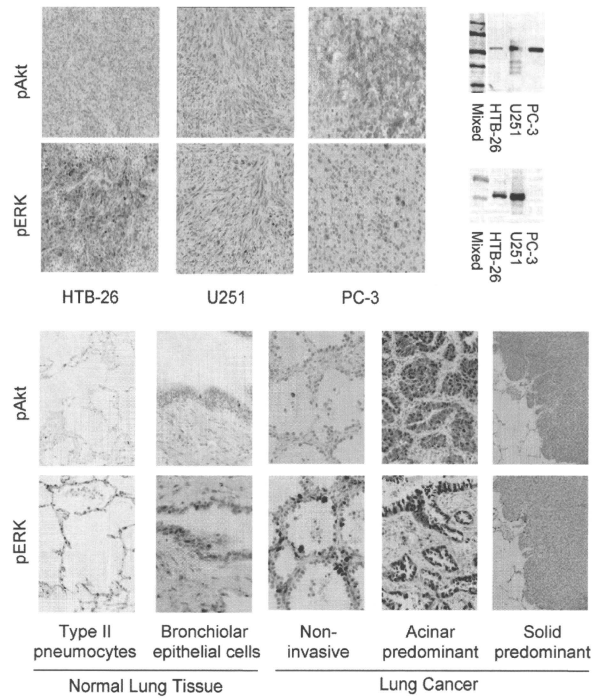

Fig. 1. Immunohistochemical staining of xenografts with specific antibody against pAkt (the upper row) and pERK1/2 (the lower row). (a) HTB-26; xenograft of breast cancer cell negative for pAkt and positive for pERK1/2; (b) U251; xenograft of glioma cell moderately positive for both pAkt and pERK1/2; (c) PC-3; xenograft of prostate cancer cell positive for pAkt and negative for pERK1/2. Almost all the cells with mitotic figure were scattered and were positive for pERK1/2. Immunohistochemical staining of resected lung specimen with specific antibody against pAkt (the upper row) and pERK1/2 (the lower row). Normal lung tissues (left side) and lung adenocarcinoma of noninvasive, acinar predominant and solid predominant histology (the right side). See that pAkt is almost homogeneously stained compared to that of pERK1/2.

immunohistochemical parameter were calculated and *p*-values for the statistical significance were given by a two-tails test checking a null hypothesis about zero Pearson's correlation coefficient between two variables.

Two-sided *p*-value below 0.05 was designated statistically significant.

3. Results

Patient characteristics of the 193 cases and differences in survival according to each clinicopathological factor or protein expression were shown in Table 1.

3.1. Patients and pathological review

The gender distribution was equal, the median age was 63 years and the median follow-up period was 2066 days (5.66 years) (ranged 133–3292 days). Pathological review revealed that more than 80% of the cases were classified as the adenocarcinoma with mixed subtype according to the current WHO classification. Therefore results based on the predominance classification together with invasive/noninvasive dichotomy were presented here. 22.8% ($n = 44$) of the cases were classified as pre- and minimally invasive adenocarcinomas. The rest (77.2%; $n = 149$) were invasive adenocarcinoma in which papillary (including micropapillary), acinar, solid patterns or patterns of other variants are predominantly recognized. The rates were 61.1% ($n = 118$), 9.9% ($n = 19$) and 6.2% ($n = 12$), respectively.

3.2. Immunohistochemical study and the EGFR/KRAS mutation status

We observed rather homogeneous and tumor-specific staining patterns for pAkt although the intensity was low, 37.8% (73/193) of surgically excised lung adenocarcinomas being positive. The positive rates for pGSK3B, pmTOR, pS6K, pFKHR and TTF-1 were 30.6% ($n = 59$), 34.7% ($n = 68$), 52.3% ($n = 101$), 40.4% ($n = 78$) and 79.8% ($n = 154$), respectively. The pERK staining pattern was characteristically heterogeneous. The rate of positive tumor cells ranging from 5% to 100% and the staining was not tumor-cell specific but rather

Table 1
Characteristics of the patients ($n = 193$) and differences* in survival rate according to each factor.

	N	5-Year survival (%)	95% CI	p-Value *
Patient and tumor characteristics				
Gender				
Male	96	71.6	0.61–0.80	0.027
Female	97	85.3	0.76–0.91	
Age				
<60	74	81.4	0.70–0.89	0.274
60 or older	119	76.5	0.67–0.83	
Smoking habit				
Never	89	83.2	0.73–0.90	0.151
Ever	104	74.3	0.64–0.82	
Stage				
I	120	94.8	0.89–0.98	<0.001
II–IV	73	48.7	0.36–0.61	
Adenocarcinoma classification				
Pre + minimally invasive**	44	96.5	0.83–0.99	<0.001
Invasive**	149	72.9	0.65–0.80	
Expression status (IHC study)				
pAkt				
–	120	85.3	0.77–0.91	0.007
+	73	68.2	0.56–0.79	
pERK				
–	124	73.8	0.65–0.81	0.058
+	69	86.4	0.76–0.93	
pGSK3B				
–	134	76.7	0.68–0.83	0.289
+	59	82.0	0.69–0.90	
pmTOR				
–	125	75.7	0.77–0.83	0.214
+	68	83.1	0.71–0.90	
pS6K				
–	92	74.1	0.63–0.82	0.260
+	101	82.3	0.73–0.89	
pFKHR				
–	115	85.6	0.77–0.91	0.006
+	78	68.1	0.56–0.77	
TTF-1				
–	39	49.4	0.32–0.65	<0.001
+	154	85.5	0.79–0.90	
Mutation status ($n = 93$)				
EGFR mutation				
–	39	69.2	0.52–0.81	0.175
+	54	84.8	0.72–0.92	
KRAS mutation				
–	86	78.7	0.68–0.86	0.774
+	7	71.4	0.26–0.92	

* Log-rank test (p-value); CI: confidence interval; N: lymph node metastasis.
** Pre + minimally invasive: adenocarcinoma in situ + lepidic pattern predominant adenocarcinoma with minimal invasion (<10% or ≤5 mm invasion); Invasive: adenocarcinoma of papillary (including micropapillary), acinar or solid pattern predominant and other variants; IHC: immunohistochemistry; TTF-1: thyroid transcription factor-1; pAkt: phosphorylated Akt; pERK: phosphorylated extracellular signal-regulated kinase; pGSK3β: phosphorylated glycogen synthase kinase 3β; pmTOR: phosphorylated mammalian target of rapamycin; pS6K: phosphorylated ribosomal protein S6 kinase; pFKHR: phosphorylated forkhead transcription factors.

ubiquitous. The contrast between positive and negative cells was excellent. With the 10% cutoff, 35.8% (69/193) of the tumors were positive for pERK (Fig. 1).

The results of mutation analysis of exons 18–21 of *EGFR* and codons 12, 13 and 61 of *KRAS* are detailed in Suppl. Table 2. *EGFR* mutations were detected in 54 cases (58.1%), among which 90% were in exons 19 and 21. Mutations of *KRAS* were seen in seven cases (7/93, 7.5%), all of which were at codon 12. The *EGFR* and *KRAS* mutations were mutually exclusive except in one case. Of note, both pAkt and pERK were strongly stained in this case with double mutation of *EGFR* and *KRAS*. Types of *EGFR* mutation did not appear to affect the pattern of pathway activation. All but one of the cases with *KRAS* mutation were strongly positive for pERK ($p = 0.009$, Table 4), whereas the *EGFR* mutation did not correlate to pERK ($p = 0.294$) nor pAkt ($p = 0.409$) expression.

3.3. Patient survival

By univariate analyses using log-rank test, gender ($p = 0.027$), stage ($p < 0.001$), adenocarcinoma classification (invasive or not) ($p < 0.001$), expression of TTF-1 ($p < 0.001$) and cytoplasmic staining of pAkt ($p = 0.007$) and pFKHR ($p = 0.006$) were significantly related

Table 2
Multivariate analysis for factors predicting poor prognostic outcome ($n = 193$).

Variable	Relative risk	95% CI	*p*-Value
Cox regression analysis			
Patient and tumor characteristics			
Female	0.48911	0.25–0.93	0.030
Age (60 or older)	1.43993	0.75–2.78	0.268
Ex or current smoker	1.58799	0.84–3.00	0.147
Stages II–IV	10.96302	5.02–23.92	<0.001
Adenocarcinoma classification (invasive*)	1.81110	1.05–2.73	0.030
Expression status (IHC study)			
TTF-1+	0.24379	0.13–0.45	<0.001
pAkt+	2.28909	1.22–4.29	0.009
pERK+	0.50813	0.25–1.04	0.051
pGSK3B+	0.68778	0.34–1.40	0.289
pmTOR+	0.64731	0.32–1.29	0.204
pS6K+	0.70277	0.38–1.30	0.260
pFKHR+	2.34981	1.26–4.38	0.007
Mutation status ($n = 93$)			
EGFR mutation	0.56279	0.24–1.31	0.180
KRAS mutation	1.23578	0.29–5.29	0.780
Cox regression analysis with step wise selection			
Female	0.54	0.27–1.06	0.074
Stages II–IV	10.542	4.61–24.1	<0.001
pAkt+	2.268	1.17–4.38	0.015
pFKHR+	1.812	0.95–3.48	0.073
TTF-1+	0.282	0.15–0.54	<0.001

* CI: confidence interval; invasive: adenocarcinoma with a frankly invasive region; TTF-1: thyroid transcription factor-1; pAkt: phosphorylated Akt; pERK: phosphorylated extracellular signal-regulated kinase; pGSK3β: phosphorylated glycogen synthase kinase 3β; pmTOR: phosphorylated mammalian target of rapamycin; pS6K: phosphorylated ribosomal protein S6 kinase; pFKHR: phosphorylated forkhead transcription factors.

to survival (Table 1). Multivariate analysis using the Cox's proportional hazard model revealed that stage ($p < 0.001$), expression of TTF-1 ($p < 0.001$) and pAkt ($p = 0.015$) are statistically significant factors for prognosis independent of any other conditions (Table 2).

3.4. Clinicopathological backgrounds of adenocarcinomas with Akt activation or TTF-1 expression

Since pAkt and TTF-1 expression was revealed to be independent prognostic factors, relationships between their positive status and other factors including clinicopathological characteristics and expression of other phosphorylated proteins are summarized in Table 3. pAkt expression was significantly associated with advanced stage (stage II-IV, $p = 0.021$) and lymph node metastasis ($p = 0.002$) but not with expression of any other signal proteins or TTF-1. TTF-1 expression, on the contrary, was significantly associated with never-smoker status ($p = 0.013$) and pre- or minimally invasive nature ($p < 0.001$) as well as with expression of pERK ($p = 0.039$) and pmTOR ($p = 0.014$). Also, TTF-1 expression was related to *EGFR* mutation ($p = 0.017$). Regarding invasiveness of tumor, the pAkt activation frequency tended to be lower in the pre- and minimally invasive adenocarcinomas (18.2%; 8/44) as compared with invasive types (43.6%; 65/149) and this tendency was highly and statistically significant ($p = 0.004$). In contrast, TTF-1 staining was more frequently seen among the pre- and minimally invasive adenocarcinomas ($p < 0.001$).

Correlation coefficients between each factor were shown in Table 4. The general tendency was largely in concert with results of survival analysis (Tables 1 and 2) and expression of Akt and TTF-1. Interestingly, activation of signal proteins of ERK had similar and statistically (or marginally) significant correlation to non-smoking ($p = 0.035$), stage I ($p = 0.029$) and noninvasive status ($p = 0.001$). GSK3B expression was related significantly to activation of ERK ($p = 0.001$), mTOR ($p < 0.001$) and S6K ($p = 0.018$). *EGFR* mutations were significantly associated with female gender ($p = 0.031$), non-smokers ($p = 0.016$), TTF-1 expression ($p = 0.008$) and pS6K expression ($p = 0.008$).

4. Discussion

By IHC applied to primary adenocarcinoma tissues and mouse xenografts of cell lines used as controls, we succeeded in demonstrating Akt activation to be an independent marker of poor prognosis. In addition, TTF-1, which is known to be a marker of type II cell differentiation, was also proved to be a significant favorable prognostic marker in correlation with activation of ERK and mTOR or *EGFR* mutation regardless of tumor stages. The expression of TTF-1 is important in outcome estimation of lung carcinoma at any tumor stage whereas Akt activation is abnormally affected according to the aggressiveness of the tumors regardless of their cell origin. While *EGFR* mutations had no correlation to activation of Akt or ERK pathways, six of seven cases with *KRAS* mutation were remarkably stained for pERK throughout the tumors. Finally, FKHR expression was established to be a marker for poor prognosis.

This study demonstrated that activated Akt was associated independently and significantly with poor prognosis ($p = 0.015$), which was in agreement with previous studies [14–19]. In fact, pAkt expression correlated with factors such as stages II-IV, positive lymph node metastasis and the invasive histology (Table 3), suggesting that Akt activation is an acquired characteristic according to tumor aggressiveness. To cast light on actual regulators and substrates of Akt that might mediate regulatory mechanisms in primary lung adenocarcinoma, we compared the activation status of Akt with upper and downstream components of the signal transduction. However our results somewhat differed from those obtained earlier with cell lines. For example, *EGFR* mutations in NSCLC cell lines were reported to selectively activate the Akt pathway [3], but in our primary tumors, this correlation between Akt and constitutively activated RTK was not significant ($p = 0.409$). Moreover, in our study, expression of pAkt did not correlate with activation of any of the downstream signal proteins such as GSK3B, mTOR, S6K and FKHR in the burgeoning list of Akt substrates implicated in oncogenesis [20,21]. Linkage of Akt with those activated downstream signal proteins has been confirmed in vitro, but the results in clinical samples have been inconsistent [18,22–24]. Certainly, Akt activation is an important factor for development and proliferation of cancer cells and perhaps a marker for targeted therapies, the exploration of this area especially in real tumors has definitely been inadequate and further study is needed.

Our multivariate analysis revealed that TTF-1 expression was also a statistically significant and independent prognostic factor ($p < 0.001$). TTF-1 is a regulator of normal lung development or maintenance of type II pneumocytes [25] and was expressed in 79.8% (154/193) of the cases. Recently, a model of lineage specific dependency on TTF-1 in a subset of adenocarcinoma, that is, the terminal respiratory unit (TRU) type adenocarcinoma, has been proposed [26]. In our study, TTF-1 staining was correlated with never-smoker ($p = 0.013$) and *EGFR* mutation status ($p = 0.039$) independently of tumor stage, which is consistent with the concept of lineage specific tumorigenesis in this subset of lung adenocarcinoma. These insights suggest the importance of the cell lineage that tumors were derived from in outcome estimation of lung carcinoma.

ERK was activated in 35.8% (69/193) of the cases in our study and was associated with pathologically early stages, the pre or minimally invasiveness and TTF-1 expression ($p = 0.039$). Normal tissues, such as type II pneumocytes or interstitial fibroblasts, were also positive with varying intensity (Fig. 1). Generally, ERK activation is known not only as the result of oncogenic dysregulation

Table 3
Backgrounds of patients with expression of a significant prognostic factors; pAkt and TTF-1 ($n = 193$).

	No. of cases with expression of pAkt and TTF-1 (%)				
	Total 193	pAkt 73 (37.8)	p-Value*	TTF-1 154 (79.8)	p-Value*
Patient and tumor characteristics					
Gender					
Male	96	33 (35.1)	0.371	73 (76.0)	0.214
Female	97	40 (42.1)		81 (83.5)	
Age					
<60	74	30 (40.5)	0.760	62 (83.8)	0.357
60 or over	119	43 (37.4)		92 (77.3)	
Smoking habit					
Never	89	37 (42.0)	0.374	78 (87.6)	0.013
Ever	104	36 (35.6)		76 (73.1)	
Stage					
I	120	37 (31.9)	0.021	99 (82.5)	0.269
II–IV	73	36 (49.3)		55 (75.3)	
N					
–	135	41 (31.3)	0.002	112 (83.0)	0.118
+	57	32 (56.1)		42 (73.7)	
Adenocarcinoma classification**					
Pre + minimally invasive	44	8 (18.2)	0.004***	44 (100.0)	<0.001
Invasive	149	65 (43.6)		110 (73.8)	
Pap-pred	118	52 (44.1)		91 (77.1)	
Acinar-pred	19	9 (47.4)		14 (73.7)	
Solid-pred and other variants	12	4 (33.3)		5 (41.7)	
Other IHC results					
pAkt					
–	120			94 (78.3)	0.851
+	73			58 (79.5)	
pERK					
–	124	51 (41.8)	0.275	93 (75.0)	0.039
+	69	22 (32.8)		61 (88.4)	
pGSK3B					
–	134	48 (36.6)	0.421	103 (76.9)	0.331
+	59	25 (43.1)		50 (84.7)	
pmTOR					
–	125	49 (39.2)	0.754	93 (74.4)	0.014
+	68	24 (35.3)		61 (89.7)	
pS6K					
–	92	39 (42.4)	0.370	73 (79.3)	1.000
+	101	34 (33.7)		81 (80.2)	
pFKHR					
–	115	39 (33.9)	0.879	87 (75.7)	0.101
+	78	34 (43.6)		67 (85.9)	
TTF-1					
–	39	15 (38.4)	0.851		
+	154	58 (37.7)			
Mutation status ($n = 93$)					
EGFR mutation					
–	39	16 (41.0)	0.409	26 (66.7)	0.017
+	54	27 (50.0)		48 (88.9)	
KRAS mutation					
–	86	41 (47.7)	0.445	70 (81.4)	0.148
+	7	2 (28.6)		4 (57.1)	

* Results of Fisher's exact test; pAkt: phosphorylated Akt; TTF-1: thyroid transcription factor-1; N; lymph node metastasis.
** Adenocarcinoma classification; see text for details; -pred: predominant; Pap: papillary pattern including micropapillary pattern; Acinar: acinar pattern; Solid: solid with mucin formation pattern.
*** Comparison between "Preinvasive + minimally invasive" vs other "invasive" carcinoma; IHC: immunohistochemistry; pERK: phosphorylated extracellular signal-regulated kinase; pGSK3B: phosphorylated glycogen synthase kinase 3B; pmTOR: phosphorylated mammalian target of rapamycin; pS6K: phosphorylated ribosomal protein S6 kinase; pFKHR: phosphorylated forkhead transcription factors.

Table 4
Correlation analysis of clinicopathological data, expression of phosphoryated proteins and mutation status.

	Gender [Female]	Age [60≦]	Smoking habit [Ever]	Stage [II-IV]	Adenocarcinoma Classification [Invasive]	TTF-1 [+]	pAkt [+]	pERK [+]	pGSK3B [+]	pmTOR [+]	pS6K [+]	pFKHR [+]	*EGFR* mutation
N=193													
Age [60≦]	-0.049	1											
Smoking habit [Ever]	-0.630 (<0.001)	0.011	1										
Stage [II-IV]	-0.063	-0.028	0.079	1									
Adenocarcinoma Classification [Invasive]	-0.021	0.058	-0.191	0.252 (<0.001)	1								
TTF-1 [+]	0.110	-0.058	-0.191 (0.009)	-0.087	-0.153 (0.039)	1							
pAkt [+]	0.083	-0.048	-0.069	0.185 (0.012)	0.107	-0.041	1						
pERK [+]	0.107	0.138	-0.156 (0.035)	-0.161 (0.029)	-0.235 (0.001)	0.178 (0.016)	-0.006	1					
pGSK3B [+]	0.098	-0.014	-0.119	-0.083	0.008	0.073	0.064	0.250 (<0.001)	1				
pmTOR [+]	0.164 (0.026)	-0.107	-0.213 (0.004)	-0.011	-0.160 (0.003)	0.203 (0.006)	-0.036	0.246 (<0.001)	0.234 (0.001)	1			
pS6K [+]	0.279 (<0.001)	0.063	-0.257 (<0.001)	-0.086	-0.134 (0.072)	0.054	-0.049	0.175 (0.018)	0.241 (0.001)	0.233 (0.001)	1		
pFKHR [+]	-0.029	-0.048	0.087	0.140	0.261 (<0.001)	-0.099	0.020	-0.138	0.040	-0.036	-0.096	1	
N=93													
EGFR mutation	0.224 (0.031)	0.040	-0.249 (0.016)	0 (1.000)	0.063	0.272 (0.008)	0.089	0.112	0.111	-0.005	0.274 (0.008)	-0.181	1
KRAS mutation	0.032	-0.024	0.125	-0.115	-0.012	-0.159	-0.101	0.268 (0.009)	0.051	0.095	0.044	0.072	-0.253 (0.014)

Correlation coefficients were calculated assuming the conditions described in the parenthesis [ ] to be observed. For abbreviations, see text. Coefficients values highlighted by underline imply statistical significance. Numbers in ( ) are *p*-values. For details of invasiveness, see text. Darkly shadowed cells imply significantly positive correlation and weakly shadowed cells imply significantly (or marginally) negative correlation. TTF-1: thyroid transcription factor-1; pAkt: phosphorylated Akt; pERK: phosphorylated extracellular signal-regulated kinase; pGSK3B: phosphorylated glycogen synthase kinase 3B; pmTOR: phosphorylated mammalian target of rapamycin; pS6K: phosphorylated ribosomal protein S6 kinase; pFKHR: phosphorylated forkhead transcription factors.

but also as an essential component of epithelial cell development or adaptation to changing circumstances [27–29]. Moreover, ERK regulation depends on very complex mechanisms, involving several intracellular parameters [30], timing or balance of the signals [31–34] and other unknown factors [35]. Considering these arguments, we may conjecture that ERK activation in lung adenocarcinoma mostly reflects the intracellular signal transduction of normal cells, which is still preserved within early-staged adenocarcinoma especially with TTF-1 expression, and that the ERK pathway is gradually switched off as the tumor cells progress to a more malignant phenotype. Also, these results shown here is in line with the recent studies of American cases [36], where the ERK pathway was more activated in earlier stages and the Akt pathway in advanced stages. In further studies, considering significant heterogeneity of ERK activation, we may use more detailed judgment criteria for immunoreactivity and whole sections of tumor, rather than tissue microarrays.

Six of seven cases with *KRAS* mutation were remarkably stained for pERK throughout the tumors, consistent with previous reports [37,38]. It is notable that the clinical impact of such *KRAS* mutation-induced ERK activation is enormous since it has already been shown in mice models that tumors with ERK activation due to *KRAS* or *BRAF* mutations can be successfully treated by an inhibitor of MEK, a signal protein upstream of ERK [39]. Our results imply so far the presence of at least two causes for ERK activation in lung adenocarcinoma, one is the vestige of normal intracellular signal and the other is the impact of *KRAS* mutation.

Two other supplementary implications were obtained from this study. Among downstream proteins of the Akt pathway, only S6K was significantly expressed in cases with the *EGFR* mutation ($p = 0.008$) and may potentially be an alternative marker for *EGFR* mutation. S6K is known to regulate ribosomal biogenesis and to play an important role in progression of G1 phase of the cell cycle [41,42]. This correlation between *EGFR* and S6K suggests again a cross talk between the Akt and ERK pathways and similar result was previously described by Conde et al. [40]. Our results also indicated cytoplasmic localization without intranuclear accumulation of pFKHR protein to be an adverse prognostic factor. FKHR is a member of a transcription factor family and represents a mammalian counterpart of DAF16, first identified at chromosomal breakpoints in human tumors [20]. Subcellular localization of FKHR is known to play an important role by regulating cell cycle and apoptosis in normal cell, which is consistent with our result. Further accumulation of cases will be needed to confirm those possibilities.

As a reference for IHC evaluation we here used mouse xenografts selected from a cell line panel repeatedly used in drug research [12,43]. This resulted in more accurate and reproducible evaluation of protein expression, implying the usefulness of tumor xenografts for clinical researches.

Conflict of interest statement

Kengo Takeuchi is a consultant for DAKO.

Acknowledgements

We thank Dr. Michiyo Okui for discussion, Ms. Kazuko Yokokawa, Ms. Miyuki Kogure, Mr. Motoyoshi Iwakoshi, Ms. Tomoyo Kakita and Ms. Yuki Togashi for their technical assistance, and Ms. Yuki Takano and Ms. Hiroko Nagano for secretarial help.

Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, from the Japan Society for the Promotion of Science, grants from the Ministry of Health, Labour and Welfare, the Smoking Research Foundation, the National Institute of Biomedical Innovation, and the Vehicle Racing Commemorative Foundation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.lungcan.2010.01.001.

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Is the Epidermal Growth Factor Receptor Status in Lung Cancers Reflected in Clinicopathologic Features?

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• *Context.*—Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors are molecular-targeted drugs that are innovatively effective for non–small cell lung carcinomas with *EGFR* mutations. Epidermal growth factor receptor is a transmembrane receptor forming dimers on ligand binding. These then stimulate signals by activating receptor autophosphorylation through tyrosine kinase activity. Autophosphorylation triggers intracellular pathways facilitating malignant conversion. The most clinically advanced EGFR inhibition strategies include small-molecule inhibition of the intracellular tyrosine kinase domain (gefitinib and erlotinib) and monoclonal antibody–mediated blockade of the extracellular ligand-binding domain (cetuximab). Lung cancers with *EGFR* mutations are prevalent among patients who are female, of Asian ethnicity, and nonsmokers; thus, they can obtain benefit from EGFR tyrosine kinase inhibitors.

Objective.—To survey histopathologic findings and examine correlations with *EGFR* mutations. We mainly focused on component cell types (hobnail, columnar, and polygonal) and presence or absence of bronchioloalveolar carcinoma elements and a micropapillary pattern. Although *EGFR* mutations can be detected by various methods, including polymerase chain reaction–Invader assay or direct sequencing, these are inconvenient.

Data Sources.—Review of the published literature.

Conclusion.—Detailed pathologic examination showed significant genotype-phenotype correlations between *EGFR* mutations and presence of a bronchioloalveolar carcinoma component, a micropapillary pattern, and the hobnail cell type. We conclude that these characteristic histologic features are good predictors of *EGFR* mutations, and patients with these features might be good candidates for and could benefit from therapy with EGFR tyrosine kinase inhibitors.

(*Arch Pathol Lab Med.* 2010;134:66–72)

The epidermal growth factor receptor (EGFR), a receptor of ligands including epidermal growth factor (EGF), is a 170-kDa glycoprotein tyrosine kinase protein that straddles the cell membrane.

Expression of EGFR is prevalent in variant normal cells including cells of epidermal, mesenchymal, and neurogenic origins. When EGF binds to EGFR, signaling pathways are activated that can lead to cell proliferation and differentiation. Epidermal growth factor receptor plays an important role in cell differentiation, development, proliferation, and maintenance. With *EGFR* gene overexpression due to mutation or structural alteration, carcinogenesis, invasion, and metastasis are facilitated.

In recent years there has been substantial interest in developing novel therapeutic agents that specifically target growth factor pathways that are dysregulated in cancer cells. Non–small cell lung cancer (NSCLC) is the most frequent cause of cancer death in the world and targeting EGFR has played a central role in advancing NSCLC research to improve patient outcome during the last several years. With the move to personalized cancer therapy, we need to understand oncologic biology at the molecular and histopathologic levels in individual lesions. In this review article, we focus on clinicopathologic features related to EGFR change and consider their indications for clinical application.

GENE MUTATIONS IN NON–SMALL CELL LUNG ADENOCARCINOMAS

Lung cancer is the leading cause of cancer death in men and women worldwide and identification of activating mutations of *EGFR* is one of the most intriguing recent discoveries in the field of lung cancer research.[1,2] Epidermal growth factor receptor mutations are present in a particular subtype of lung adenocarcinomas, and cancers with this mutation have been shown to be highly sensitive to chemical inhibitors of the kinase activity of EGFR. This subtype is prevalent among patients who are female, of Japanese and other Asian ethnicity, and nonsmokers.[2–4] K-ras is a downstream mediator of EGFR-induced cell signaling, and K-ras mutations confer constitutive activation of the signaling pathways without EGFR activation. Growing evidence indicates that K-ras mutations are also important in the development of lung carcinomas.[5] Very recently, we found a novel transforming fusion gene resulting from linkage between the echinoderm microtubule-associated protein like 4 (*EML4*) and anaplastic lymphoma

Accepted for publication February 20, 2009.

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The authors have no relevant financial interest in the products or companies described in this article.

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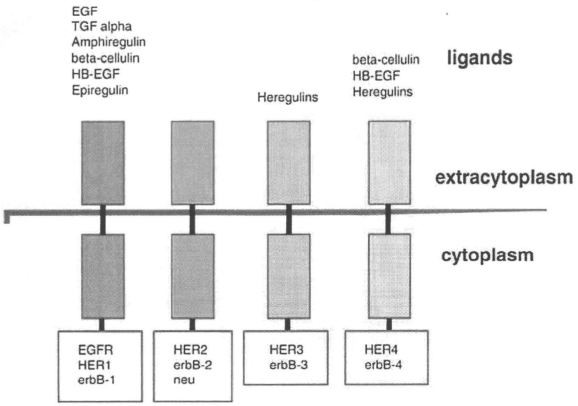

Figure 1. *The epidermal growth factor receptor (EGFR) family proteins and their ligands. TGF, transforming growth factor; HB-EGF, heparin-binding epidermal growth factor–like growth factor; HER, human epidermal growth factor receptor.*

kinase (*ALK*) genes in NSCLCs.[6] This translocation promotes strong tyrosine kinase activity, which is a prominent feature of ALK, leading to intensive oncogenesis in NSCLCs. Cancers featuring *EML4-ALK* fusion thus constitute a subtype of NSCLCs that might be highly sensitive to ALK inhibitors. Interestingly, *EGFR* mutation, K-ras mutation, and *EML4-ALK* translocation are mutually exclusive.[7,8] Furthermore, lung cancers with each of these alterations appear to have their own particular clinicopathologic characteristics.

DISCOVERY OF EGFR

In 1975, the existence of the EGF-specific receptor was first reported on the cell membrane of the fibroblast.[9] Thereafter, from work with the A431 human cancer cell line, EGFR was initially defined as a 170-kDa protein.[10] In 1984, the sequence of v-*erbB*, an oncogene of the avian erythroblastic leukemia virus, was reported to be extremely similar to that of EGFR.[11] Gene products of oncogene *erbB* and *EGFR* subsequently turned out to be identical proteins. Thereafter, it was found that human genes corresponding to v-*erbB* were not only *EGFR* but also human epidermal growth factor receptor 2 (*HER2*), these 2 now being referred to as *ERBB1* and *ERBB2*, respectively.

STRUCTURE AND FUNCTION OF EGFR

Growth factors belong to a family of polypeptides that have been shown to stimulate proliferation and/or differentiation in both normal and malignant cells. One of the first growth factors discovered was EGF. Later studies showed that this protein binds to the cell surface growth factor receptor EGFR, thereby either inducing cell proliferation or differentiation in mammalian cells.

The binding of a ligand to EGFR induces conformational changes within the receptor, which increase the catalytic activity of its intrinsic tyrosine kinase, resulting in the autophosphorylation that is necessary for biologic activity. Epidermal growth factor receptor is a 170-kDa transmembrane glycoprotein that binds to specific ligands. The erbB family cell-signaling process uses EGF-like ligands that include cell-signaling transforming growth factor α (TGF-α), amphiregulin, heparin-binding EGF, epiregulin, heregulin, neuregulin, and betacellulin. Epidermal growth factor receptor is known to bind with particularly high affinity to EGF, amphiregulin, and TGF-α.

As noted above, EGFR is a member of the erbB family of receptor tyrosine kinase proteins, now known to also include HER2/*neu* (erbB2), HER3 (erbB3), and HER4 (erbB4). These receptors are all composed of an extracellular ligand-binding domain, a transmembrane lipophilic domain, and an intracellular tyrosine kinase domain and, with the exception of HER2, all bind to receptor-specific ligands (Figure 1). Phosphorylation of the tyrosine kinase domain followed by homodimerization or heterodimerization between receptors of the same family leads to protein activation on the cell surface. In cancer cells, this is believed to promote signaling cascades, cell growth, differentiation, cell survival, cell cycle progression, and angiogenesis.

GENETIC STRUCTURE OF EGFR

The approximately 200-kb human *EGFR* gene, comprising 28 exons and 27 introns, exists on the short arm of chromosome 7 (7p12). Exons 1 to 16 encode the extracellular domain, while exon 17 codes for the transmembrane domain, and exons 18 to 28 are responsible for the intracellular domains. The tyrosine kinase domain is encoded by exons 18 to 24, while the C-terminal domain is encoded by exons 25 to 28.

ACTIVATION OF EGFR DOWNSTREAM SIGNALING

Receptor tyrosine kinases, such as EGFR, transmit extracellular signals of growth factors into the intracytoplasmic region and transmit their stimulus to the nuclei by signal transduction. As a result, transcriptional upregulation follows, leading to protein synthesis and transformation of cell functions or cellular architecture.

As signaling pathways of EGFR, the Ras/Raf/MAPK (mitogen-activated protein kinase) pathway, the PI3K (phosphatidylinositol-3-kinase)/Akt pathway, and the Jak (Janus kinase)/ STAT (signal transducers and activator of transcription) pathway are all important. As a result of

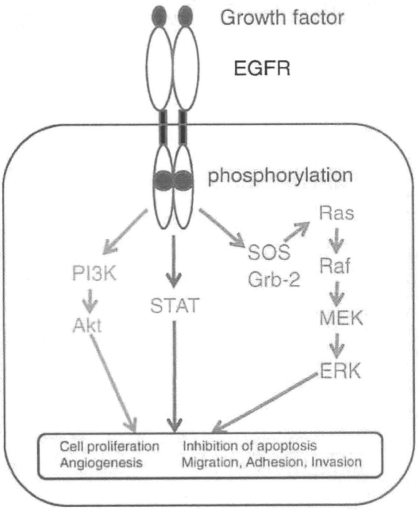

Figure 2. *Schematic illustration of the epidermal growth factor receptor (EGFR) and downstream signaling pathways. Binding of a receptor-specific ligand leads to phosphorylation of EGFR and signaling through the mitogen-activated protein kinase (MAPK) pathway (green), signal transducers and activator of transcription (STAT) pathway (blue), and phosphatidylinositol-3-kinase (PI3K)/Akt pathway (orange). These pathways promote cell proliferation, angiogenesis, migration, adhesion, and/or invasion, while inhibiting apoptosis. SOS, son of sevenless; Grb-2, growth factor receptor–bound protein 2; Ras and Raf are well-known oncoproteins; MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinase.*

Table 1. Epidermal Growth Factor Receptor (EGFR) Expression in Various Tumor Types

Tumor Type	Tumors Expressing EGFR, %
Head and neck	80–100
Renal	50–90
Lung	40–80
Breast	14–91
Colon	25–77
Ovarian	35–70
Prostate	39–47
Glioma	40–63
Pancreas	30–50
Bladder	31–48

the signal transduction, cell differentiation or cell proliferation are promoted. The Ras/Raf/MAPK pathway mainly promotes cell proliferation and survival, while the PI3K/Akt pathway is mainly associated with cell growth, inhibition of apoptosis, invasion, or migration (Figure 2).

EGFR OVEREXPRESSION

In a wide range of solid cancers, EGFR overexpression has been detected to varying degrees (Table).[12] Reported values are 30% to 38% for gastric adenocarcinomas,[13,14] 30% to 62% for pancreatic cancers,[15,16] and 100% for undifferentiated thyroid carcinomas.[17] Although the prognostic significance of EGFR expression remains unclear, as reports on these issues are contradictory, a retrospective review of EGFR studies determined that EGFR expression levels are highly predictive of clinical outcome for patients with ovarian, cervical, bladder, esophageal, and head and neck cancers. They are of moderate prognostic value for gastric, colorectal, breast, and endometrial cancers and of relatively low prognostic value for NSCLCs.[18]

EXTRACELLULAR MUTATION OF *EGFR*

In 1988, it was found that human glioblastoma multiforme cells carried amplified *c-erbB* genes that bore short deletion mutations within the ligand-binding domain of the EGFR. The products of these mutated *c-erbB* genes were about 30 kDa smaller than the normal 170-kDa EGFR, and cancer cell membrane fractions containing the 140-kDa abnormal EGFR showed a significant elevation of tyrosine kinase activity without any ligand.[19] This mutation type was referred to as EGFRvIII. There is no ligand binding site and the result is constant activation without any ligand binding.[20] EGFRvIII is associated with cell proliferation and malignancy in various neoplasms involving breast cancers, small cell lung cancers, gliomas, and prostatic cancers.[21]

MUTATIONS OF INTRACYTOPLASMIC DOMAIN OF *EGFR* GENE

In 2004, mutations of intracytoplasmic domain of *EGFR* gene were found in NSCLCs, and NSCLCs with such mutations were reduced in size by gefitinib, a chemical inhibitor of the kinase activity of EGFR.[1,2] In the gene coding for the receptor, mutations are divided into 4 major types: point mutations in exon 18, deletions in exon 19, insertions in exon 20, and point mutations in exon 21. Particularly, the 2 most frequent mutations are deletion around codons 746 to 750 of exon 19 and transversion of T to G in codon 858 of exon 21, with an amino acid change from leucine to arginine (L858R). These 2 mutations account for approximately 90% of intracytoplasmic mutations of EGFR (Figure 3).[22] They both cause conformational change in the ATP-binding domain, which results in constant activation of EGFR without ligand binding. However, affinity for gefitinib is upregulated, so that the cancer cells are susceptible to induction of apoptosis by this agent and to reduction in cancer size.[23] The 2 *EGFR* mutations have been found to be present in normal lung tissue around cancers,[24] and mice transgenic for the mutated *EGFR* gene develop lung cancers.[25] The results thus suggest that *EGFR* mutation is involved at an early stage of neoplasia in the lung.

EGFR MUTATIONS GENERATING GEFITINIB TOLERANCE

In addition to the EGFR mutations increasing sensitivity to gefitinib, as mentioned above, secondary mutations can occur so that cancers become tolerant. Substitution in codon 790, with a resulting amino acid shift from threonine to methionine (T790M),[1] or in codon 761, resulting in change from asparaginic acid to tyrosine (D761Y),[26] are reported to be gefitinib tolerance–inducing mutations. T790M has been detected in about half of the NSCLCs exhibiting acquired gefitinib tolerance.[26] Alteration of the gefitinib binding site in the EGFR cytoplasmic domain is presumably involved.

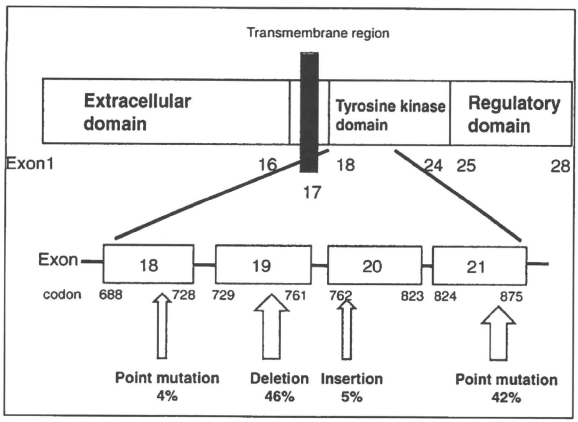

Figure 3. *Distribution of mutations in the epidermal growth factor receptor.*

DRUGS TARGETING EGFR

The most clinically advanced EGFR inhibition strategies include small-molecule inhibition of the intracellular tyrosine kinase domain and monoclonal antibody–mediated blockade of the extracellular ligand-binding domain. Gefitinib and erlotinib are oral anticancer drugs, inhibiting tyrosine kinase domain. Their cytoreductive effects are to some extent dependent on intracytoplasmic mutations of EGFR, as noted above, and they have been found to be useful in the treatment of NSCLCs. Cetuximab is a monoclonal antibody, binding to the ligand-binding site of EGFR and blocking its dimerization and activation. It is also effective for the wild-type EGFR, with applications in the treatment of colorectal as well as head and neck cancers.

EFFECT OF CANCER REDUCTION, COMBINATION USAGE OF CHEMOTHERAPY, AND LIFE PROLONGATION

From 2000 to 2001, 2 phase II studies of pretreated advanced NSCLCs (Iressa Dose Evaluation in Advanced Lung Cancer [IDEAL] 1 study[27] and IDEAL 2 study[28]) were performed. The positive response rate to gefitinib was 9% to 19% and the 1-year survival rate was 21% to 36%. Cancer reduction effects were most prevalent in Asian nonsmoking females with adenocarcinomas. Cancers with EGFR mutations demonstrated significant cytoreductive effects to treatment,[1,2] and this response is predominantly seen in persons with adenocarcinoma, who are nonsmokers, of female sex, and of Asian ethnicity.

From 2000 to 2001, as a first treatment for advanced NSCLCs, gefitinib was given in combination to standard treatment involving the platinum-containing drugs. Although the other drugs included gemcitabine and cisplatin[29] or paclitaxel and carboplatin,[30] significant combination effects were not obtained.

In 28 countries, not including Japan, a phase III study has been performed for 1692 cases of posttreatment advanced NSCLCs (Iressa Survival Evaluation in Lung Cancer).[31] For either all lung cancers or lung adenocarcinomas, gefitinib (versus placebo) could not significantly prolong survival time for patients. However, on subset analysis, gefitinib did significantly enhance survival in Asian persons and nonsmokers.

SIDE EFFECTS OF EGFR TYROSINE KINASE INHIBITION (GEFITINIB)

Major clinical problems caused by gefitinib are acute lung damage and interstitial pneumonia, the latter being the most significant side effect.[32] An epidemiologic investigation by the West Japan Thoracic Oncology Group, which used approximately 2000 cases, revealed an incidence rate of 3.2% to 3.5% and a death rate of 1.2% to 1.4%. Generally, ineffectiveness of steroid therapy makes the condition serious. Male sex, the existence of lung fibrosis before treatment, and a smoking habit were identified as risk factors for development of interstitial lung diseases related to gefitinib therapy. Thus, the effective treatment group for gefitinib and the high-risk group for interstitial lung disease with gefitinib are widely dissociated. This means that it is essential to preselect patients for gefitinib therapy.

EGFR MUTATIONS AND CLINICOPATHOLOGIC FEATURES

Lung cancers with EGFR mutations are prevalent among patients who are young, of female sex, never-smokers, and of East Asian ethnicity.[2–4,33–35]

Correlations between morphology and EGFR mutations in lung adenocarcinomas have been investigated previously. Concerning histopathology, a bronchioloalveolar carcinoma (BAC) histologic feature and well-differentiated to moderately differentiated grades were earlier reported to predict responsiveness to the EGFR tyrosine kinase (TK) inhibitor and the presence of EGFR mutations[33,34] The finding that the hobnail cell type and a micropapillary morphology can predict a higher incidence of EGFR mutations in lung adenocarcinomas has been reported more recently.[36]