isting biomarkers (predictability, reliability, specificity e.g.), whereas the latter is to select biomarkers without any hypothesis, by DNA microarray or proteomics. At the same time, validation of the selected biomarkers is necessary. Currently, the hypothesis-free approach seems to be the trend.

In general, biomarkers in the hypothesis-driven approach are relatively easy to understand, and are based on biological evidence. They can be expected to be more easily applied clinically. However, there is a limitation : only pre-existing biomarkers can be used. On the other hand, in the case of biomarkers in the hypothesis-free approach, it is difficult to understand underlying biological mechanisms and it is difficult to directly apply these markers clinically. However, novel biomarkers can be discovered by this approach.

When considering a new prospective study using microarray gene expression profiling, it is of importance to pay attention to some points, as follows. The investigators should recognize the role of quality assurance and perform the study accordingly. Regarding the data of DNA expression for Cancer Diagnostics, the guidelines proposed by the NCI-EORTC Working Group are helpful.[3] For the development of classifications based on the gene expression profile, the following points must be taken into considera-

tion. 1) A common therapy is essential for identical populations. Are the results reasonable enough to establish a therapeutic policy ? Will the new classification be generally used based on the cost-benefit balance, by comparing the selection of the therapies and the cost for mis-classified ? These points should be discussed preliminarily during the process of designing of the study. For further evaluation, internal validation is necessary to prove the accuracy of the new classification in comparison with the pre-existing prognostic factors. The validation process includes 1) transfer to other platforms that are commonly used in clinical situations. (For example, will the classification identified by DNA chip analysis be valid for transfer to that by RT-PCR or immunohistochemical (ICH) examination), 2) confirmation of the reproducibility of the classification by the new platform (RT-PCR or ICH), and 3) independent validation in a prospective study. In addition, the investigators should recognize "multiplicity" of the comprehensive data sets, such as those of gene expression. Many researchers have reported classifiers to predict the prognosis of patients with cancers. For example, a 17-gene signature associated with metastasis was identified by a DNA chip analysis by Ramaswamy et al..[4] (Fig. 4)

Several researchers have attempted the same

Table 3   The 17-gene signature associated with metastasis

| Gene	Gene name	GenBank ID
Upregulated in metastases		
SNRPF	Small nuclear ribonucleoprotein F	A1032612
EIF4EL3	Elongation initiation factor 4E-like 3	AF038957
HNRPAB	Heterogeneous nuclear ribonucleoprotein A/B	M65028
DHPS	Deoxyhypusine synthase	U79262
PTTG1	Securin	AA203476
COL1A1	Type 1 collagen, α1	Y15915
COL1A2	Type 1 collagen, α2	J03464
LMNB1	Lamin B1	I 37747
Downregulated in metastases		
ACTG2	Actin, γ2	D00654
MYLK	Myosin light chain kinase	U48959
MYH11	Myosin, heavy chain 11	AF001548
CNN1	Calponin 1	D17408
HLA-DPB1	MHC Class II, DPβ1	M83664
RUNX1	Runt-related transcription factor 1	D43969
MT3	Metallothionein 3	S72043
NR4A1	Nuclear hormone receptor 1R3	L13740
RBM5	RNA binding motif 5	AF091263
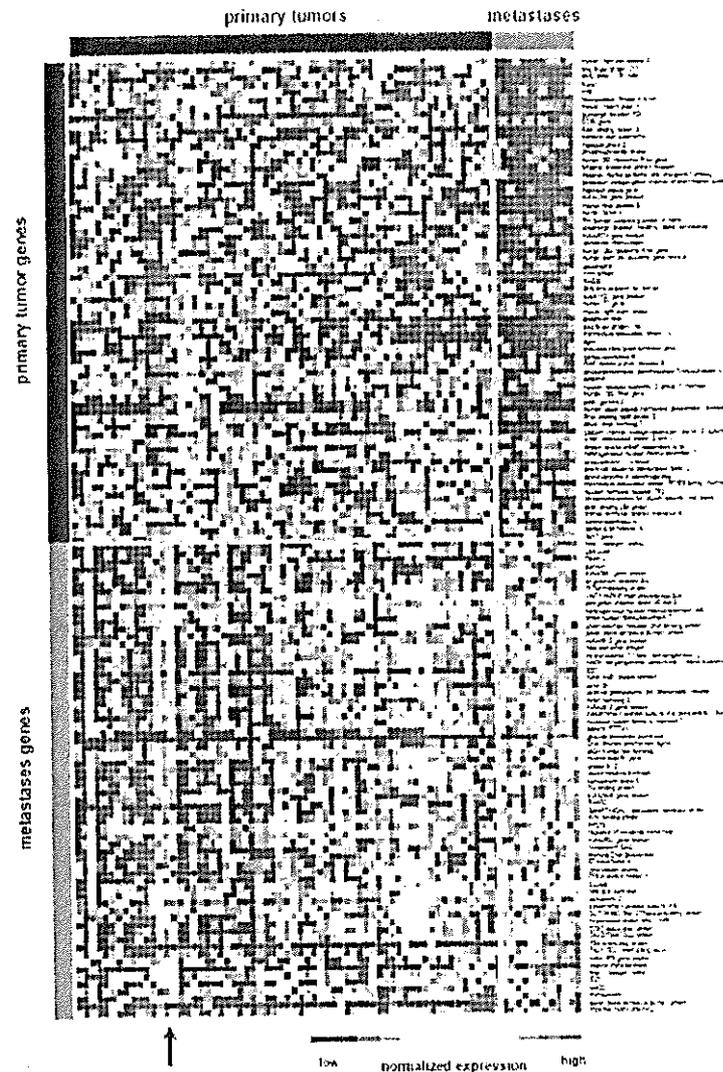
Fig. 4   Genes associated with metastases.

Columns represent human tumor samples (64 primary and 12 metastatic adenocarcinomas); rows represent the 128 genes (64 overexpressed and 64 underexpressed in metastases) that best distinguished the primary from the metastatic tumors using a weighted-voting algorithm in leave-one-out cross-validation (cross-validation accuracy=80%, $P=0.012$ by permutation testing; Colorgram depicts high (red) and low (blue) relative levels of gene expression A 'striped' pattern was observed in some primary tumors (arrow), indicating the presence of a gene-expression program associated with metastases (Ramaswamy et al., Nature Genetics 2002)

approach and obtained gene sets that predict prognosis of patients with breast cancer. Sorlie selected a set of 456 genes[5] (PNAS 2001). Van't Veer selected a set of 231 genes determining the prognosis and validated it using the independent data of 295 patients.[5] However, in relation to their selected genes, a few genes were overlapped. This discrepancy might be due to the following reasons : 1) validation in different clinical backgrounds, such as disease, histology, response criteria, and treatment, 2) difference in the assay

methods used for RNA purification and in the methods used for gene amplification, and/or 3) difference in the analytical process used, such as standardizations and algorithms. How should future biomarker studies be considered ? Future biomarker studies should include : 1) a prospective correlative study between markers and clinical features (survival and response e.g.), 2) barebones sample size, and 3) validation on another platform.[7]

Another problem is that the selected markers

usually contain many functionally unknown markers. Therefore, it is difficult to discuss the implications of biomarkers without the availability of biological information. At the same time, it is necessary to analyze the functions of each biomarker, which requires much effort. Therefore, investigators should start biomarker (pharmacogenomic) studies in the early phase. Statistically, algorithms and data sets containing the biological information should be constructed. In addition, standardization of these analytical methods is essential. For clinical side application ?, adequate prospective clinical studies are required. It is thus of utmost importance to establish better communication between clinical researchers, basic researchers and bio-statisticians from the planning stage.

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Development and characterization of an antibody specifically recognizing a mutant EGFR (L858R) protein expressed frequently in non-small cell lung cancer

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Abstract

L858R point mutation in exon 21 of the EGFR gene, accounting for approximately 40% of non-small cell lung cancer (NSCLC)-associated EGFR mutations, has been known to hyper-respond to gefitinib, a selective EGFR tyrosine kinase inhibitor. From this view point, it is important to detect EGFR mutations. Immunohistochemistry (IHC) is commonly used to analyze the molecular status of several clinical specimens. We have developed specific antibodies recognizing the mutant EGFR (L858R) protein and characterized the antibodies by ELISA, Western blot, immunocyto- chemistry, and immunohistochemistry. Using any of these evaluation methods, we found an antibody, AbyD02889, which could detect the EGFR (L858R) protein with specificity. AbyD02889 may be a useful tool to detect the EGFR (L858R) mutation of NSCLC in the clinical situation. IHC using the mutant-specific anti-EGFR antibody will be a powerful assay to predict or select subpopulation sensitive to EGFR-TKI.
Key words : NSCLC, mutant EGFR, HuCAL antibody

Introduction

Lung cancer is a major cause of cancer-related mortality worldwide and is expected to remain a major health problem for the foreseeable future.[1] Targeting the epidermal growth factor receptor (EGFR) is one appealing therapeutic strategy for non-small cell lung cancer (NSCLC) because constitutively-active types of EGFR mutations, sometimes together with their strong expression, have been believed to contribute to the pathological features of NSCLC such as disease progression, or unregulated cell growth[2] In the clinical situation, NSCLC tumors with such mutations have been reported to hyper-respond significantly to gefitinib, a selective EGFR tyrosine kinase inhibitor.[3,4] Such EGFR mutations consisted of small in-frame deletions or substitutions clustered around the ATP-binding site in exons 18, 19, and 21 of the EGFR, and the mutations increased the affinity of the enzyme for ATP and gefitinib. Some investigators subsequently found that these EGFR mutations were strong determinants of the tumor response to an EGFR tyrosine kinase inhibitor.[5-7] The two studies demonstrated two major EGFR mutations (E746 A750del in exon 19 and L858R in exon 21) occupying approximately 90% of the NSCLC-

associated EGFR mutations, in both trials using surgical tissue to detect the EGFR mutations.[6,8]

Our laboratory has also been interested in the clinical relationship between the somatic EGFR mutations and responsiveness of NSCLC-tumors to EGFR tyrosine kinase inhibitors. Kilmura reported that the Scorpion ARMS method could detect the EGFR mutations using serum or pleural effusion from NSCLC patients with a high sensitivity, reliability, and less invasiveness even though only a small amount of genomic DNA was contained in the serum or pleural effusion sample.[9,10] In addition to these approaches to detect EGFR mutations using genomic DNA, it is considered important and useful to specifically detect the mutant EGFR proteins which are final products by the somatic mutations in cancer cells. From this view point, development of a specific antibody recognizing a mutant EGFR protein becomes a useful alternative method of detection to PCR-based genomic diagnosis, which will provide us with even more information about the mutation. For example, using the mutant-specific antibody and immunocytochemical technique, we will be able to conveniently detect EGFR mutations in only one cancer cell.

MorphoSys (Munich, Germany) provides an uniquely powerful technology of antibody generation. In the HuCAL® libraries, the structural diversity of the human antibody repertoire is represented by seven heavy chain and seven light chain variable region genes, giving rise to 49 frameworks in the master library. Highly variable genetic cassettes (CDRs, complementarity determining regions) are then superimposed on these frameworks to mimic the entire human antibody repertoire (Figure 1A). More than 10 billion functional human antibody specificities in Fab format have already been prefabricated and are available in phage libraries.[11-13]

Using this technology, we developed a specific antibody for the mutant EGFR (L858R). Herein we report the characterization of the antibody and discuss the feasibility of using the antibody for NSCLC.

Materials and Methods

Expression constructs

A eukaryotic expression vector, pcDNA3.1 (+) (Invitrogen, Carlsbad, CA), was used as a backbone vector to produce pcDNA-IG, which

was constructed by insertion of an IRES-EGFP (enhanced green fluorescence protein following internal ribosome entry sequence) fragment at the NotI-XhoI sites of pcDNA3.1 (+). pcDNA-IG expressed the gene of interest together with EGFP and allowed us to ascertain the protein expression indirectly by monitoring the EGFP expression. Full length cDNA of wild-type EGFR and its mutant EGFR (L858R) were amplified by RT-PCR from a human embryonal kidney cell line (HEK293) and a non-small cell lung cancer cell line,[11-15] respectively. A High Fidelity RNA PCR Kit (TaKaRa, Shiga, Japan) was used for the RT-PCR and the following primer sets were synthesized (forward, CGCTAGCCCCCTGACTCCGTC-CAGTATTGA ; reverse, CCCCTGACTCC-GTCCAGTATTGA). The PCR products were amplified again using PyrobestTM DNA polymerase (TaKaRa) with the primer sets (forward, CGCTAGCCCCCTGACTCCGTC-CAGTATTGA ; reverse, CGAAGCTTTGCT-CCAATAAATTCACTGC). This amplified DNA encoding wild-type and mutant EGFR included NheI and HindIII at the 5'- and 3'-ends, respectively. These two PCR products were subcloned into a pCR BluntII-TOPO vector (Invitrogen) and their sequences were confirmed with an ABI 310 capillary sequencer (Applied Biosystem).

Reverse and forward oligonucleotides encoding the myc-tag sequence (EQKLISEEDLN) were designed and synthesized as follows: forward, AGCTTGAACAGAAGCTGATCT-CAGAGGAGGACCTGAATTGAC ; reverse, TCGAGTCAATTCAGGTCCTCCTCT-GAGATCAGCTTCTGTTCA. Two oligos were annealed under at the following conditions : 95°C for 2 min, 80°C for 2 min, 55°C and 37°C for 2 min. This annealing procedure generated the ds-oligos including HindIII- and NotI-cut cohesive ends, at the 5'- and 3'-ends, respectively. These ds-oligos were inserted in the HindIII-NotI sites of pcDNA-IG. Subsequently, each cDNA of wild-type or mutant EGFR, that was cut out from the pCR BluntII-TOPO vector with NheI and HindIII, was transferred to the NheI-HindIII sites of pcDNA-IG. Finally, two vectors expressing myc-tagged wild-type or mutant EGFR proteins with EGFP were constructed and designated as pcDNA-EGFR (WT)-myc-IG and pcDNA-EGFR (L858R)-myc-IG, respectively.

Cell culture and transfection

HEK293 (a human embryonal kidney cell line) and 11-18 (a non-small cell lung cancer cell line) cells were maintained in RPMI-1640 medium supplemented with 10 % fetal bovine serum (FBS). pcDNA-EGFR (WT)-myc-IG or pcDNA-EGFR (L858R)-myc-IG was transfected into the HEK293 cells using the FuGene6 transfection reagent (Roche Diagnostics, Basel, Switzerland). Briefly, 80% confluent cells cultured on a 10 cm dish were transiently transfected with 6 μg of vector. Forty-eight hours after transfection, the cells were washed with phosphate buffered saline (PBS) and the cell lysates were prepared for immunoblotting analysis. For immunostaining, the transfected cells were trypsinized once, replated on a poly-L-lysine (PLL) (SIGMA-ALDRICH, St. Louis, MO)-coated 24 well plate, and then used for the examination.

ELISA

The specificity of each HuCAL antibody (AbyD02889, AbyD02890, AbyD02991) was checked by ELISA. Briefly, a 96-well microtiter plate was coated with 20 μg/ml of EGFR (WT), EGFR (L858R), CD33-6xHis, Ubiquitin, Stat, and FITC proteins which were diluted in PBS with or without either transferring (Trf) or bovine serum albumin (BSA). After incubation at 37°C for 1 h, the plate was washed three times with PBS. Then, the proteins were proved with each HuCAL antibody at 1 ng/ml followed by incubation with horseradish peroxidase (HRP)-conjugated anti-His antibody (Santa Cruz Biotechnologies). One hundred μl of the substrate solution were added per well. After sufficient color development, 100 μl of stop solution to the wells. The absorbance of each well was read at 450 nm with a plate reader.

Immunoprecipitation and Immunoblotting

The two GST-fused recombinant proteins with cytoplasmic wild-type EGFR and its L858R mutant were purchased from Upstate Biotech (Lake Placid, NY). The transiently transfected HEK293 cells with either pcDNA-EGFR (WT)-myc-IG or pcDNA-EGFR (L858R)-myc-IG were lysed 48 h later with a lysis buffer containing 1% triton X, 50 mM HEPES (pH 7.4), 5 mM EDTA, 50 mM NaCl, 10 mM Na pyrophosphate, 50 mM NaF, 1 mM Na orthovanadate, and protease inhibitor mix, complete™ (Roche). Five hundred micrograms of cell lysate were immunoprecipitated by incubation with 2 μg of anti-myc antibody (Roche) for 3 h followed by further

incubation with protein-G agarose (Santa Cruz Biotechnologies) for 1 h. The recombinant proteins and immunoprecipitated samples were separated with SDS-PAGE and blotted on a PVDF membrane. The membrane was probed with HuCAL antibodies, monoclonal anti-EGFR antibody (Cell Signaling, Beverly, MA), or monoclonal anti-pY20 antibody (Cell Signaling) followed by incubation with a monoclonal or polyclonal HRP-conjugated second antibody (Cell Signaling, Beverly, MA). An ECL detection system was then used for visualization. GST-tagged cytoplasmic wild-type and mutant EGFR (L858R) proteins were purchased from Upstate Biotech. For probing with His-tagged anti-EGFR (L858R) HuCAL antibodies, a monoclonal HRP-conjugated anti-His antibody (Santa Cruz Biotechnologies) was used as the second antibody.

Immunocytochemistry and Immunohistochemistry

The 11-18 and HEK293 cells were plated on a PLL-coated 24-well plate at 5,000 cells/well. For assay of the transfected cells, the 48 h-incubated cells after transfection were trypsinized and replated. The cells were fixed with 4% paraformaldehyde for 30 min. The cells were then permeabilized and blocked with a PBS buffer containing 0.3% Triton X and 10% normal goat serum for 1 h and probed with the HuCAL antibodies at 20 μg/ml followed by visualization using an FITC- or rhodamine-conjugated anti-His antibody (Santa Cruz Biotechnologies) as the second antibody. Fluorescence microscopic examination was carried out using a KEYENCE microscopic system (Woodcliff Lake, NJ). For immunohistochemistry, an HRP-conjugated anti-His antibody was used as the second antibody followed by DAB staining.

Results

Generation of monoclonal antibodies against mutant EGFR (L858R)

We used the recombinant antibodies technology provided by MorphoSys (MorphoSys). An antigen (the peptide of mutant EGFR (L858R)) was designed and synthesized. The antigen was screened against the HuCAL GOLD® library (MorphoSys) with its more than 15 billion antibody specificities, which enabled us to develop monoclonal antibodies rapidly. The antigen enters the automated panning process

(AutoPan™, provided by MorphoSys), where it is immobilized for screening against an antibody-displaying phage. Three candidates (AbyD02889, AbyD02890, and AbyD02891) from the screening process were obtained and affinity-purified. We used the monovalent format of the Fab fragment tagged with Myc-His at the C-terminus (Figure 1A) and checked the specificity of these three antibodies with an ELISA for EGFR (WT), EGFR (L858R)-BSA, and EGFR (L858R)-Trf (Figure 1B). Two antibodies, AbyD02889 and AbyD02890, recognized EGFR (L858R) specifically, whereas AbyD02891 bound to both the wild-type and mutant EGFR.

Specificity of HuCAL antibodies against recombinant GST-fused wild type and mutant EGFR proteins

Figure 2A shows a schematic representation of the cytoplasmic domains of wild-type and L858R-mutant EGFR fused with glutathione S-transferase (GST). Using these two antigens, we tested the specificity of the HuCAL antibodies. Both AbyD02889 and AbyD02890 antibodies

Fig. 2 Immunoblot analysis of two recombinant GST-fused EGFR proteins, GST-EGFR(WT) and GST-EGFR(L858R), using AbyD02889 and AbyD02890 (A) Cytoplasmic domains of wild-type and L858R-mutant EGFR were fused with glutathione S-transferase (GST) and used for immunoblot analysis as the antigens against AbyD02889 and AbyD02890. (B) Checking the specificity and affinity of AbyD02889 and AbyD0289050 against two GST-fused EGFR proteins. Fifty, 5, and 0.5 ng of each protein were separated by SDS-PAGE and blotted on a PVDF membrane followed by probing using AbyD02889 or AbyD02890 antibody at 5 μg/ml
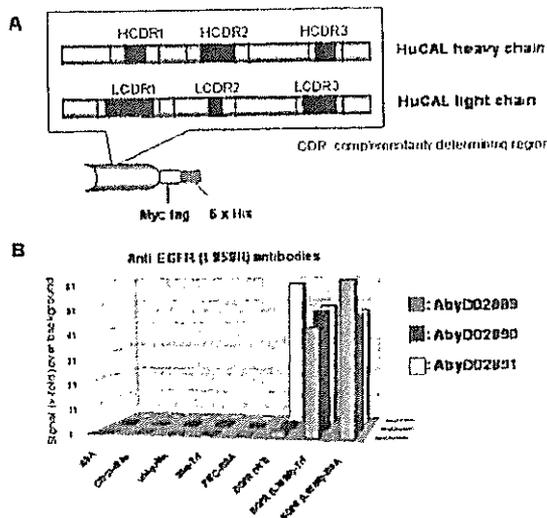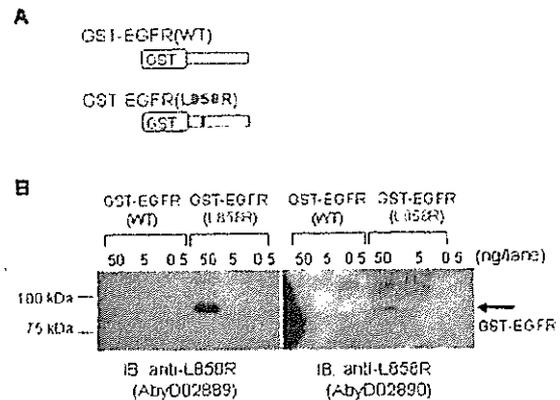
Fig. 1 Generation of monoclonal antibodies against a mutant EGFR (L858R)
(A) A representation of the structure of anti EGFR (L858R) antibodies The structural diversity of the human antibody repertoire is represented by heavy and light chain variable region genes. Highly variable genetic cassettes (CDRs complementarity determining regions) are then superimposed on these frameworks to mimic the entire human antibody repertoire The monovalent format of the Fab fragment we used is tagged with Myc-His at the C-terminus (B) Characterization of three candidates (AbyD02889, AbyD02890, and AbyD02891) by ELISA The specificity of the three was checked by an ELISA for EGFR (WT) EGFR (L858R)-BSA and EGFR (L858R) Trf
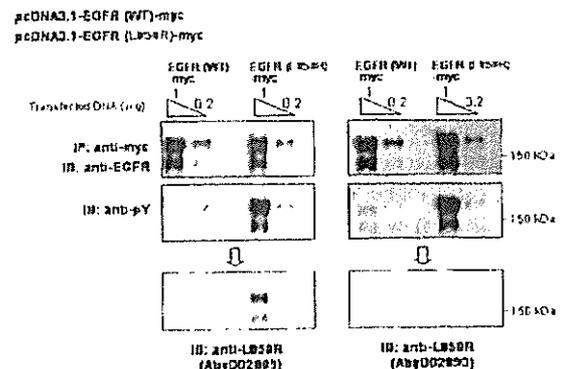
Fig. 3 Checking the specificity and affinity of AbyD02889 and AbyD02890 against the full length wild-type or L858R-mutant EGFR protein expressed in the HEK293 cells One of 0.2 μg of either pcDNA3.1-EGFR (WT)-myc or pcDNA3.1-EGFR (L858R)-myc were transiently transfected into the HEK293 cells (2×10⁵ cells/6 cm well) Five hundred μg of cell lysate were immunoprecipitated with anti-myc followed by immunoblotting The membrane was probed with a commercially available anti EGFR antibody (top) antibody AbyD02889 or AbyD02890

recognized 50 ng mutant EGFR protein specifically, while they were not able to bind to wild-type EGFR protein at all (Figure 2B). In addition, the affinity of AbyD02889 for the mutant protein was clearly demonstrated to be higher than that of AbyD02890.

Specific recognition of AbyD02889 against the full length mutant EGFR (L858R) protein expressed in HEK293 cells

One or 0.2 μg of either pcDNA3.1-EGFR (WT)-myc or pcDNA3.1-EGFR (L858R)-myc was transiently transfected into the 2x10⁶ HEK293 cells/6 cm well. Immunoprecipitated EGFR (L858R) protein with anti-myc antibody (in the upper panels of Figure 3) was phosphorylated at a much higher rate than wild-type EGFR protein (in the middle panels). After denuding the anti-pY antibody, the membrane was reproved with AbyD02889 and AbyD02890. AbyD02889 specifically recognized the EGFR (L858R) protein but AbyD02890 did not (in the lower panels). These findings together with the

results in Figure 2 suggested that AbyD02889 specifically recognized the EGFR (L858R) protein either produced in bacteria or expressed in human cells.

Immunocytochemical evaluation of the specificity of AbyD02889 and AbyD02890

Next, we checked the feasibility of the use of HuCAL antibodies for immunocytochemistry. The HEK293 cells were transfected with pcDNA3.1 (Figure 4, panels a, d, g, and j), pcDNA3.1-EGFR (WT)-myc (Figure 4, panels b, e, h, and k), or pcDNA3.1-EGFR (L858R)-myc (Figure 4, panels c, f, l, and l). EGFR (L858R)-transfected HEK293 cells (293-L858R) were positive for AbyD02889 (Figure 4, panel c) but negative for AbyD0890 (Figure 4, panel i), and EGFR (WT)-transfected cells were negative for both antibodies (Figure 4, panels b and h). Then, we analyzed 11-18 cells harboring an intrinsic EGFR (L858R) mutation using AbyD02889. The 11-18 cells were positive for AbyD02889 (Figure 5, panel b) but the HEK293
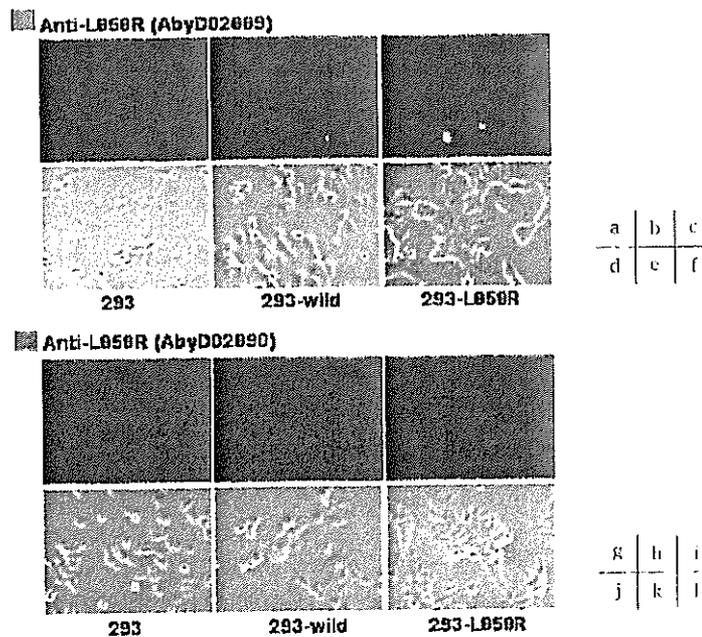

Fig. 4   Evaluation of the specificity of AbyD02889 and AbyD02890 by immunocytochemical analysis. The HEK293 cells transfected with pcDNA3.1 (a, d, g, and j), pcDNA3.1-EGFR (WT)-myc (b, e, h, and k), or pcDNA3.1-EGFR (L858R)-myc (c, f, l, and l) were examined immunocytochemically using the HuCAL antibodies. Forty eight h after transfection, the cells were replated on a PLL-coated 24-well plate at 5,000 cells/well and further incubated for 24 h. After fixation, permealization, and blocking, the cells were probed with the HuCAL antibodies followed by visualization using a rhodamine-conjugated anti His antibody as the second antibody. A fluorescence microscopic examination was carried out. The fluorescence microscopic views of the cells probed with AbyD02889 and AbyD02890 are shown in the upper (a, b, and c) and lower panels (g, h, and i) respectively. The light microscopic views are shown in d, e, f, j, k, and l.
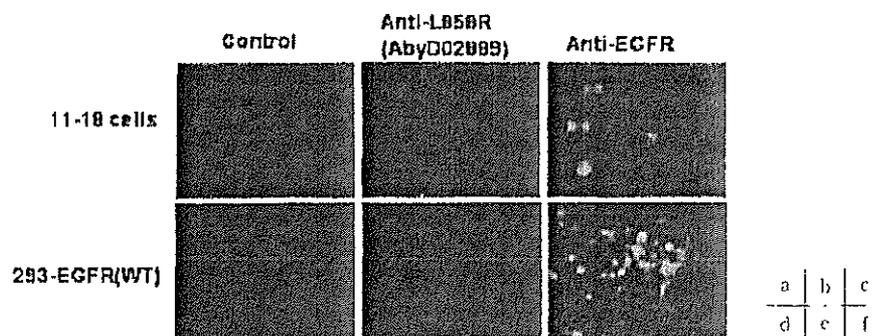
Fig. 5 Specificity of AbyD02889 against EGFR (L858R) protein intrinsically expressed in the 11-18 cells. The 11-18 cells and HEK293-expressing EGFR (WT)-myc (293-EGFR(WT)) were ectopically plated on a PLL-coated 24-well plate at 5,000 cells/well. After fixation, permialization, and blocking, the cells were probed with the AbyD02889 (b and e) and anti-EGFR antibodies (Cell Signaling) (c and f) followed by probing with the FITC-conjugated anti-His antibody as a second antibody. The signal was examined with fluorescence microscopy. The fluorescence views of 11-18 cells and 293-EGFR(WT) cells are shown in the upper (a, b, and c) and lower panels (g, h, and I), respectively
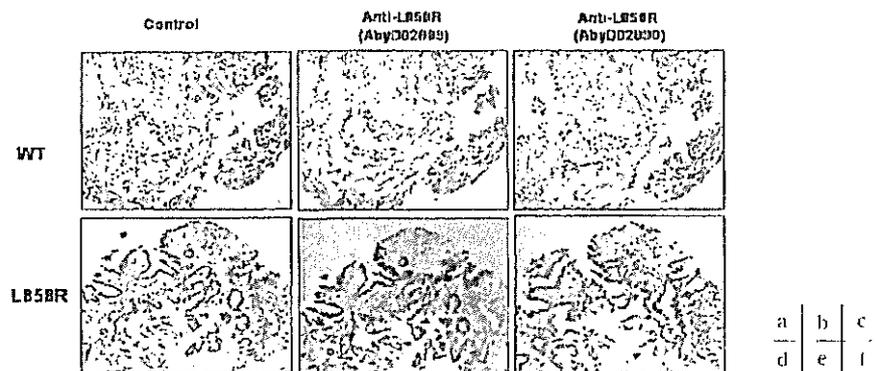

Fig. 6 Immunohistochemical analysis of two clinical specimens of non-small cell lung cancer whose EGFR status had been confirmed by the direct sequencing method
The upper and lower three panels show DAB staining by AbyD02889 and AbyD02890 for tissue sections of EGFR (WT)- and EGFR (L858R)-expressing NSCLC, respectively

cells transfected with the wild-type EGFR (293-EGFR (WT)) were negative for the antibody. On the contrary, an anti-EGFR antibody (from Cell Signaling) detected both the 11-18 and 293-EGFR (WT) cells (Figure 5, panels c and f). These results showed that the mutant EGFR protein expressed in human cells could be detected with specificity by AbyD0889.

Immunohistochemical evaluation of AbyD02889 and AbyD02890

Finally, we evaluated the immunohistochemical assay for two clinical specimens of NSCLC whose EGFR status had been confirmed by direct sequencing. The upper sections of an NSCLC tumor with no EGFR mutation were negative for both AbyD02889 and AbyD02890 (Figure 6, panels b and c) while the lower sec-

tions with the L858R mutation were positive for AbyD02889 but negative for AbyD02890, suggesting that AbyD02889 could specifically detect the EGFR (L858R)-expressing cells even in a paraffin embedded section and might be useful for the immunohistochemical examination of EGFR mutations.

Discussion

We developed an antibody specifically recognizing a mutant EGFR (L858R) protein by screening a HuCAL phage library. We found that this specific antibody (AbyD02889) could specifically detect the mutant protein and was available for ELISA, immunoblotting, immunocytochemistry, and immunohistochemistry. Here, we

would like to discuss the feasibility of the use of this antibody in the cancer research field and in the clinical situation.

Recent cancer research has shown trends toward the discovery of a molecular target which is necessary to maintain tumor survival or growth.[14] This may possibly lead to the development of a specific inhibitor for molecular targets characteristic of various tumor species, which may become one promising therapeutic strategy for cancer. Typical cases of this strategy are the currently successful results of tyrosine kinase inhibitors such as Iressa, tarceva, and glivec.[15-19] Gefitinib has been reported to be effective especially against NSCLC patients with E746 A750del in exon 19 or L858R in exon 21, which account for approximately 90% of the NSCLC-associated EGFR mutations.[8,9] In other words, the development of Iressa has made a revolutionary contribution to lung cancer treatment. The EGFR mutation in Japanese NSCLC patients has been reported to be relatively high in frequency, although there are differences among the various human racial types.[20,21]

Under these circumstances, it is important to know how to select the group of patients in whom the benefit of Iressa may be maximized. Based on this view, we have developed the Scorpion ARMS method, a highly-sensitive PCR-based detection method of gene mutation, and have reported its reliability and feasibility for clinical use.[9,10] In addition to the high sensitivity, this method has another great advantage that contamination of the wild-type EGFR gene derived from normal tissue surrounding the tumor does not interfere with its sensitivity or specificity. However, because there were a few false negative or positive cases,[9] it is still impossible to say that this method is perfect. For example, when we examine the genome DNA containing a lower amount of the mutant EGFR gene than the lower detectable limit (this is considered to be caused by the situation that only a small number of tumor cells having EGFR mutation exist in the tissue sample), the Scorpion-ARMS technique may possibly fail to detect this mutation. Although this is considered as one limitation of the ARMS technology, we still have to make a greater effort to improve the precise detection of the mutation. One strategy is to detect the protein of the mutant EGFR using a mutant-specific antibody, which would enable us to improve the detectability through

the use of the antibody and the Scorpion-ARMS method in combination. Form this point, we stress the significance of the mutant EGFR (L858R)-specific antibody and would like to discuss its feasibility.

This antibody enables us to examine the mutation using one tumor cell, which is a great advantage in addition to its contribution to improvement of the detectability of the mutation. Actually, Figures 4 and 5 show that only the cells expressing mutant EGFR (L858R) protein were stained with the mutant-specific antibody. One tumor cell is the smallest tumor sample necessary for examination. Using the mutant-specific antibody we have developed, it is therefore possible to detect a single cell mutation, meaning that we can diagnose the EGFR mutation in one tumor cell derived from the smallest clinical sample with a less invasive approach. This antibody offers the potential to make a large contribution to the clinical evaluation of the EGFR mutation. Furthermore, this antibody shows promising importance when considering tumor oncogenesis and progression from the aspect of the research field. At present, it remains controversial whether a hit on the EGFR gene causes the development of cancer or if one heteropopulation of cancer cells in the tumor acquires the EGFR mutation. This discussion is considered to be related largely to the clinical responsiveness of cancer to Iressa, and its prognosis. According to the latter hypothesis of oncogenesis, the tumor cells with wild-type EGFR, to which Iressa may be less effective, will survive and grow even while Iressa responds well to the cells harboring the EGFR mutation. There we will find that the mutation-positive and negative tumor cells co-localize in one tumor mass using our EGFR (L858R)-specific antibody. Finally, we would like to say that our EGFR (L858R)-specific antibody will be a useful tool to obtain more important information on NSCLC in the clinical situation or even in the research field as well. Our current research strategy is focused on developing a specific antibody against deletional EGFR mutants.

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Gefitinib Efficacy Associated with Multiple Expression of HER Family in Non-small Cell Lung Cancer

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Abstract. The aim of this study was to compare the relationship between HER family expression and clinical response to gefitinib. Patients and Methods: Tissues from thirty-one non-small cell lung cancer (NSCLC) patients treated with a monotherapy of gefitinib were analyzed. Expressions of HER family in 31 tumors were examined by immunohistochemistry. Results: The total expressions were 21 for EGFR (68%), 24 for HER2 (77%), 17 for HER3 (55%) and 4 for HER4 (13%). Fourteen out of 31 (45%) demonstrated triple expression of EGFR and HER2, as well as HER3 or HER4. A significantly better response rate (RR) and time to progression (TTP) were observed for the group with the triple expression than for the other groups (RR 50 vs. 11%; p<0.05, median TTP 4.29 vs. 1.2 months; p<0.05). Conclusion: Multiple expression of the HER family might be related with the clinical response to gefitinib and EGFR mutation status.

The epidermal growth factor receptor (EGFR/HER1) is a promising target for anticancer therapy. Gefitinib (ZD 1839, Iressa; AstraZeneca, London, UK) is an orally active, selective EGFR-tyrosine kinase inhibitor (1), which showed promise in a recent clinical trial of non-small cell cancer (NSCLC) cases, in terms of rapid symptom improvements (2-7) and clinically meaningful benefit in some patients (5, 7). Thus, the selection of individuals who may demonstrate a response to gefitinib is important. The degree of EGFR expression seems not to directly determine the response, although gefitinib is considered to be a targeted therapy by virtue of its selective inhibition of EGFR tyrosine kinase (8-10). Recent reports show that specific

missense and deletion mutations in the tyrosine kinase domain of the EGFR gene (11-13) are associated with EGFR tyrosine kinase inhibitor sensitivity. Although these EGFR mutations can account for almost all objective responses to tyrosine kinase inhibitors, the clinical benefit observed with these drugs and the survival benefit cannot be explained only by the presence of mutations.

The HER family includes the following four distinct receptors: EGFR, HER2 (ErbB-2), HER3 (ErbB-3) and HER4 (ErbB-4) and recent preclinical studies indicated that gefitinib (ErbB-4) causes reduced phosphorylation levels of not only EGFR, but also of HER2 and HER3 (14), inducing the formation of inactive EGFR/HER2 and EGFR/HER3 heterodimers (15). Based on these data, the co-expression profile of HER family receptors (especially the additional expression of HER2 and HER3) was hypothesized to play an important role in determining the efficacy of gefitinib in NSCLC cases. The relationship between the co-expression status of HER family members and gefitinib efficacy was evaluated with regard to response rate (RR), time to progression (TTP) and overall survival (OS).

Patients and Methods

Between September 2002 and January 2004, 31 advanced or recurrent NSCLC patients from whom tumor tissues were available, were treated with 250 mg of gefitinib monotherapy until disease progression at our institution. The medical records, pathology slides and imaging studies of these patients were retrospectively reviewed. The study was conducted after obtaining approval of the appropriate ethical review boards following recommendations of the Declaration of Helsinki for biomedical research involving human subjects.

For all patients, archival paraffin blocks of transbrochial lung biopsy (TBLB) specimens taken at the time of initial diagnosis (n=14) or tumor tissue specimens obtained by surgical resection (n=17) were sectioned for staining with antibodies against EGFR, HER2, HER3 and HER4 using an EGFR pharmDx kit (DAKO), Herceptest (DAKO), anti-HER3 (Chemicon) and anti-HER4 (Chemicon), respectively, with the Autostainer (DAKO).

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The results of the immunostaining were reviewed by an experienced pathologist (Y.I.). For HER2 staining, only moderate to strong cell membrane-specific immunostaining was considered positive, whereas cytoplamic staining was also taken into account for the other receptors.

Genetic analysis of the *EGFR* gene was performed on the 17 frozen tumor specimens obtained by surgical resection. Genomic DNA was extracted from 1-2 mm³ tumor specimens using REDExtract-N-AmpTM Tissue PCR Kit (Sigma) and the DNA was purified with a QIAmpDNA blood mini kit.

Genetic analysis of the *EGFR* gene was performed by PCR amplification of exons 18, 19 and 21. The following primers, specifically designed for this study, were used for PCR amplification: exon 18 (forward, 5'-AGGTGACCCTTGTCTCTGTGTTCT-3'; reverse, 3'-CACCSGACCATGAGAGGCCCTGCG-5'), exon 19 (forward, 5'-GA TCACTGGGCAGCATGTGGCACC-3'; reverse, 3'- TGGACCCCCAC ACAGCAAAGCAAAGCAGA-5'), exon 21 (forward, 5'-TTCCCATG ATGATCTGTC-3'; reverse, 3'-ATGCTGGCTGACCTAAA -5'). PCR was performed in a total volume of 20 µL, containing reaction buffer, 1.5 mmol/L MgCl₂, 0.2 mmol/L dNTPs, 100 nmol/L each primer, 0.5 units AmpliTaq (Biosystems) and 4 µL genomic DNA. Thermal cycling conditions included 3 min at 94°C, followed by 35 cycles of 94°C for 20 sec, 68°C for 40 sec and 72°C for 3 min.

After completion of the PCR reaction, the products were denatured (5 min at 90°C), immediately cooled on ice and loaded onto a nondenaturing polyacrylamide gel. The concentration of acrylamide was 1%-14% gradient. Electrophoresis was carried out for 3 h at 0°C at 72V/cm. The shifted bands were removed from the gel, and the recovered DNA was amplified in duplicate and subjected to bidirectional dye-terminator sequencing using the same primers used for amplification. Sequencing fragments were detected by capillary electrophoresis. SSCP and sequencing were performed by Hitachi Hitechnology Co. (Tokyo, Japan).

All 31 patients were evaluated for responses to gefitinib using WHO criteria (16). TTP and OS were measured from the date of initial gefitinib treatment to the date of disease progression and to the date of death or last follow-up examination, respectively, and were estimated using the Kaplan-Meier method (17).

Since recent clinical studies suggested that females with no smoking history and an adenocarcinoma were positive predictors for gefitinib responses (5, 7, 18), these were evaluated as potential prognostic factors for gefitinib sensitivity. Age, gender, performance status, histology type, number of prior chemotherapy, prior platinum or docetaxel use, smoking history and HER family-expression were analyzed using the Fisher's exact test and the Students *t*-test. Differences in TTP and OS between the two groups were tested using the log-rank test. All statistical analyses were performed using SPSS Version 8 statistical software (SPSS, Inc., IL, USA).

Results

Patients characterization. Of the 31 patients, eleven (35%) were females and 74% of the patients had adenocarcinomas. Eleven patients (35%) had never smoked (Table I). The median age was 62 years (range, 51 to 77 years). Eight patients (25%) had no prior chemotherapy and the remainder had received platinum-based chemotherapy (Table I).

Immunohistochmical staining of HER family in tumors. Total positive staining included 21 for EGFR (68%), 24 for HER2

Table I. *Patient characteristics.*

Variable	No. of patients	%
Portial response		
yes	9	29
no	22	71
Gender		
male	20	65
female	11	35
Age, years		
median	62	
range	51 - 77	
Smoking history		
never	11	35
former/current	20	65
Histology		
adenocarcinoma	23	74
non-adenocarcinoma	8	25
Stage		
III	5	16
IV	12	39
recurrence after surgery	14	45
Performance status		
0.1	18	58
>2	13	42
No. of prior chemotherapy regimens		
0	8	25
1	14	45
2	7	23
3	2	6
Prior platinum		
yes	23	74
no	8	26
Prior docetaxel		
yes	7	23
no	24	77

Table II. *EGFR/HER2/HER3/HER4 expression status.*

Status	No. of patients	%
EGFR		
negative	10	32
positive	21	68
HER2		
negative	7	23
positive	24	77
HER3		
negative	14	45
positive	17	55
HER4		
negative	27	87
positive	4	13
Co-expesion		
no expression of EGFR	10	32
mono-expression of EGFR	4	13
double-expression of EGFR/HER2	3	10
triple-expression of EGFR/ HER2/HER3 or HER4 +	14	45

(77%), 17 for HER3 (55%) and 4 for HER4 (13%) (Table II). Fourteen out of 31 (45%) samples demonstrated triple expression of HER family members (13 were positive for EGFR/HER2/HER3 and 1 for EGFR/HER2/HER4). Three (10%) double-expressed EGFR /HER2, 4 (13%) mono-expressed EGFR and 10 (32%) exhibited no expression of EGFR (Table II).

Clinical response to therapy. The response to treatment was evaluated in all 31 patients. Nine partial responses (PR; 29%) were observed. The results of univariate analysis of the significance of potential prognostic factors for gefitinib sensitivity using Fisher's exact test and a *t*-test for age are provided in Table III. An objective response was observed in 6 out of 11 females and in 3 out of 20 males ($p<0.05$), 6 out of 11 non-smokers and 3 of 20 current or former smokers ($p<0.05$), 8 out of 23 adenocarcinoma and 1 out of 8 non-adenocarcinoma ($p=0.37$) cases (Table III).

No correlation was found between the EGFR-expression status and gefitinib efficacy. However, there was a significant difference in the gefitinib response between the group with triple expression of EGFR/HER2 /HER3 or HER4 and the remainder (50% *vs.* 11%, $p=0.043$) (Table III). There was also a significant difference in TTP between these groups (TTP; 4.3 *vs.* 1.2 months, $p=0.0449$, Figure 1A). Median OS time of the group with triple expression was longer time than that of the others remainder, but was not significant (15.3 *vs.* 6.7 months, $p=0.099$, Figure 1B).

EGFR mutations. The genomic status of the TK domain of the *EGFR* gene was evaluated in 17 frozen primary NSCLC tumor specimens. Exons 18, 19 and 21 were subjected to mutational analysis. PCR amplification followed by SSCP analysis was used since SSCP analysis is more sensitive than direct sequencing (19). A total of 6 of the shifted bands were found and were directly sequenced. Four mutations were located in exon 19, 1 in exon 21, and 1 in exon 18. Of the 6 mutations identified, 4 were in frame deletions in exon 19, and 2 were aminoacidic substitutions in exons 21 and 18. The deletions "E746-A750 del", "L747-S752" and "L747-S752 del, P753S" in exon 19, the leucine to arginine mutation (L858R) in exon 21 and "E709D, T710del" in exon 18 were found.

EGFR mutations were more frequently found in adenocarcinomas than non-adenocarcinomas (6 out of 12 adenocarcinomas and 0 out of 5 non-adenocarcinomas). There were no obvious differences in *EGFR* mutation status with gender or smoking history (3/6 females and 3/11 males; 3/5 non-smokers and 3/12 current or former smokers). The clinical responses to gefitinib in 6 cases with *EGFR* mutations were 3 partial response (PR) and 3 stable disease. No PR was observed in the 11 cases without *EGFR* mutations.

In addition, all 6 cases with *EGFR* mutations showed triple expression of EGFR/HER2/HER3 or HER4 and no *EGFR* mutation was detected in non-triple expression cases; $p=0.035$.

Table III. *Univariate analysis of features associated with sensitivity to gefitinib.*

Variable	Response (n=9) No. of patients	No response (n=22) No. of patients	P
Gender			
male	3	17	0.037
female	6	5	
Age, years			
median	62	63	0.97
range	53 - 74	51 - 77	
Histology			
adenocarcinoma	8	15	0.37
non-adenocarcinoma	1	7	
No. of prior chemotherapy regimens			
0	1	7	0.37
>1	8	15	
Prior platinum			
yes	8	15	0.38
no	1	7	
Prior docetaxel			
yes	1	6	0.64
no	8	16	
Performance status			
0,1	5	13	0.99
>2	4	9	
Smoking history			
never	6	5	0.037
former/current	3	17	
HER family expression status			
EGFR			
negative	1	9	
positive	8	13	0.21
HER2			
negative	1	6	0.64
positive	8	16	
HER3			
negative	3	11	0.46
positive	6	11	
HER4			
negative	7	20	0.56
positive	2	2	
Co-expression of HER family			
triple expression of EGFR/HER2/HER3 or HER4+	7	7	0.043
Other	2	15	

Discussion

We studied the correlation between HER family expression status and sensitivity to gefitinib monotherapy in patients with advanced NSCLC and we showed that sensitivity to gefitinib is related to the triple expression of EGFR, HER2 and HER3 or HER4.

Although inhibition of EGFR tyrosine kinase is considered an essential mode of action of gefitinib, previous studies
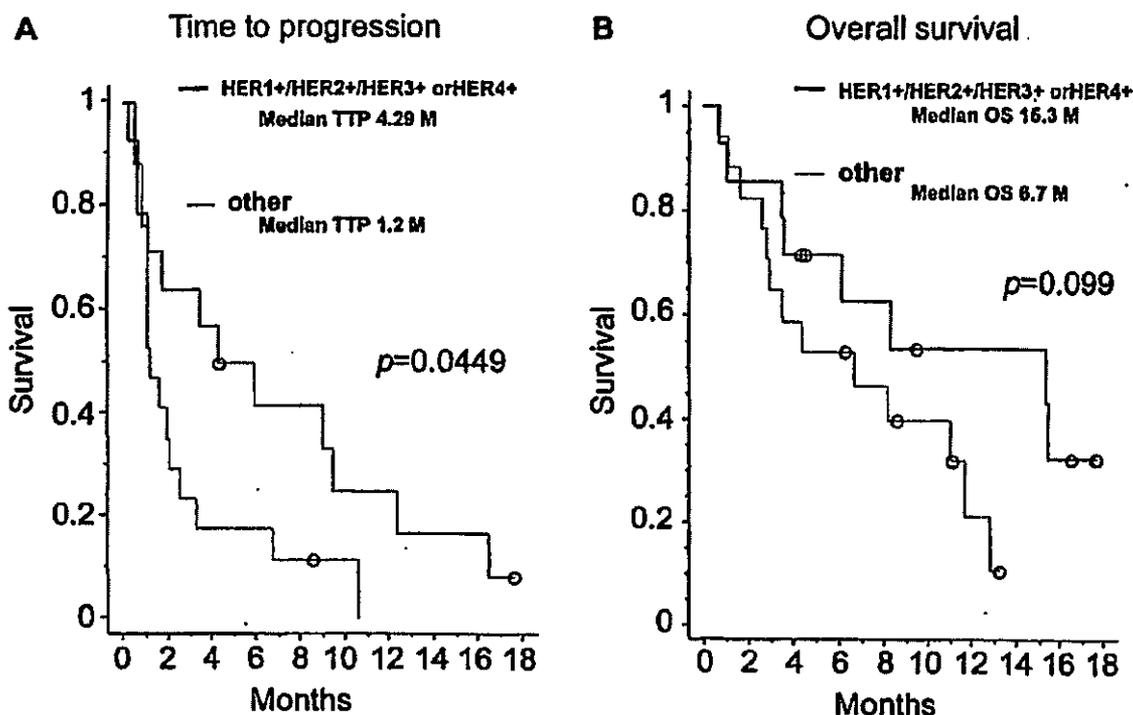
A Time to progression   **B** Overall survival

Figure 1. (A) Time to progression (TTP) and triple expression of HER family (EGFR1+/HER2+/HER3+ or /HER4+); (B) Overall survival (OS) and triple expression of HER family (EGFR+/HER2+/HER3+ or /HER4+).

indicated that expression of EGFR does not itself determine sensitivity to gefitinib therapy (8-10, 14, 20, 21). We also found no correlation between the EGFR-expression status and gefitinib efficacy. However, there was a significant difference in the gefitinib response between the group with triple expression of EGFR/HER2/HER3 or HER4 and the remainder (50% vs. 11%, p=0.0439). There was also significant difference in TTP between these groups (4.3 vs. 1.2 months, respectively; p=0.0449). The results are, thus, in line with those of several recent studies which indicated that all of the HER family members are targeted by gefitinib. Preclinical studies indicate that heterodimer formation is a factor impacting on sensitivity. Gefitinib causes reduced basal phosphorylation of EGFR/HER2, EGFR/HER3 and HER2/HER3 and this might correlate with the antitumor activity of this agent (14, 15, 22).

Cappuzzo et al. reported no correlation between co-expression of EGFR and HER2 in NSCLC patients and the results of treatment with gefitinib with regard to RR, TTP and OS (8). However, these authors did not evaluate the expressions of HER3 and HER4, and HER3 positive rate which is relatively high (>50%) in this study. This might be critical for sensitivity to gefitinib. In addition, the RR in our study was relatively high compared with their value (29% vs. 15.9%, respectively) and an ethnic difference may account for this difference.

Sensitivity to gefitinib appears to be greatly influenced by the presence of activating mutations within the kinase domains (11-13) and the mutations were more frequent in tumors from Japanese and other East Asian patients (23-25). EGFR gene mutations in exons 18, 19, 21 were analyzed by SSCP in this study and a comparable frequency of EGFR mutations was detected by the SSCP methods in a previous report (6 out of 17 cases; 35%). EGFR mutations were detected in cases with triple expression of EGFR/HER2 /HER3 or HER4, but no mutation was found in cases without triple expression of HER family receptors. Although these EGFR mutations can account for almost all objective responses obtained with gefitinib, the clinical benefits, such as long stable disease, cannot be explained only by the presence of mutations.

Recently, Hirata et al. reported that NSCLC cells trasfected with the HER2 gene (LK2/HER2) were approximately 5-fold more sensitive to gefitinib than LK2/mock cells and cell survival and death were dependent on HER2/HER3 signaling. However, the sensitivity was about 20-fold lower in the LK2/HER2 cells than in the PC9 cells, which harbor in-frame deletion mutation of EGFR (E746-A750) in exon 19 (22). These results support our findings that multiple expressions of HER family members may contribute to gefitinib efficacy and multiple expressions of HER family members may play more important roles in cases without EGFR mutations than in case with mutations of this gene.

This small pilot study is not enough to conclude that triple expression of HER family members is strong predictive factor for response to gefitinb. Further large-scale prospective trials are necessary to confirm these results.

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INVITED REVIEW SERIES: LUNG CANCER

Clinical aspects of epidermal growth factor receptor inhibitors: Benefit and risk

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Abstract: Gefitinib and erlotinib are small molecules that selectively inhibit epidermal growth factor receptor (EGFR) tyrosine kinase activity. Developmental studies of either drug have failed to show synergistic effects when combined with cytotoxic drugs as the first line treatment in patients with advanced non-small cell lung cancer, but erlotinib has shown survival prolongation when compared with best supportive care in patients with recurrence. Female gender, adenocarcinoma histology and lack of smoking history are considered to be clinical factors predicting response. Being positive for EGFR mutations in exons 18–24 in cancer cells has a strong correlation with response. On the other hand, preceding idiopathic pulmonary fibrosis, male gender and history of smoking appear to be risk factors for EGFR tyrosine kinase inhibitor-induced interstitial lung disease in the Japanese population. Reports on these factors predicting response or risk for interstitial lung disease have attracted great interest in the relation between cancer genetics and drugs, as well as the relation between ethnicity and genetics. In clinical practice, EGFR tyrosine kinase inhibitor should be prescribed with careful consideration and it is essential to assess benefit and risk of the drug.

Key words: interstitial lung disease, lung cancer.

INTRODUCTION

Lung cancer is the leading cause of cancer-related death, with 1.18 million deaths worldwide.[1] Incidence and mortality rates are increasing because the disease is very much influenced by past exposure to tobacco smoking.[1] Eighty-five per cent of tumours are non-small cell lung cancer (NSCLC), the others, small cell lung cancers. More than half of NSCLC patients are diagnosed at an advanced stage at which mainly systemic chemotherapy is recommended.

For patients with previously untreated advanced NSCLC, combination chemotherapy with cisplatin or carboplatin and third generation agents, such as docetaxel, gemcitabine, irinotecan, paclitaxel or vinorelbine, have yielded a response in 30–40% of the patients, 7–12 months of median survival times

(MST) and 30–40% achieved 1-year survival.[2,5] When these tumours become refractory to the first-line chemotherapy, docetaxel[6,7] and pemetrexed[8] are reportedly effective cytotoxic agents as second-line treatments, with response rates of about 10%, an 8-months MST and 30% achieving 1-year survival with symptom palliation.

In addition to these cytotoxic agents, two molecular targeting agents have been approved for the treatment of advanced NSCLC, gefitinib and erlotinib. Both agents are orally active epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI), small molecules belonging to the quinazolinamine class, which inhibit EGFR tyrosine kinase activity by competing for the ATP-binding domain, thereby inhibiting cellular proliferation, angiogenesis and consequently reducing tumour invasion and metastasis (Fig. 1).

Gefitinib was a first molecular targeting agent approved for lung cancer in the world. Because of ever higher response rates for recurrent disease, gefitinib was made public based on to the results of phase II trials. To date, it has, however, failed to prolong survival in NSCLC patients, and the problem of interstitial lung disease (ILD), especially in Japan, has also been recognized. In this review, the current status of
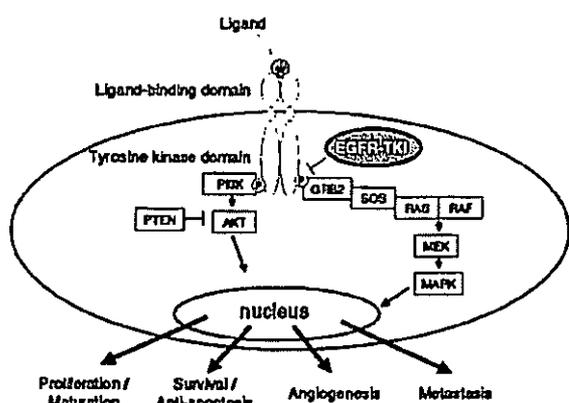
Figure 1 Epidermal growth factor receptor (EGFR) signal transduction, its biological consequences and EGFR tyrosine kinase inhibition (TKI).

the gefitinib and erlotinib will be discussed especially in terms of benefits and risks in clinical practice.

DEVELOPMENT

Two EGFR-TKIs, gefitinib and erlotinib, have been developed using similar procedures. Both drugs were well tolerated and had higher response rates in single agent phase I and phase II studies, but failed to indicate synergistic effects in phase III studies with combination of cytotoxic agents. Results of single agent phase III studies for patients with recurrent or resistant disease differed between the two drugs. Although erlotinib apparently prolonged survival, gefitinib did not.

Phase I trials

Four phase I trials of gefitinib were performed in 252 patients with a variety of solid tumours, including NSCLC, head and neck cancer and colorectal cancer.[9][12] Major adverse events were rash and diarrhoea. These events were generally mild and tolerable at doses not exceeding 600 mg/day and 700 mg/day came to be the maximal tolerated dose (MTD). Because doses of 150–800 mg/day were associated to tumour responses, 250 and 500 mg/day were selected for subsequent phase II trials. In the case of erlotinib, phase I trials identified a dose of 150 mg/day for further clinical development. Adverse events were similar to those of gefitinib and the incidence and severity of the adverse events generally increased as the dose increased.

Second-line phase II trials

Among single-agent phase II studies, one erlotinib and two gefitinib studies were conducted in patients

with NSCLC. Two large scale multicentre phase II trials of gefitinib were performed; Iressa™ dose evaluation in advanced lung cancer (IDEAL)-1 in Japan, Europe and Australia,[13] and IDEAL-2 in North America.[14] Eligibility criteria of the IDEAL-1 included having failed only one prior platinum-containing regimen, whereas the IDEAL-2 criteria were to have failed a platinum-containing regimen and docetaxel. Patients were randomized to gefitinib 250 or 500 mg/day. In the IDEAL trials, response rates ranged from 9 to 19% and severe toxicities were relatively uncommon. Treatment related toxicities, that is, diarrhoea, rash, acne, dry skin, nausea and vomiting, were slightly more severe and more frequent on 500 mg/day than on 250 mg/day. Because no additional response was observed with 500 mg/day, gefitinib at a dose of 250 mg/day was approved in Japan and the USA for treatment of advanced NSCLC.

A randomized phase II study was also performed with erlotinib in patients with previously treated advanced NSCLC.[15] The study, a comparison with best supportive care, revealed 150 mg/day of the drug to produce a 12.3% of objective response rate in patients with previously treated advanced NSCLC. MST was 8.4 months and the 1-year survival rate was 40% with no grade 4 toxicity.

First-line combination phase III trials

In a preclinical study, EGFR-TKI showed an additive effect on antitumour activity with no toxicity increase when combined with cytotoxic agents.[16][18] On the basis of these data, four randomized trials were conducted with gefitinib; Iressa™ NSCLC trial assessing combination treatment (INTACT)-1, 2,[19,20] and with erlotinib; TALENT[21] and TRIBUTE,[22] in chemotherapy-naïve patients with advanced NSCLC to compare chemotherapy plus EGFR-TKIs to chemotherapy alone.

In the INTACT-1 and TALENT trials, the chemotherapy regimen consisted of cisplatin and gemcitabine. In the INTACT-2 and TRIBUTE trials, the chemotherapy regimen was carboplatin and paclitaxel. Unfortunately, none of those studies showed any definitive benefit of adding an EGFR-TKI to standard chemotherapy in patients with NSCLC. These trials failed to support the concept of synergistic preclinical studies and to show additive or synergistic effects when combined with platinum-based chemotherapy as a first-line treatment for NSCLC.

Second-line phase III trials

To investigate the survival benefit of EGFR-TKIs as single agents, two large scale placebo controlled phase III trials were conducted as second- or third-line treatment for the patients with NSCLC. Iressa™ survival evaluation in lung cancer (ISEL) trial was planned to compare gefitinib with a placebo with 1692 patients.[23] Although the results of the study showed a response in the gefitinib group, there was no survival prolongation effect with gefitinib. MST

was 5.6 months in the gefitinib arm and 5.1 months in the placebo arm. On the other hand, the BR.21 trial investigated erlotinib as compared with a placebo.[4] Results of the trial included 731 patients showing a 9% of response rate in the erlotinib arm and less than 1% in the placebo arm. In terms of survival, 2-month prolongation of MST was achieved in the erlotinib arm, 6.7 months compared with 4.7 months in the placebo arm. Based on these data, erlotinib was approved in the USA and European countries.

Although study results were similar in phase I and phase II trials, only erlotinib, not gefitinib, produced a survival benefit compared with the placebo. The reason for the difference may be explained partly by the administered dose of gefitinib possibly being lower. In a phase I trial of gefitinib, a dose of 250 mg/day was less toxic and as effective as a dose of 500 mg/day but the MTD in the trial was 700 mg/day. On the other hand, a dose of 150 mg of erlotinib is nearly the MTD in a phase I trial. Another explanation is an issue of the difference in the response to previous treatment between participants in two trials. In terms of the best response, 18% of the patients in the gefitinib group had responded and 45% had progressed in the ISEL trial. By contrast, 38% of the patients in the erlotinib group had responded and 28% had progressed in BR.21 trial.

EGFR-TKI-INDUCED INTERSTITIAL LUNG DISEASE

Incidence

Although toxicities, like myelosuppression and vomiting, were not dose limiting for patients receiving gefitinib, a proportion of Japanese patients experienced ILD.[25-27] This type of adverse event has also been reported from Korea[28] and Taiwan.[29] In a large scale surveillance by the West Japan Thoracic Oncology Group (WJTOG), among 1976 patients, 70 patients (3.5%) were identified as having ILD after a panel review of 102 patients who were suspected by their physicians, and 31 patients (1.6%) who had died due to the event.[30] Another large scale post marketing surveillance conducted in Japan, an analysis involving 3322 patients, found that the incidence of ILD was 5.8%, and mortality due to ILD 2.3%.[31] Other smaller but detailed studies reported similar ratios of 5.4%

and 4.5% for incidence, 3.6% and 2.4% for mortality.[32,33] Report of the incidence of ILD in first-line single agent treatment is limited. In a phase II study of chemo-naïve patients with advanced NSCLC, 4 of 37 patients (11%) died due to severe ILD.[34] In this study, only a CXR was required to exclude preceding interstitial pneumonia or pulmonary fibrosis.

A Korean report indicated two of 65 patients (3%) to have ILD.[28] Another study in Taiwan, of patients with brain metastases reported four of 76 patients (6%) to have experienced non-lethal ILD.[29] Regional differences seem to exist because relatively higher ratios of pulmonary involvement have only been reported in East Asian countries. The incidence of ILD is reportedly only 1.0–1.1% in the USA or Europe.[19,20,35,36] The ISEL study conducted in both Europe and Asia was the only study in which a difference in incidence between ethnicities could be compared directly. East and South-east Asian patients tend to suffer more ILD in 3–4% of the patients although the frequency of ILD in all population was 1%. However, there were no differences in incidence between patients receiving gefitinib versus a placebo.[23]

Risk factor

Several Japanese studies have reported risk factors for ILD. In the WJTOG surveillance, the presence of idiopathic pulmonary fibrosis (IPF), male gender and history of smoking were independent predictive factors for developing ILD.[30] In other studies, multivariate analysis revealed that preceding IPF, poor performance status (PS), smoking history, prior history of irradiation or chemotherapy were independent risk factors for ILD.[31-33,37] In these studies, the most striking factor was pre-existing IPF, with a higher odds ratio. Factors associated with a poor prognosis have been analysed. These included a short interval from initiation of gefitinib treatment to the onset of ILD, an acute interstitial pneumonia (AIP) pattern on CT, the presence of preceding IPF, male gender and a poor PS.[30,33,37] (Table 1)

Diagnosis and patterns

Most patients with EGFR-TKI-induced ILD experienced symptoms such as coughing, increasing

Table 1 Factors associated to EGFR-TKI-induced interstitial lung disease

	Risk factors for ILD	Factors associated to poor prognosis after ILD
Definitive	Preceding IPF	
Possible	Male gender	AIP patterns
	History of cigarette smoking	Preceding IPF
	Poor PS	Early onset of ILD
	Prior chemotherapy	Male gender
	Japanese/East Asian ethnicity	Squamous cell carcinoma

AIP, acute interstitial pneumonia; EGFR-TKI, epidermal growth factor receptor tyrosine kinase inhibitor; ILD, interstitial lung disease; IPF, idiopathic pulmonary fibrosis; PS, performance status.

Table 2 Predictive markers for response to EGFR-TKI

	Clinical	Molecular
Definitive	Female gender adenocarcinoma Lack of smoking history Japanese/East Asian	Positive EGFR mutation
Possible	Better PS No preceding IPF Skin eruption as adverse event	Increased EGFR gene copy number Positive p-Akt expression Negative K-ras mutation

EGFR-TKI, epidermal growth factor receptor tyrosine kinase inhibitor; IPF, idiopathic pulmonary fibrosis; PS, performance status.

dyspnoea and fever. It is difficult to diagnose ILD and differentiate it from other respiratory conditions that produce similar symptoms, such as infections, and cancer progression. According to the surveillance, suspicion of ILD by the attending physician was refuted by an expert panel consisting of pulmonologists and radiologists in 15.7–31.4% of patients.[30,31,33,37] These findings indicate that a diagnosis of ILD might not always have been correct in other reports.

As well as other pulmonary infiltrative disease, it must be emphasized that accurate diagnosis before the start of the treatment is necessary. When any sign or symptom or CXR abnormality appears, CT, especially high-resolution CT (HRCT), is recommended to diagnose interstitial shadows on the CXR. Screening for respiratory tract infection is also essential, including culture and polymerase chain reaction examination for pneumocystis carinii and aspergillus, for example. Transbronchial biopsy or BAL may contribute to making a correct diagnosis, and may be the key to the mechanism of EGFR-TKI-induced ILD.

Some reports have tried to classify radiological patterns and clinical course.[31,33,36,38] There seem to be four patterns. About a half of the ILD patients showed non-specific ground-glass attenuation on CT or HRCT without lung volume loss on CXR.[33,36,38] This group and two other small groups, including cryptogenic organizing pneumonia-like pattern and acute eosinophilic pneumonia pattern, seemed to correlate with a fair prognosis or better response to steroid therapy.[35] The remaining one third of the patients showed AIP-like pattern with extensive bilateral ground-glass attenuation or airspace consolidation with traction bronchiectasis on CT and lung volume loss on CXR. The prognosis of AIP-like pattern patients were very poor with 75–100% dying early.[33,36] In some patients, the histopathology at autopsy revealed diffuse alveolar damage.[25,31] These findings may support the EGFR-TKI-induced ILD hypothesis that EGFR inhibitor suppresses lung injury repair and results in irreversible alveolar damage.

PROGNOSTIC FACTORS

According to the trials and surveillance of EGFR-TKIs used as first-line treatments or for refractory cases, as single agents or combined with other therapies,

several factors have been proposed to predict response, long-term prognosis, or risk for ILD.

In summary, female gender, adenocarcinoma, lack of smoking history, being Japanese or another East Asian ethnic group are considered to be clinical factors predicting response. In addition, better PS and lack of preceding pulmonary fibrosis and skin rash while taking the drug could be predictive factors.[13,14,30,32,39] (Table 2)

Based on molecular analysis, being positive for mutations in EGFR exons 18–24, which encode the kinase domain of the protein, strongly predict response to EGFR-TKI, especially in the Asian population.[40-46] Other molecular factors include an increased EGFR gene copy number,[47-49] p-Akt expression[28,50] and lack of K-ras mutation,[51,52] but are still controversial. To clarify whether being positive for EGFR mutations correlates with better survival, some prospective studies are now underway.

In addition to these molecular factors associated with primary response or resistance, a second mutation T790M in exon 20 is reported in acquired resistance to EGFR-TKI.[53,54]

A numbers of research reports and practical experience from Asian countries support the favourable 'benefit to risk' balance of treating NSCLC patients. However, these results still lack survival advantages, and there is the problem of ILD, which appears to limit the use in patients with advanced NSCLC in taking EGFR-TKIs, even as second- or third-line treatments. It is necessary to assess benefit and risk individually before prescribing the drug and to give the patients adequate information to make an informed decision. Even in East Asian countries, gefitinib should be used only in clinical trials or for well-assessed patients.

SUMMARY

It remains unclear why gefitinib appears to produce a greater response in Asian patients than in patients from the rest of the world. Now, several genetic studies are starting to provide clarification of the mechanism underlying the differences in response and adverse events between ethnicities.

The benefits of using EGFR-TKI as the first-line treatment for NSCLC also remain unclear. There are