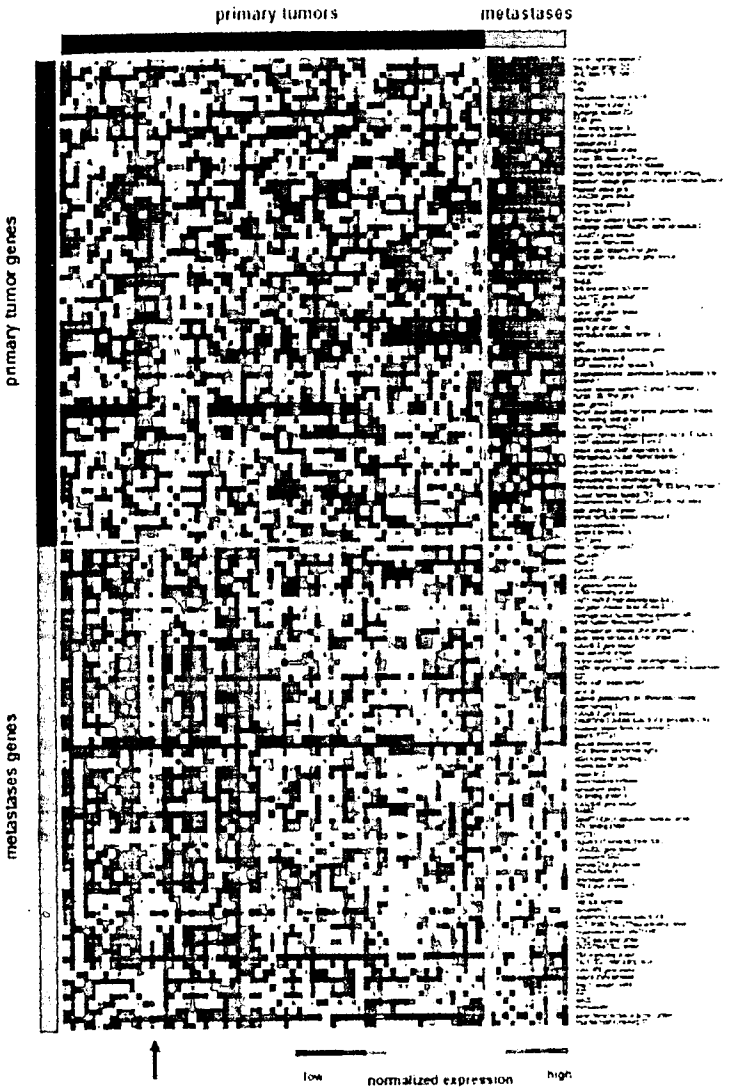

Fig. 4 . Genes associated with metastases.
Columns represent human tumor samples (64 primary and 12 metastatic adenocarcinomas); rows represent the 128 genes (64 overexpressed and 64 underexpressed in metastases) that best distinguished the primary from the metastatic tumors using a weighted-voting algorithm 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. Sorile 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.[6] 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, immunocytochemistry, 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-

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

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,[11-18] 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 H1-18 (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 ug/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 H1-18 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

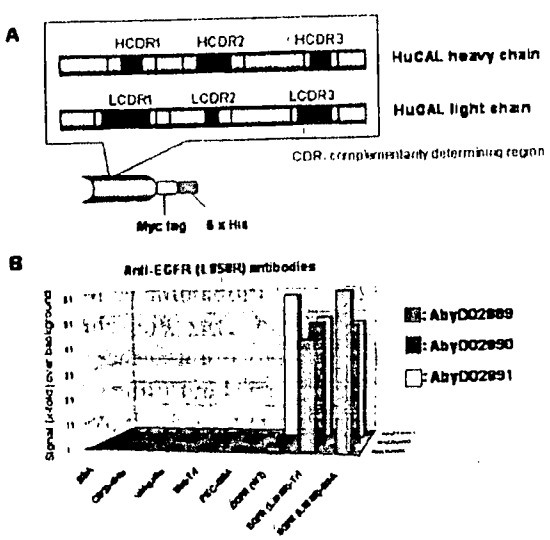

Fig. 1 Generation of monoclonal antibodies against a mutant EGFR (L858R)
(A) A representation of the structure of anti-EGFR (L858R) antibodies. The structural diversity of the human antibody repertoire is represented by heavy and light chain variable region genes. Highly variable genetic cassettes (CDRs: 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.

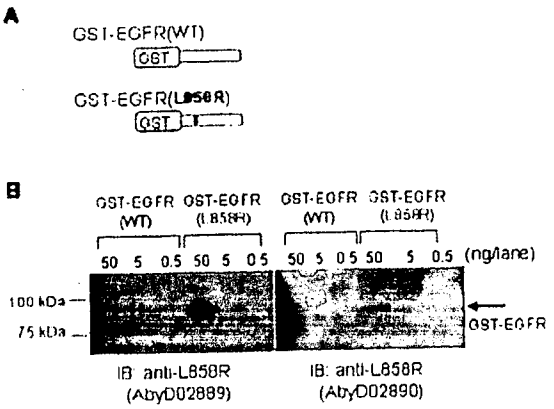

Fig. 2 Immunoblot analysis of two recombinant GST-fused EGFR proteins, GST-EGFR(WT) and GST-EGFR(L858R), using AbyD02889 and AbyD02890.
(A) Cytoplasmic domains of wild-type and L858R-mutant EGFR were fused with glutathione S-transferase (GST) and used for immunoblot analysis as the antigens against AbyD02889 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.

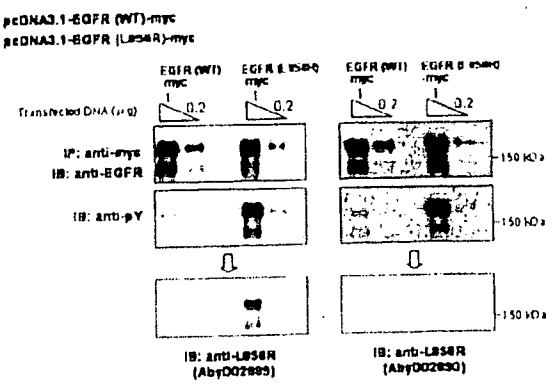

Fig. 3 Checking the specificity and affinity of AbyD02889 and AbyD02890 against the full length wild-type or L858R-mutant EGFR protein expressed in the HEK293 cells. One or 0.2 μg of either pcDNA3.1-EGFR (WT)-myc or pcDNA3.1-EGFR (L858R)-myc were transiently transfected into the HEK293 cells (2×10^6 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, anti-pY 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 $2x10^6$ 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, I, 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 H-18 cells harboring an intrinsic EGFR (L858R) mutation using AbyD02889. The H-18 cells were positive for AbyD02889 (Figure 5, panel b) but the HEK293

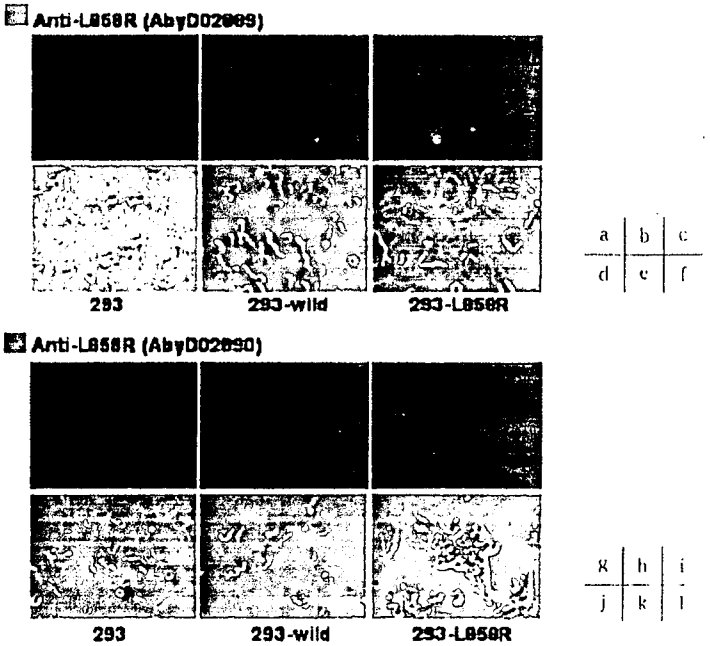

Fig. 4 Evaluation of the specificity of AbyD02889 and AbyD02890 by immunocytochemical analysis
The HEK293 cells transfected with pcDNA3.1 (a, d, g, and j), pcDNA3.1-EGFR (WT)-myc (b, e, h, and k), or pcDNA3.1-EGFR (L858R)-myc (c, f, I, and l) were examined immunocytochemically using the HuCAL antibodies. 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, permialization, 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.

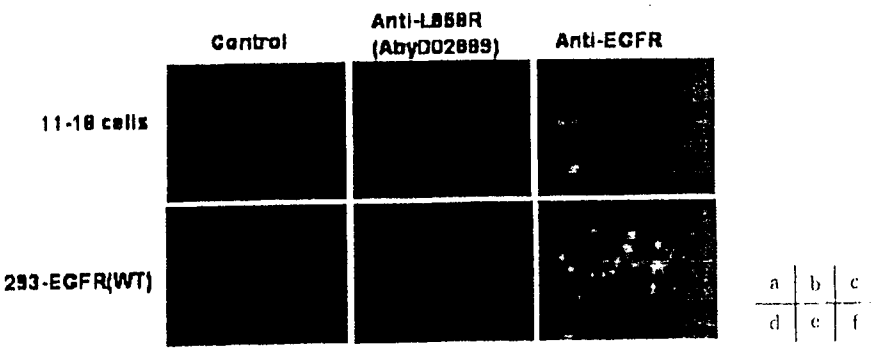

Fig. 5 Specificity of AbyD02889 against EGFR (L858R) protein intrinsically expressed in the 11-18 cells. The 11-18 cells and HEK293-expressing EGFR (WT)-myc (293-EGFR(WT)) were ectopically plated on a PLL-coated 24-well plate at 5,000 cells/well. After fixation, permialization, and blocking, the cells 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.

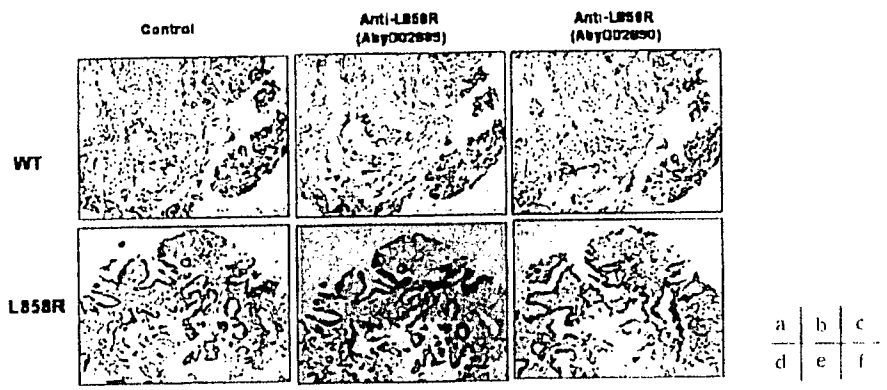

Fig. 6 Immunohistochemical analysis of two clinical specimens of non-small cell lung cancer whose EGFR status had been confirmed by the direct sequencing method.
The upper and lower three panels show DAB staining by AbyD02889 and AbyD02890 for tissue sections of EGFR (WT)- and EGFR (L858R)-expressing 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 sections 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.[8,6] 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. From 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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EGFR mutation in various tissues

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Abstract Somatic mutations have been demonstrated in various tumors. *EGFR* mutations were first demonstrated in adenocarcinoma of the lung, and a large-scale retrospective study has clearly shown that these mutations are specifically observed in this form of the disease. Recently, possible occurrence of *EGFR* mutations in other tumor types including ovarian and colorectal malignancies has been reported. This raises the possibility of application of EGFR-specific tyrosine kinase inhibitors (EGFR-TKI) to the treatment of these malignancies, although broad success in this venture would depend on the frequency of such mutations. In this article, we discuss somatic mutations in various tumors as well as potential application of TKI to their treatment. Ethnic difference in the frequency of somatic mutations is another area of interest since it is closely related to clinical response to EGFR-TKIs. Preliminary studies have revealed such ethnic variations regarding *EGFR* mutation and gene amplification. Ethnic difference of transcriptional regulation of *EGFR* has also been demonstrated. We recently found a biomarker related to clinical response to EGFR-TKI that might explain the ethnic differences in response to this therapy. Various tyrosine kinases are known targets of TKIs. Thus genomics of individual patients may allow personalized target-based therapeutics.

Keywords EGFR mutation · Tyrosine kinase inhibitor · Ethnicity · HLA

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EGFR mutation in various cancers

Somatic mutations have been demonstrated in various tumors. *EGFR* mutations were first demonstrated in adenocarcinoma of the lung, and a large-scale retrospective study has clearly shown that these mutations are specifically observed in this form of the disease [10]. However, extensive analysis of somatic mutation in various tumors subsequently demonstrated the existence of *EGFR* somatic mutation in many human tumors such as colorectal and head and neck cancer, renal cell carcinoma, prostate cancer, and cholangiocarcinoma [4, 7, 8]. Gwak et al. [5] reported *EGFR* mutation in cholangiocarcinoma and found that it was detectable in 13.6% (3/22) of patients. The type of mutation was deletion of exon 19. This is commonly observed in intrahepatic and poorly differentiated tumors. These and other researchers also reported this *EGFR* mutation in squamous cell head and neck carcinoma [7], and Cohen's group demonstrated a new mutation on *erb2* and gene amplification in this disease [3]. The mutation has also been reported in persistent ovarian and primary peritoneal carcinoma in clinical phase II trials of gefitinib [14]. Similar types of mutation have been reported in lung cancers, although these seem to be of minor occurrence [4]. Thus somatic mutations of *EGFR* exist in various tumors. Because of limited samples, it

remains unknown whether *EGFR* mutation in cancer is correlated with clinical response to EGFR-specific tyrosine kinase inhibitors (EGFR-TKI). *EGFR* mutation in other types of tumors than lung cancer seems correlated with immunohistochemical expression but correlation with gene amplification is unknown [14]. Functional aspects of *EGFR* mutation in other types of tumors are also only partially understood. To clarify the significance of somatic mutations in various tumors, tissue banking is necessary. In addition, validated and standardized analytical methods and cross-validation are important to give consistent results. We should also consider how to conduct clinical trials of target-based drugs for less common tumors based on biological data.

Ethnic difference in *EGFR* mutation

Ethnic difference in *EGFR* mutation is another important topic. It is considered that ethnic differences may determine both the frequency of *EGFR* mutation and response to TKi [2]. However, although it has not been fully discussed whether these differences are due to ethnic or merely geographical divides, ethnicity can explain differences in clinical response because of the data acquired in Asian–US patients. It is also considered that differences among the regions of Asia might be obtained: patterns of *EGFR* mutation may differ between Japanese, Chinese, Korean, South Indian, and Turkish individuals [16]. Expanding genome databases should eventually pinpoint the contribution of ethnicity in this regard. Already there is some evidence related to ethnic differences. A CA repeat exists in exon 1 of *EGFR*, related to transcriptional level of this gene. The length of CA repeat varies and is related to ethnicity [9]. Japanese have longer CA repeat compared with Caucasians. Moreover, intron 1 polymorphism reportedly mediates response to EGFR-TKI [1].

What are the differences among the types of *EGFR* mutation? The deletion mutation in exon 19 and point mutation L858R in exon 21 are the two major mutations. Previously, we speculated that the deletion mutation is more frequently detected in Japanese and Asian lung cancer patients as compared with Caucasians. However, recent data seem to refute ethnic difference in the types of *EGFR* mutations [12].

A predictive biomarker related to ethnic difference of sensitivity to gefitinib

Ethnic difference might also exist in sensitivity to drugs. In most such cases, gene polymorphism including microsatellite polymorphism and single nucleotide polymorphism may explain ethnic difference of response to drugs.

Using microarray technique, we analyzed gene expression profiles of peripheral mononuclear cells in lung cancer patients receiving gefitinib as a first-line monotherapy. Our results revealed that HLA genotype was closely related to response to this agent. On the other hand, large ethnic difference of HLA genotype was recognized. Previous reports have demonstrated that HLA genotype plays a role in the metabolism of certain drugs and may be a prognostic factor in malignancies such as gastric, ovarian, and cervical cancers [6, 11, 13, 15, 17]. We hypothesize that HLA subtype may be related to response to gefitinib and might explain ethnic differences. Cross-validation study of this HLA biomarker is ongoing.

Ethnic difference of gefitinib toxicity profile

Subpopulation analysis of gefitinib's toxicity in the ISEL study revealed that only southwest Asian and Taiwanese patients exhibited high ratios of interstitial lung disease (ILD) while on this therapy [16]. However, ILD might not have been induced by gefitinib. More interestingly, the data indicated that Indian–British patients experienced severe (grade 3) skin toxicity along with higher response to gefitinib. Although these phenomena are based on subpopulation analysis, we can speculate that ethnic difference might guide toxicity as well as clinical response to EGFR-TKI. Genomic and biomarker research is necessary to further elucidate these preliminary findings.

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EGFR mutation status in tumour-derived DNA from pleural effusion fluid is a practical basis for predicting the response to gefitinib

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Epidermal growth factor receptor (EGFR) mutations are strong determinants of tumour response to EGFR tyrosine kinase inhibitors in non-small-cell lung cancer (NSCLC). Pleural effusion is a common complication of lung cancer. In this study, we assessed the feasibility of detection of *EGFR* mutations in samples of pleural effusion fluid. We obtained 43 samples, which was the cell-free supernatant of pleural fluid, from Japanese NSCLC patients, and examined them for *EGFR* mutations. The *epidermal growth factor receptor* mutation status was determined by a direct sequencing method (exons 18–21 in *EGFR*). *EGFR* mutations were detected in 11 cases (E746_A750del in seven cases, E746_T751del insA in one case, L747_T751del in one case, and L858R in two cases). The *EGFR* mutations were observed more frequently in women and non-smokers. A comparison between the *EGFR* mutant status and the response to gefitinib in the 27 patients who received gefitinib revealed that all seven patients with partial response and one of the seven patients with stable disease had an *EGFR* mutation. No *EGFR* mutations were detected in the patients with progressive disease. The results suggest that DNA in pleural effusion fluid can be used to detect *EGFR* mutations and that the *EGFR* mutation status may be useful as a predictor of the response to gefitinib.

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Lung cancer is a major cause of cancer-related mortality worldwide and is expected to remain a major health problem for the foreseeable future (Parkin *et al*, 2005). Most patients have advanced disease at the time of diagnosis. The initial therapy for advanced non-small-cell lung cancer (NSCLC) is systemic chemotherapy with a two-drug combination regimen, which often includes a platinum agent, but the median survival of patients treated with such regimens has ranged from only 8 to 10 months. Little improvement in the efficacy of chemotherapy has been made in the last 20 years (Breathnach *et al*, 2001; Kelly *et al*, 2001; Schiller *et al*, 2002).

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Targeting epidermal growth factor receptor (EGFR) is an appealing strategy for the treatment of NSCLC, because EGFR has been found to be expressed, sometimes strongly, in NSCLC (Franklin *et al*, 2002). Gefitinib ('Iressa', AstraZeneca) is a small molecule and selective EGFR tyrosine kinase inhibitor that has shown antitumour activity in NSCLC patients as a single agent in phase II trials (Fukuoka *et al*, 2003). Adding gefitinib to chemotherapy in phase III studies of patients with untreated advanced NSCLC did not significantly improve the outcome over chemotherapy alone (Giaccone *et al*, 2004; Herbst *et al*, 2004), and a possible explanation for the failure to observe any added benefit in these trials is that the patients had not been screened or selected for their ability to derive any clinical benefit from an EGFR inhibitor.

An association between mutations in sites of *EGFR* tyrosine kinase in NSCLC and hyper-responsiveness to gefitinib has recently been reported (Lynch *et al*, 2004; Paez *et al*, 2004). The mutations consisted of small in-frame deletions or substitutions clustered around the ATP-binding site in exons 18, 19, and 21 of *EGFR*, and the mutations increased the affinity of the enzyme for ATP and gefitinib (Lynch *et al*, 2004). Some investigators subsequently found that *EGFR* mutations are one of the strong determinants of tumour response to EGFR tyrosine kinase inhibitors (Pao *et al*, 2004; Han *et al*, 2005). The investigators

used surgical tissue to detect the *EGFR* mutations in their studies, but most patients who require gefitinib therapy are diagnosed at an advanced stage of the disease and are inoperable. As it is often difficult to obtain a sufficient tumour sample from patients with inoperable NSCLC to detect *EGFR* mutations by direct sequencing, a method of detecting *EGFR* mutations in other specimens needed to be established.

Malignant pleural effusion is a common complication of lung cancer. It is present in approximately 15% of patients at the time of diagnosis (Pass *et al*, 2005) and in 10–50% of patients during the course of the disease (Fenton and David Richardson, 1995). In about half of NSCLC patients with a pleural effusion, the effusion fluid is cytologically positive at the first time examined, and ultimately most effusions are determined to be malignant. As pleural effusion fluid sampling is usually easy, non-invasive, and repeatable, we hypothesised that tumour-derived DNA in the pleural effusion fluid of NSCLC patients would be a source of useful information on the status of the *EGFR* gene and could allow prediction of the response to gefitinib. Some investigators have reported that pleural effusion fluid is a useful clinical specimen for searching for point mutations in oncogenes, such as *K-ras*, *rho A*, *p53*, and *FHIT* (Nakamoto *et al*, 2001; Lee *et al*, 2004). As the two trials were small, the results regarding the sensitivity and specificity of detection of the mutations in pleural effusion as a diagnostic method were unclear. Detection of *EGFR* mutations in pleural effusion fluid has been described in one case report, and the patient responded to gefitinib (Huang *et al*, 2005). The results in that patient encouraged us to hypothesise that the *EGFR* mutation status determined in pleural effusion fluid is useful for predicting the responsiveness to EGFR tyrosine kinase inhibitors.

In the present study, we attempted to detect *EGFR* mutations in pleural effusion fluid and to clarify the usefulness of their detection as a predictor of the response to gefitinib.

PATIENTS AND METHODS

Patients

The subjects were NSCLC patients who had a pleural effusion at the time of diagnosis. The diagnosis of NSCLC was based on the histological or cytological findings, and the histological type was determined according to the WHO criteria (Travis *et al*, 1999). Patients' records consisted of age, gender, smoking habit, histological type, and treatment. Smoking status was collected from the patients' records. Patients were divided into three groups according to their smoking status: never smokers (<100 cigarettes/lifetime), former smokers ($\geqslant 100$ cigarettes/lifetime, no smoking at present), and current smokers ($\geqslant 100$ cigarettes/lifetime). The response of the patients treated with gefitinib was evaluated every 4 or 8 weeks in accordance with the 'Response Evaluation Criteria in Solid Tumours (RECIST)' guidelines. (Therasse *et al*, 2000). Partial response (PR) and stable disease (SD) were confirmed by a sustained 4-week follow-up. This study was approved by the Institutional Review Board of the National Cancer Center Hospital and of Kanazawa University Hospital, and written informed consent was obtained from all participants. No research results were entered into the patient's records or released to the patient or the patient's physician.

Collection of pleural effusion fluid and DNA purification

The pleural effusion fluid was collected into heparinised tubes between 29 March 2005 and 30 January 2006. No particular collection method was used. A 2-ml sample of the fluid was centrifuged at 250 *g* for 10 min at room temperature, and the supernatant was collected and stored at −80°C until DNA extraction. DNA was extracted from 1 ml of the supernatant with a Qiamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the blood and body fluid spin protocol in the manufacturer's instructions, with the following protocol modifications. The same column was used repeatedly until the whole sample had been processed. The DNA obtained was eluted in 50 μl of sterile bi-distilled buffer, and the extracted DNA was stored at −20°C until used. The amounts of DNA extracted were estimated with spectrophotometry.

Polymerase chain reaction amplification and direct sequencing

Exons 18, 19, 20, and 21 of the *EGFR* gene were amplified by polymerase chain reaction (PCR). The primers were designed based on the report by Lynch *et al* (2004). Genomic PCR of 1 μl of template DNA was performed in 25 μl volumes containing 0.75 U of Ampli Taq Gold DNA polymerase (Perkin-Elmer, Roche Molecular Systems Inc., Branchburg, NJ, USA), 2.5 μl of PCR buffer, 0.8 μM dNTP, 0.5 μM of each primer, and different concentrations of $MgCl_2$, depending on the polymorphic marker. The first PCR analyses were performed in a volume of 25 μl by 25 cycles consisting of a denaturation step at 94°C for 45 s, a primer annealing step at 58°C for 30 s, and an elongation step at 72°C for 30 s. The final step at 72°C was extended for 10 min. Nested PCR was performed with 20 cycles under the same conditions as the first PCR. Sequencing of each sample was performed in duplicate with an ABI prism 310 (Applied Biosystems, Foster City, CA, USA). PCR products were sequenced in both sense and antisense directions. *Epidermal growth factor receptor* mutations detected in the initial round of sequencing were confirmed by subsequent rounds of independent PCR and sequencing reactions. Only specimens in which a mutation was identified in both rounds were recorded as mutation-positive. The sequences were compared with the GenBank-archived human sequence for *EGFR* (accession number: AY588246). The nucleic acid and protein coordinates used to name the mutations are based on NM_005228.3 and NP_005219.2, respectively.

Statistical analyses

This study was carried out as exploratory research for detecting *EGFR* mutations from pleural effusion fluid and clarifying the relationship between the mutation status and clinical manifestations. The number of enrolled patients was therefore not precalculated. Patient characteristics, including gender, tumour histology, and smoking habit were tabulated according to their mutation status. Fisher's exact test was used to test for associations between the presence of *EGFR* mutations and the patients' characteristics. The relationship between response to gefitinib and the mutation status was evaluated individually.

RESULTS

Patients and pleural effusion specimens

Forty-three patients were enrolled in this study (Table 1). Two hundred and sixty-two patients were seen with stage IIIB and IV at our institutions in the period of this study. Forty-three of the 262 patients were enrolled in this study. The enrolled patients were not all of the patients with pleural effusion because written informed consent was not obtained from any patients with pleural effusion. Their median age was 62 years (range, 39–82 years), and there were 21 females (53.8%) and 17 never smokers (43.6%). The histological and/or cytological diagnosis was adenocarcinoma in 39 patients, and squamous cell

Table 1 Patient characteristics and *EGFR* mutation status

	(n)	EGFR mutation (n)
No. of patients	43	11 (25.6%)
Age (years)		
Median	63	
Range	39–82	
Gender		
Male	22	4 (18.2%)
Female	21	7 (33.3%)
Smoking habit		
Current	9	2 (22.2%)
Former	16	2 (12.5%)
Never	18	7 (38.9%)
Histology		
Adenocarcinoma	39	11 (28.2%)
Squamous cell carcinoma	1	0 (0%)
Large cell carcinoma	1	0 (0%)
Unclassified	2	0 (0%)
No. of patients treated with gefitinib	27	8 (29.6%)
PR	7	7 (14.3%)
SD	7	1 (0%)
PD	13	0 (0%)

EGFR = epidermal growth factor receptor; PD = progressive disease; PR = partial response; SD = stable disease.

carcinoma and large cell carcinoma in one each, and unclassified NSCLC in two patients. Non-small-cell lung cancer cells in the pleural effusion samples of 40 of the patients were identified cytologically. There were no malignant cells in the pleural effusion fluid of the other three patients. We have no data of the proportion of malignant cells and normal cells. Twenty-seven patients were treated with gefitinib (250 mg day^{-1}) and evaluated for a response. Eight of the 27 patients were treated with gefitinib as an initial treatment and the other 19 patients were treated with the agent as a second or third line. The others were treated with systematic chemotherapy, including a platinum agent. The results of the evaluation showed that seven of the 27 patients who received gefitinib therapy had a PR and seven had SD. The other 13 patients had progressive disease (PD). DNA was extracted from all 43 samples of pleural effusion fluid. Amounts of the DNA extracted were detectable from 27 samples at a concentration up to 144.0 ng ml^{-1}. Amounts from 16 samples were under the detectable limit.

Detection of *EGFR* mutations in pleural effusion fluid

Direct sequencing of PCR products in exons 18–21 of *EGFR* in the pleural effusion fluid of all patients allowed their mutation status to be determined. Heterozygous mutations were identified in 11 (25.6%) of the 43 patients (Table 1). Nine mutations were deletional mutations located in exon 19 (E746_A750del in seven, L746_T751del insA in one, L747_T751del in one), and two were substitution mutations located in exon 21 (L858R) (Table 2) (Figure 1). No mutations were detected in exon 18 or 20. The E746_A750 deletion and L858R substitution mutations were the most common (9 out of 11 mutations. 81.8%) and are well-known hotspot mutations described previously (Kosaka *et al*, 2004; Pao *et al*, 2004). No more than one mutation was identified per patient, and no EGFR mutations were detected in pleural effusion fluid that did not contain malignant cells.

Table 2 Site of mutations in exons 18–21 of *EGFR*

Nucleotide changes	Amino-acid changes	No. of patients
2481_2495del	E746_A750del	6
2482_2496del	E746_A750del	1
2483_2497del	E746_T753del insA	1
2486_2500del	L747_T751del	1
2819T>G	L858R	2

EGFR = epidermal growth factor receptor; del = deletion; ins = insertion. The numbering of the mutation sites was based on NM_005228.3 (nucleotide) and NP_005219.2 (amino acid).

Epidermal growth factor receptor mutation status and patients' characteristics

EGFR mutations were detected more frequently in the samples from females (7 out of 21, 33.3% of females, 4 out of 18, 22.2% of males; $P = 0.310$) and non-smokers (7 out of 17, 41.1% of non-smokers, 4 out of 22, 18.1% of current or former smokers; $P = 0.156$), although the differences were not statistically significant (Table 3). Of the 11 mutations, 63.6% were in women and 63.6% were in non-smokers. All of the patients with mutations had adenocarcinoma. No *EGFR* mutations were found in any of the patients with squamous carcinoma or large cell carcinoma. A comparison between the *EGFR* mutant status and the response to gefitinib showed that all seven patients with a PR and one of the seven patients with SD had an *EGFR* mutation. No *EGFR* mutations were detected in any of the patients with PD (Table 4). We have no response data from the 16 patients who had never treated with gefitinib, and we have not evaluated the relationship between the response to chemotherapy and the EGFR mutation status in pleural effusion fluid.

DISCUSSION

This is the first report of an analysis of the *EGFR* mutation status in DNA obtained from the pleural effusion fluid of a series of NSCLC patients and evaluation of the relationship between the mutation status and the clinical response to gefitinib. It is interesting that all patients who achieved a PR to gefitinib had the *EGFR* mutations. We hypothesised that the mutation status in DNA extracted from pleural effusion fluid would allow prediction of the clinical outcome of gefitinib therapy in NSCLC patients, and we therefore expected the pleural effusion fluid to be a practical source of DNA for detection of *EGFR* mutations. The sites of *EGFR* mutations found in this study are identical to these reported in previous studies (Kosaka *et al*, 2004; Pao *et al*, 2004). The main mutations found were in-frame deletions in exon 19 and the missense mutation L858R in exon 21. No patients had more than one mutation. It was possible to determine the mutation status of *EGFR* by using the DNA in only 1.0 ml of pleural effusion fluid, even though the concentration of the extracted DNA specimens was in most cases below the concentration detectable by spectrophotometry (data not shown). The results of the comparison between the mutation status and clinical manifestations in this study confirmed the finding in previous studies that *EGFR* mutations are frequently present in small sub-groups of NSCLC patients, such as females and never smokers, although the differences were not statistically significant. It is well known that *EGFR* mutations are frequently observed in adenocarcinomas. As 36 of the 39 patients (92.3%) enrolled in this study had adenocarcinoma, we could not evaluate differences in the frequency of the *EGFR* mutations according to the histological type. Pleural effusion occurs in lung carcinoma of all cell types, but

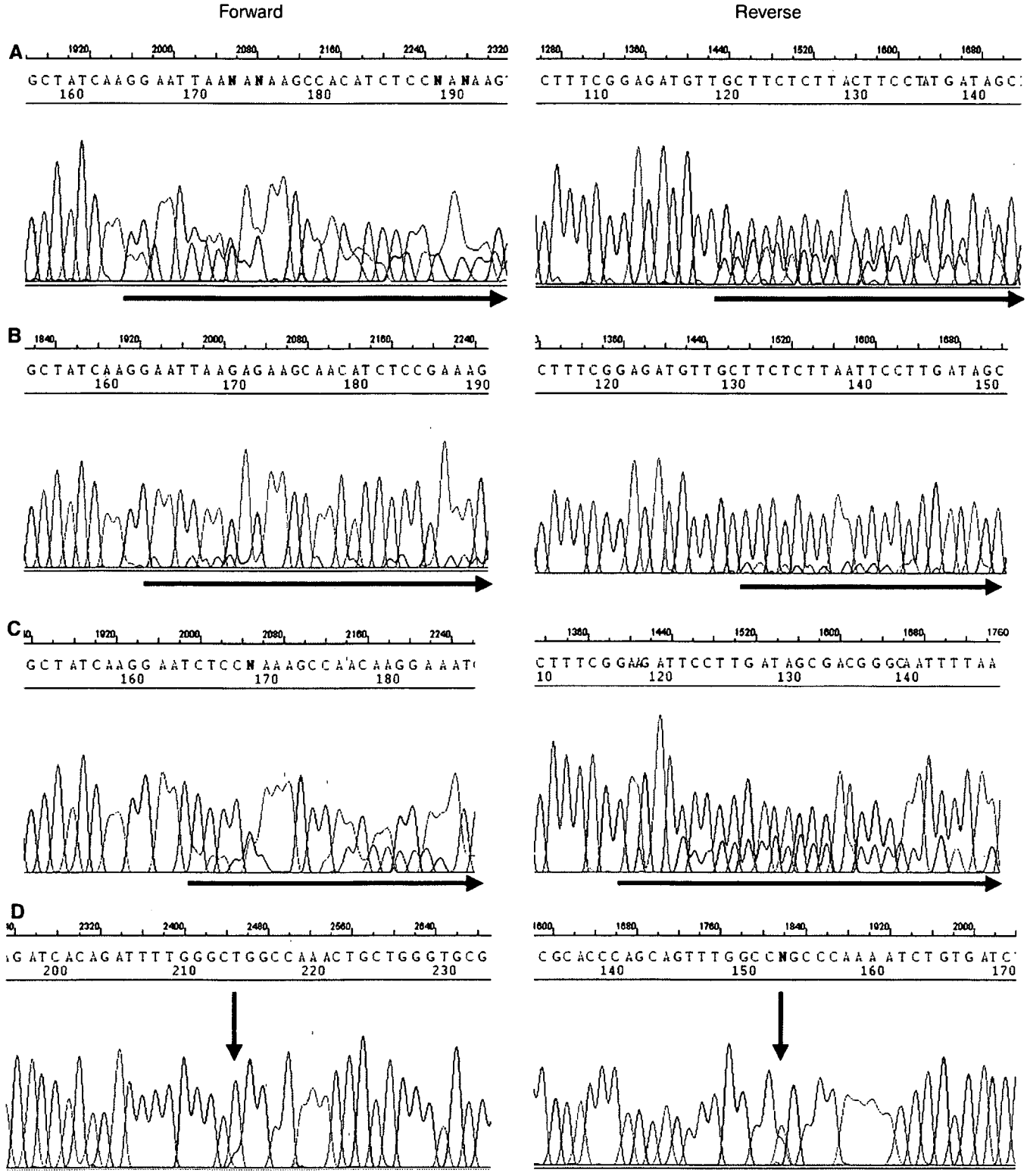

Figure 1 The wave figures of the nucleotide sequence of the *EGFR* gene with heterozygous mutations obtained by direct sequencing (see 'Patients and Methods') are shown. Horizontal arrows in both the sense and the antisense directions are shown to demonstrate the two breakpoints of the deletion. The patients in **A**, **B**, and **C** have inframe deletions in exon 19 (Figure **A**, E746_A750del; **B**, E746_T753del insA; **C**, L747_T751del; **D**, L858R). The double peaks (vertical arrows) represent the heterozygous missense mutations resulting in an amino acid substitution of L858R in exon 19 (Figure **D**).

appears to be most frequent in adenocarcinoma (Chernow and Shahn, 1997).

This study had several limitations. First, we could not compare the results of the *EGFR* mutation status in the pleural effusion fluid to the mutation status in tumour tissue. Forty of the 43 patients enrolled were cytologically diagnosed as having NSCLC from pleural effusion fluid specimens. As the DNA extracted from pleural effusion fluid consisted of DNA derived from both tumour cells and normal cells, the *EGFR* mutation status needs to be evaluated in a pair of DNA specimens from the tumour and pleural

Table 3 Frequency of *EGFR* mutations in DNA from the pleural effusion fluid of NSCLC patients according to (A) gender, (B) histology, (C) smoking habit, and (D) response to gefitinib

(A) Gender and *EGFR* mutation status

	EGFR mutation		
	+	−	
Female	7	14	
Male	4	18	$P=0.310$

(B) Histology and *EGFR* mutation status

	EGFR mutation		
	+	−	
Ad	11	28	
Non-Ad	0	4	$P=0.558$

(C) Smoking habit and *EGFR* mutation status

	EGFR mutation		
	+	−	
Never	7	11	
Current/former	4	21	$P=0.156$

Ad = adenocarcinoma; EGFR = epidermal growth factor receptor; + = mutation-positive; − = no mutations. (A)(B)(C); a total of 43 samples were evaluated.

effusion fluid to confirm the usefulness of the mutation status determined from pleural effusion fluid. However, it is sometimes difficult to obtain tumour samples from patients with advanced NSCLC, and even more so from patients diagnosed as having NSCLC using methods other than the histological examination of tumour tissue, such as on the basis of pleural effusion or sputum cytology. Second, direct sequencing may be not able to provide satisfactory results for detection of *EGFR* mutations in mixed samples of mutated and wild DNA. Although direct sequencing has generally been used to detect *EGFR* mutations in previous studies, detection of a mutation by this method requires at least 30% of the mutated DNA in a sample (Bosari *et al*, 1995; Fan *et al*, 2001). Lung cancers are very heterogeneous, and as normal cells, such as inflammatory cells or mesothelial cells, are contained in the pleural effusion fluid of lung cancer patients, in addition to tumour cells, a small amount of mutated DNA in pleural effusion fluid can be missed by direct sequencing. Unfortunately, we have no data at the present time on whether EGFR mutations were detectable in pleural effusion samples with either a few malignant cells, a small proportion of malignant cells with normal mesothelial cells, or cytologically negative samples. To establish a method for the detection of EGFR mutations from pleural effusion fluid, the mutation detectable proportion of malignant cells to normal cells in pleural fluid should be elucidated. We are planning an additional study using cytological examination to clarify the mutation detectable proportion as a next step. When pleural fluid is used as the material for detection of *EGFR* mutations, a patient with an *EGFR* mutation may be diagnosed as having wild-type *EGFR* because of the two limitations described above. Although we expected a high frequency of detection of *EGFR* mutations in this study because of the high proportion of adenocarcinomas (92.3%), we detected *EGFR* mutations in only 28.2% of the patients enrolled, a lower frequency than in two previous reports on Japanese NSCLC patients (Takano *et al*, 2005; Asano *et al*, 2006). Patients with false-negative results, meaning that no *EGFR* mutations were detected in a patient with an *EGFR* mutation, were not excluded from this study. Some investigators have tried to improve the sensitivity of detection of

Table 4 *EGFR* mutation status in patients who received gefitinib therapy

Age (years)	Gender	Smoking	Histology	EGFR mutation status	Response to gefitinib
62	F	Never	Ad	E747_P753insS	PR
58	F	Never	Ad	E746_A750del	PR
80	F	Never	Ad	E746_A750del	PR
61	M	Never	Ad	E746_A750del	PR
65	M	Former	Ad	E746_A750del	PR
60	M	Current	Ad	E746_A750del	PR
66	F	Never	Ad	E747_T750del	PR
76	F	Never	Ad	Wild	SD
57	F	Former	Ad	Wild	SD
40	F	Never	Ad	Wild	SD
72	F	Never	Ad	Wild	SD
58	F	Former	Ad	Wild	SD
66	F	Never	Ad	Wild	SD
65	F	Former	Ad	L858R	SD
39	F	Never	Ad	Wild	PD
69	M	Former	Ad	Wild	PD
72	F	Never	Ad	Wild	PD
74	F	Never	Ad	Wild	PD
67	M	Former	Ad	Wild	PD
62	M	Former	SCC	Wild	PD
59	F	Current	Ad	Wild	PD
77	M	Current	Ad	Wild	PD
82	F	Never	Ad	Wild	PD
66	F	Never	Ad	Wild	PD
56	M	Current	Ad	Wild	PD
61	M	Former	Ad	Wild	PD
65	M	Former	Ad	Wild	PD

Ad = adenocarcinoma; EGFR = epidermal growth factor receptor; F = female; M = male; NSCLC = unclassified NSCLC; PD = progressive disease; PR = partial response; SCC = squamous cell carcinoma; SD = stable disease.

EGFR mutations in samples containing a mixture of tumour and normal cells. Wookey *et al* (2005) reported findings that the ARMS method was superior to the direct sequencing method and WAVE method for detecting *EGFR* mutations. Other groups have reported that LightCycler PCR assay (Sasaki *et al*, 2005), SSCP assay (Marchetti *et al*, 2005), and enriched PCR assay (Asano *et al*) are more sensitive than direct sequencing and are more rapid. A standardised method of detecting *EGFR* mutations needs to be established as soon as possible.

The final limitation in the present study is that it remains unclear whether there is any survival benefits associated with gefitinib therapy in those patients enrolled with *EGFR* mutations. The relationship between the *EGFR* mutation status determined in pleural effusion fluid and the gefitinib response in a portion of the patients enrolled supports the pleural effusion fluid *EGFR* mutation status as useful for predicting the response to gefitinib. The relationship between the *EGFR* mutation status determined in the pleural effusion fluid and the gefitinib response in the remaining patients and the survival benefit of gefitinib therapy in the patients with *EGFR* mutations are currently being evaluated, and confirmation of the results is expected in the very near future.

In conclusion, our results suggest that the DNA in pleural effusion fluid can be used to detect *EGFR* mutations and that the *EGFR* mutation status determined may be useful as a predictive factor of response to gefitinib.

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INVITED REVIEW SERIES: LUNG CANCER

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

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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

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