

Overexpression of EGFR pathway-related genes in the circulation is highly correlated with EGFR mutations and overexpression in paired cancer tissue from patients with non-small cell lung cancer

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Abstract. Epidermal growth factor receptor (EGFR)-directed tyrosine kinase inhibitors (TKIs) have been established as a treatment option in patients with advanced non-small cell lung cancer (NSCLC). Clinically, PCR and RFLP are commonly used to evaluate the efficacy of TKIs, and these methods require cancer tissues to proceed. In the event a peripheral blood test is able to replace current evaluation methods, a greater clinical application advantage may be achieved. Therefore, in this study, we selected 30 EGFR pathway-related genes and constructed activated EGFR chips to identify overexpression of EGFR pathway-related genes from the peripheral blood of 72 NSCLC patients and 100 normal subjects. According to ROC curve analysis, the best chip interpretation cutoff value was 11 genes. Correlation analysis showed high significance among EGFR mutations, overexpression and the overexpression of EGFR pathway-related genes ($p < 0.0001$). The potential application of this new technique may provide an accurate, instantaneous and convenient drug evaluation tool.

Introduction

The epidermal growth factor receptor (EGFR) is a membrane spanning glycoprotein that is normally under tight regulatory control. Activation of EGFR signaling may induce cell

migration, proliferation, invasion, angiogenesis and inhibition of apoptosis (1,2). There are several mechanisms by which EGFR becomes oncogenic. i) Increased EGFR levels are prevalent in many cancer types leading to aberrant EGFR signaling. ii) EGFR is activated by autocrine and/or paracrine growth factor loops. iii) Heterodimerization with other EGFR family members and cross-talk with heterologous receptor systems serve as another mechanism for oncogenic activation of EGFR. iv) Defective receptor downregulation can also lead to cellular transformation, and v) the activating mutations promote EGFR signaling (3). Dysregulated EGFR is frequently associated with overexpression of the EGFR, and therefore has been implicated as a major mechanism in the pathogenesis of several epithelial malignancies including non-small cell lung (NSCLC), colorectal, gastric, breast and endometrial cancers (4). A number of studies have found that cancer patients with activated EGFR are more likely to experience a more aggressive progression and are correlated with poor clinical outcomes (4). Thus, the inhibition of EGFR cellular action has been intensely studied as a therapeutic target.

Gefitinib and erlotinib are small-molecule inhibitors of EGFR that prevent EGFR autophosphorylation and downstream signaling (5,6). To date, clinical trials have been carried out in which patients have been treated with these drugs either as single agents or in combination with other anticancer drugs (7-9). Several significant factors affect sensitivity or resistance to tyrosine kinase inhibitors (TKIs) of EGFR and are associated with polymorphisms of EGFR, amplified EGFR gene and/or elevated EGFR mRNA expression (9-11). However, patient cancer tissues are required for clinical analysis of EGFR mutations, amplification and mRNA expression. When tissues are absent, efficacy prediction targets are lost.

Recently, we developed a colorimetric membrane array method which simultaneously detects the expression levels of a multiple mRNA marker in the peripheral blood used in

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circulating tumor cells (12). This platform of colorimetric membrane array test positively detected circulating activated K-ras in all of the NSCLC patients with K-ras mutations. Our previous results suggest that the K-ras oncogene membrane array can serve as a tool for the detection of the K-ras oncogene in the circulation (13). Activating mutations in the kinase domain of EGFR have been described in advanced NSCLC. These mutations also increase the kinase activity of the EGFR, leading to the hyperactivation of downstream genes, which in turn activates the intracellular signal transduction cascades, mainly the mitogen-activated protein kinase (MAPK), phosphoinositol 3 kinase (PI3K-AKT) and STAT pathways (14-16). In patients with these activating EGFR mutations, the single-agent response rates to either gefitinib or erlotinib [EGFR tyrosine kinase inhibitors (TKIs)] range from 60 to 80%, which is remarkable in this disease (15).

Therefore, in this study, we used membrane array to assess the overexpression of EGFR pathway-related genes in the circulation of NSCLC patients. We also analyzed EGFR mutations, overexpression and overexpression of EGFR pathway-related genes in patient tissues and evaluated the consistency of outcomes with those from blood samples. The main goal of this study was to develop an innovative and sensitive diagnostic method for the rapid screening of peripheral blood of lung cancer patients. The potential application of this newly established method may provide an accurate and convenient means of monitoring the efficacy of therapies managing this malignancy.

Materials and methods

Specimen collections. This study was approved by the Institutional Review Board of Fooyin University Hospital, PingTung Christian Hospital and Kaohsiung Medical University Hospital. Seventy-two patients with pathology-confirmed NSCLC who had undergone surgical resection or biopsy between January 2006 and December 2007, were enrolled in this study. All samples were collected immediately after surgical resection, frozen instantly in liquid nitrogen, and then stored at -80°C until analyzed. Peripheral blood was collected from the patients before surgery and chemotherapy. Whole blood (4 ml) was drawn into a tube with sodium citrate and preserved at 4°C for activated EGFR detection. Written informed consent was obtained from all subjects and/or guardians for the use of their samples. Clinical stages and pathological features of the primary tumors were defined according to the criteria of the American Joint Commission on Cancer.

DNA extraction. All samples were collected immediately after surgical resection, frozen instantly in liquid nitrogen, and then stored at -80°C until analyzed. Genomic DNA was isolated from frozen primary NSCLC and the paired normal tissues using proteinase-K (Stratagene, La Jolla, CA) digestion and the phenol/chloroform extraction procedure according to the method by Sambrook *et al.*

Direct sequencing. To identify mutations of the EGFR genes, the oligonucleotide primers for EGFR exons 18-21 (Table I)

were used to perform polymerase chain reaction (PCR) analysis. The PCR products were purified by the Qiaex II Gel Extraction Kit and then subjected to sequencing using a double-strand cycle sequencing system. The purified products were then sequenced directly with T7 promoter/IRD800, which was a T7 promoter primer (Table I) labeled with a heptamethine cyanine dye or DNA polymerase incorporating IRD-labeled dATP for the sequencing reaction. Upon completion of the sequencing reaction, 4 ml of formamide loading buffer was added to the reaction mixture. The samples were then heated to 95°C for 5 min, snap-cooled, and loaded onto the sequencing gel. An automated DNA electrophoresis system (Model 4200; LI-COR) with a laser diode emission at 785 nm and fluorescence detection between 815 and 835 nm was used to detect and analyze the sequencing ladders. An aliquot of 1.5 ml from each sequencing sample was loaded onto a sequencing gel (41 cm x 25 cm x 0.2 mm) consisting of 6% Long Ranger Matrix (AT Biochem; Malvern, PA, USA) and 7 M urea in TBE buffer (133 mM Tris base, 44 mM boric acid and 2.5 mM EDTA, pH 9.0 at 50°C). Following the loading of samples, electrophoresis was carried out at a constant voltage of 2000 V with heating of the gel to 50°C . Data collection and image analysis were performed using IBM486 (Model 90) with the Base Image IR software included with the model 4200 DNA sequencer.

RNA extraction and RT-PCR. Total RNA was purified from peripheral blood using a QIAmp[®] RNA Blood Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. First-strand cDNA was synthesized from total RNA by using an RT-PCR kit. Reverse transcription was carried out in a reaction mixture consisting of 1X Transcription Optimized 5X Buffer, 25 $\mu\text{g}/\text{ml}$ Oligo(dT)15 Primer, 100 mmol/l PCR Nucleotide Mix, 200 $\mu\text{mol}/\text{l}$ M-MLV Reverse Transcriptase, and 0.5 μl of Recombinant RNasin[®] Ribonuclease Inhibitor (Promega Corp., USA). The reaction mixtures with RNA were incubated at 42°C for longer than 2 h, heated to 95°C for 5 min and then stored at 4°C until analysis.

Northern blot analysis. Twenty micrograms of total RNA was denatured with 6.5% formamide and 50% formaldehyde for 15 min at 55°C and separated by electrophoresis through 1.2% agarose gels containing 1.1% formaldehyde. Then RNA was transferred onto a nylon membrane (Schleicher & Schuell, Dassel, Germany) and fixed. The blots were successively hybridized with human EGFR cDNA. The hybridized filters were then washed twice with washing buffer (solution I: 3X SSC, 0.5% SDS; solution II: 0.5X SSC, 0.1% SDS) at 65°C to wash off the unbound probe. The membrane was exposed to Kodak X-film at -70°C for autoradiography. We used the GAPDH or β -actin signal to normalize data for the above mRNA. All of the probes were confirmed by direct sequencing.

Oligo membrane array preparation. We used Vector NTI to design oligonucleotide probe sequences for the target genes, and β -actin served as an internal control (Table I). The newly synthesized oligonucleotide fragments were dissolved in DI-water to a concentration of 20 mM and then applied to a



nucleotide sequences of primers used for DNA sequencing and probes for membrane array.

| Gene | Sequence | |
|--------------|---|------------------------|
| SOS | TCATGGTACCTGGTCTTGGGTTTGTATGGACGAACACCAGGAGATTTTAGG | |
| GRB | ACATCACCACAGGGGACCAGAAGTGGAGAGAAGACAGAGAGTTCTGCCGA | |
| Raf | ACAAGGCTGTTTGTGTTTGTGTTTGTAGAGAAAACAAGGCTGGCCCTGCG | |
| MEK | CATGGCAGTGAAGGGCTCTGGCTAGATTTGGATGTCAACTGCTGAGTTCT | |
| ERK | TTGCTGTAAACAAACCCAGATGCAGGATCAACCCTTCTCAGCGGCAGTCG | |
| VEGF | CCTGGGGGGACAGAAGTGTGGTTTCAATGGTGTGAGGACATAGGTCCTT | |
| COX2 | ACAAACCCCGTACAGTTCTCTCTGAGGCACTAGCCTCTTTGCATCCATCT | |
| PTGE2 | CATACACTTGCTGGGCCTCCTTCTCGTTGAGCATGAGCCAGTACTTATTG | |
| RAC | CATCTGGGCCGTGAACTCCTCATCAAATACCTGGTGTGAGTCTCCGACG | |
| RHO | CAGAATGGAGAATGGACTAGCAACTGCTGAAGATGGGCTTGTCTGGCAAG | |
| MEKK1 | GGCAAGAGTGCATCCTTTAAAGCTTGACATGAGAGACTTGGAAACAATC | |
| JNK | ATGATGGATGCTGAGAGCCATTGATCACTGCTGCACCTGTGCTAAAGGAG | |
| PLC | CACTAAAGACAATTCCCAATCCTGAGTGGGTGGCAGAGACTCCTGCGATG | |
| DAG | CTGGGTCCACCCACCCACCCTCAATCAGGGAATGGATACATTATTGCGAA | |
| PKC | ACGCTTGGCTTGAAGTCTTACACCCAGGCTCAACGATGGAGTTTGCATT | |
| PI3K | GCTGTTGAACTGCAGTGCACCTTTCAAGCCGCCTTTGCACTGAATTTGCA | |
| PIP2 | ACCTGAAGCTCTCAGAACGAATTGGTGGTAGAATGCAGCTATCGCAGCTC | |
| PIP3 | AGCAGCACCAAAGCTTCTAGCTGGGTTGATACCAGTTCCAGTAATGGGGA | |
| PTEN | CCCTTCGGAAACCTCTCTTAGCCAAGTCAAACTTATCTGTTGCCACAAG | |
| AKT | GCCAGCATACCATAGTGAGGTTGCATCTGGTGCCACCAGGTTGAACTGAG | |
| CDC42 | GAGGATGTGAATCATTCCACAGATGTCTGTCTTCTAGCACCACCCCTCGT | |
| SGK | TGATCAGGCATACCACACTCACACGACGGTTCACACAGCATATCCACAAG | |
| BAD | AACATGCTCTGGGCTGTGAGGACAAGATGTTACGTAGTCAAGGCACAGCT | |
| FKHR | GCCTTTCCCTGGACTTCACTGTTCTCAGAGAGCTACCAAGGATTCATGAC | |
| GSK3B | CTTGACAGTCTTGTAGTGGTGAAGTTGAAGAGTGCAGGTGTGTCTCGCC | |
| MDM2 | GAAGCAGAACCCTTGAACACAGCCAGGAGGCAGAGGTTGCAGTGAGCCA | |
| JAK2 | ATGAACACCAGCCCTCATGTGTGTAACACATCACACAAGATGCCAAGGTA | |
| STAT3 | GGGCGAACCCCTGTTTCTTATAGAGAAGGTCGTCTCCCTTAAATTCAGAG | |
| STAT5B | TGTGGTTTGCAGGAACAAGGAGGAGAGAAGATAGAACGCAGAGAGCGA | |
| PDK | CAGGATCTTGGCCTCTTAAATGAGGGCACTGGACTAACTGCCCATTCACA | |
| | Forward primer | Reverse primer |
| EGFR exon 18 | GCTGAGGTGACCCTTGTCTC | ACAGCTTGCAAGGACTCTGG |
| EGFR exon 19 | CCCAGTGTCCCTCACCTTC | CCACACAGCAAAGCAGAAAC |
| EGFR exon 20 | TTCTGGCCACCATGCGA | CCGTATCTCCCTTCCCTGATTA |
| EGFR exon 21 | TGATCTGTCCCTCACAGCAG | TCAGGAAAATGCTGGCTGAC |

BioJet Plus 3000 nanoliter dispense system (BioDot, Irvine, CA), which blotted sequentially the 30 target genes, 1 TB gene, and 1 housekeeping gene (β -actin) (50 nl per spot and 1.5 mm between spots) on a Nytran[®] SuperCharge nylon membrane in triplicate. DMSO was also dispensed onto the membrane as a blank control. After rapid drying and cross-linking procedures, preparation of the breast cancer diagnostic membrane array was carried out.

Preparation of biotin-labeled cDNA targets and hybridization. First-strand cDNA targets for hybridization were produced by using SuperScript II Reverse Transcriptase (Gibco-BRL) in the presence of biotin-labeled UTP (Roche Diagnostics

GmbH, Penzberg, Germany). After procedures of pre-hybridization and blocking, the gene chips were subjected to hybridization. The lifts were covered with the Express Hyb Hybridization Solution (BD Biosciences, Palo Alto, CA, USA) containing DIG-11-UTP-labeled cDNA probes, and then incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche Diagnostics). For hybridization, the arrays were incubated at 42°C for 12 h in a humidified chamber. After washing, the arrays were exposed to light. For signal detection, the gene chips were incubated in chromogen solution containing nitroblue-tetrazolium and 5-bromo-4-chloro-3-indoyl-phosphate (NBT/BCIP) (Zymed, CA, USA) for 15 min. The hybridized arrays were then

Table II. Results of the mutation, overexpression and membrane array analysis of EGFR.

| EGFR mutation | EGFR overexpression | | Membrane array analysis | |
|---------------|---------------------|----|-------------------------|----|
| | P | N | P | N |
| WT | 10 | 45 | 16 | 39 |
| 18,G719C | 4 | 0 | 4 | 0 |
| 18,E709K | 4 | 0 | 4 | 0 |
| 18,N700D | 1 | 0 | 1 | 0 |
| 20,V756M | 1 | 0 | 1 | 0 |
| 20,R766C | 1 | 0 | 1 | 0 |
| 20,V769M | 1 | 0 | 1 | 0 |
| 20,S768I | 2 | 0 | 2 | 0 |
| 20,R803W | 2 | 0 | 2 | 0 |
| 19,L747-T751 | 0 | 1 | 1 | 0 |
| Total | 26 | 46 | 33 | 39 |

N, negative; P, positive; WT, wild-type.

scanned with an Epson Perfection 1670 flat bed scanner (Seiko Epson Corp., Nagano-ken, Japan). Subsequent quantification analysis of the intensity of each spot was carried out by using AlphaEase[®] FC software (Alpha Innotech Corp., San Leandro, CA, USA). Spots consistently varying by a factor ≥ 2 were considered to be differentially expressed. For each sample, membrane array hybridization was performed in triplicate to ensure the reproducibility of the results.

Statistical analysis. All data were analyzed by using the Statistical Package for the Social Sciences version 12.0 software (SPSS Inc., Chicago, IL). Correlations between EGFR mutant and wild-type and between EGFR mutations and EGFR activation were compared by using the Chi-square test. Receiver operating characteristic (ROC) curve analyses were carried out to determine cut-off value, sensitivity, and specificity for each mRNA marker. For a particular marker, a test was regarded as positive when the expression ratio of the marker was higher than its cut-off value.

Results

We collected 72 sets of peripheral blood, cancer tissue and neighboring normal tissue samples from lung cancer patients. In order to investigate EGFR mutations in lung cancer tissues, direct sequencing was performed on EGFR exons 18-21. The results showed that 17 (23.61%) had mutations, including G719C, E709K and N700D of exon 18, L747-T751 deletion of exon 19 and V756M, R766C, V769M, S768I, and R803W of exon 20. The mutation hotspots were close to 18G719C and 18E709K; 4 cases were found on each of the two sites (Table II).

Northern blotting was used to analyze EGFR mRNA expression in samples of the lung cancer patients. There were 26 (36.11%) samples exhibiting EGFR overexpression.

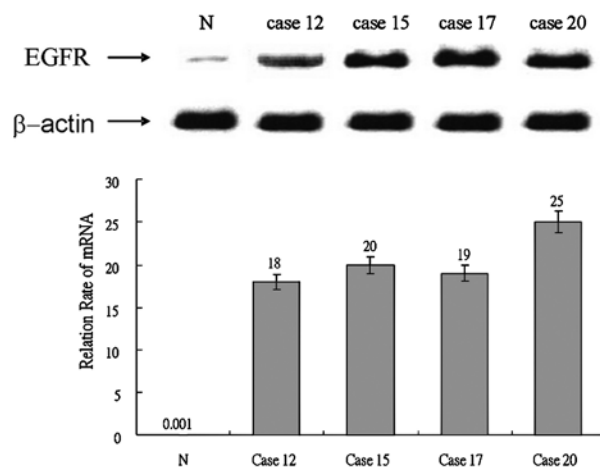


Figure 1. Northern blot analysis of EGFR mRNA expression in patient samples of lung cancer. EGFR mRNA was significantly overexpressed in cases 12, 15, 17 and 20 compared to normal tissues after calibrating with β -actin expression.

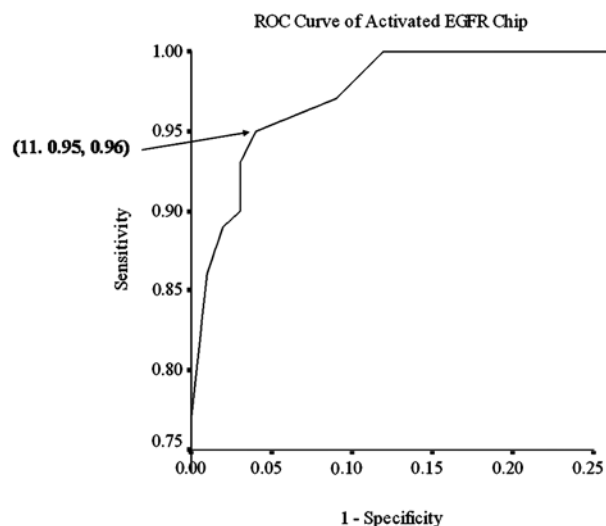


Figure 2. ROC curve analysis of membrane array data of 72 paired samples. The optimal cutoff point for the number of differentially expressed genes was 11. A membrane on which ≥ 11 of the 30 genes were expressed 2-fold higher than normal levels was considered to be positive and vice versa. At this cutoff point, the sensitivity and specificity of membrane arrays were 95 and 96%, respectively.

Significant EGFR mRNA overexpression was observed in cases 12, 15, 17 and 20 compared to normal tissues after calibrating with β -actin expression (Fig. 1).

On the other hand, we used the membrane array to analyze overexpression of EGFR pathway-related genes in 72 sets of peripheral blood and cancer tissue samples. We used the ROC curve to analyze 72 pairs of membrane array data, and determined that the optimal cutoff point for the number of differentially expressed genes was 11 (Fig. 2). In other words, the activated EGFR chip found ≥ 11 positive signals out of the 30 genes, expressing 2-fold higher than normal levels (Fig. 3). Based on this standard, among all 72 NSCLC peripheral blood samples, 33 (45.83%) were positive with EGFR activation in the membrane array analysis (Table II).

| | | | | | | | |
|-------|------|-------|-------|--------|------|------|---------|
| SOS | GRB2 | Raf | MEK | ERK | VEGF | COX2 | PGE2 |
| SOS | GRB2 | Raf | MEK | ERK | VEGF | COX2 | PGE2 |
| SOS | GRB2 | Raf | MEK | ERK | VEGF | COX2 | PGE2 |
| RAC | RHO | MEKK1 | JNK | PLC | DAG | PKC | P13K |
| RAC | RHO | MEKK1 | JNK | PLC | DAG | PKC | P13K |
| RAC | RHO | MEKK1 | JNK | PLC | DAG | PKC | P13K |
| PIP2 | PIP3 | PTEN | AKT | CDC42 | SGK | BAD | FKHR |
| PIP2 | PIP3 | PTEN | AKT | CDC42 | SGK | BAD | FKHR |
| PIP2 | PIP3 | PTEN | AKT | CDC42 | SGK | BAD | FKHR |
| GSK3B | MDM2 | JAK2 | STAT3 | STAT5b | PDK | TB | B-actin |
| GSK3B | MDM2 | JAK2 | STAT3 | STAT5b | PDK | TB | B-actin |
| GSK3B | MDM2 | JAK2 | STAT3 | STAT5b | PDK | TB | B-actin |

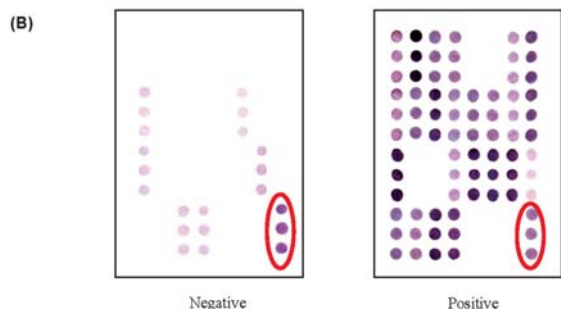


Figure 3. (A) Schematic representation of the membrane array with 30 target genes, 1 TB gene as a negative control, and 1 housekeeping gene (β-actin) as an internal control. (B) Images of membrane array. Left diagram: experimental results of the lung cancer patients with non-activated EGFR. Right diagram: experimental results of the lung cancer patients with overexpression of EGFR pathway-related genes.

Furthermore, we analyzed the correlation among EGFR mutations, overexpression of cancer tissues, and overexpression of EGFR pathway-related genes in blood samples. The statistical analysis showed that 22.22% (16/72) of the cancer tissues had both EGFR overexpression and mutations (Table III). One hundred percent of the 17 patient samples with EGFR mutations (Table IV) and the 26 patient samples with EGFR overexpression (Table V) exhibited overexpression of EGFR pathway-related genes in both cancer tissue and peripheral blood analysis with the activated EGFR chip. A high correlation ($p < 0.0001$) was found among these three expression levels (Table III-V). There were no statistical correlations between EGFR alterations and the clinical characteristics of the lung cancer patients including gender, size of tumor and metastasis (Table VI).

Discussion

Epidermal growth factor receptor (EGFR) is frequently amplified and/or mutated in a number of human tumors, and abnormal signaling from this receptor is believed to contribute to the malignant phenotype observed in these tumors (17). This has inspired the development of specific pharmacological inhibitors of the EGFR tyrosine kinase such as gefitinib, which disrupts EGFR kinase activity by reversibly binding within the ATP-binding pocket of the EGFR protein (18). Response rates of advanced NSCLC patients to these

Table III. Correlation analysis between EGFR mutations and EGFR overexpression in lung cancer tissues.

| | | EGFR mutations | | Total | P-value |
|---------------------|---|----------------|----|-------|---------|
| | | WT | M | | |
| EGFR overexpression | N | 45 | 1 | 46 | 0.000 |
| | P | 10 | 16 | 26 | |
| Total | | 55 | 17 | 72 | |

WT, wild-type; M, mutation; N, negative; P, positive.

Table IV. Correlation analysis between EGFR mutations and EGFR membrane array in lung cancer tissues.

| | | EGFR mutations | | Total | P-value |
|---------------------|---|----------------|----|-------|---------|
| | | WT | M | | |
| EGFR membrane array | N | 39 | 0 | 39 | 0.000 |
| | P | 16 | 17 | 33 | |
| Total | | 55 | 17 | 72 | |

WT, wild-type; M, mutation; N, negative; P, positive.

Table V. Correlation analysis between EGFR overexpression and EGFR membrane array in lung cancer tissues.

| | | EGFR overexpression | | Total | P-value |
|---------------------|---|---------------------|----|-------|---------|
| | | N | P | | |
| EGFR membrane array | N | 39 | 0 | 39 | 0.000 |
| | P | 7 | 26 | 33 | |
| Total | | 46 | 26 | 72 | |

N, negative; P, positive.

drug treatments were 30-40% in Asian populations (19) and 10% in other ethnic groups (20).

Lynch *et al* (21), Paez *et al* (22) and Pao *et al* (23) reported that many NSCLC tumor tissues obtained from patients who responded to gefitinib or erlotinib treatment harbored somatic mutations in the tyrosine kinase domain of the EGFR gene. In addition, Dziadziuszko *et al* (10) and Hirsch *et al* (11) reported that patients with amplified EGFR gene and/or elevated EGFR mRNA expression have higher response rates and improved survival than those with low

Table VI. Correlation analysis between the EGFR mutation, overexpression and membrane array in lung cancer tissues and clinical characteristics of the NSCLC patients.

| | EGFR mutations | | | EGFR overexpression | | | EGFR membrane array | | |
|-------------------------|----------------|----|---------|---------------------|----|---------|---------------------|----|---------|
| | WT | M | P-value | N | P | P-value | N | P | P-value |
| Total | 55 | 17 | | 46 | 26 | | 39 | 33 | |
| Gender | | | | | | | | | |
| Male | 34 | 8 | 0.281 | 29 | 13 | 0.281 | 27 | 15 | 0.041 |
| Female | 21 | 9 | | 17 | 13 | | 12 | 18 | |
| Histology | | | | | | | | | |
| Adenocarcinoma | 36 | 14 | 0.07 | 29 | 21 | 0.133 | 24 | 26 | 0.206 |
| Adenosquamous carcinoma | 2 | 2 | | 2 | 2 | | 2 | 2 | |
| Squamous cell carcinoma | 17 | 1 | | 15 | 3 | | 13 | 5 | |
| Tumor size (T) | | | | | | | | | |
| T1 | 11 | 5 | 0.199 | 9 | 7 | 0.281 | 9 | 7 | 0.307 |
| T2 | 22 | 10 | | 18 | 14 | | 14 | 18 | |
| T3 | 11 | 1 | | 9 | 3 | | 7 | 5 | |
| T4 | 11 | 1 | | 10 | 2 | | 9 | 3 | |
| Lymph node (N) | | | | | | | | | |
| N0 | 25 | 7 | 0.456 | 19 | 13 | 0.914 | 16 | 16 | 0.531 |
| N1 | 5 | 4 | | 6 | 3 | | 4 | 5 | |
| N2 | 15 | 4 | | 13 | 6 | | 13 | 6 | |
| N3 | 10 | 2 | | 8 | 4 | | 6 | 6 | |
| Metastasis (M) | | | | | | | | | |
| M0 | 38 | 11 | 0.735 | 29 | 20 | 0.225 | 27 | 22 | 0.816 |
| M1 | 17 | 6 | | 17 | 6 | | 12 | 11 | |
| TNM stage | | | | | | | | | |
| I | 16 | 5 | 0.885 | 11 | 10 | 0.429 | 10 | 11 | 0.614 |
| II | 7 | 1 | | 6 | 2 | | 6 | 2 | |
| III | 15 | 5 | | 12 | 8 | | 11 | 9 | |
| IV | 17 | 6 | | 17 | 6 | | 12 | 11 | |

WT, wild-type; M, mutation; N, negative; P, positive.

EGFR copy numbers and/or mRNA expression levels upon treatment with TKIs. Although the methods described may predict the efficacy of TKIs in NSCLC patients, clinically, cancer tissues are still required for EGFR mutation, amplification, and mRNA expression analysis. When tissues are absent, the prediction target for the efficacy is lost. In addition, cancer tissues cannot provide instantaneous evaluation of the efficacy after patient treatment with TKIs.

In our previous study, K-ras oncogene membrane array was successfully established; the downstream genes of K-ras were used to evaluate K-ras activity with blood samples of cancer patients. The sensitivity, specificity and accuracy of the diagnostic membrane array were 83.7, 90.9 and 86.8%, respectively (12). Blood samples were easier to obtain and provided instant gene expression monitoring; EGFR auto-phosphorylation also activated downstream signaling pathways, including the Ras/Raf/mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3'-kinase (PI3K)-Akt pathways. Cappuzzo *et al* confirmed that phospho-Akt-positive patients had a better response rate than patients with

P-Akt-negative tumors to gefitinib treatment (24). Therefore, we used a membrane array to assess EGFR mutations and overexpression and the overexpression of EGFR downstream genes using NSCLC cancer tissues. The results were then compared to the blood analysis of these expression levels, showing high consistency. We believe that EGFR pathway-related genes in patient blood may be used to predict EGFR alterations in tissues as a therapeutic target predictor for drug efficacy.

In our study, rare mutations including S768I and V769M were found, and similar mutation patterns were also reported in Japanese NSCLC patients suggesting that these two mutation patterns might be detected only in Asian populations (25,26). In addition, S768I and V769L mutations may be associated with resistance to gefitinib since such mutations are refractory to EGF-induced ubiquitination and degradation (27). In our study, small deletions centered around 5 codons in exon 19 (amino acid residues 747-751), similar to previous studies which found that deletions in exon 19 are the most common types of mutations identified in all of the large



mutation studies of NSCLC patients, were clearly activated and correlated with sensitivity to tyrosine kinase inhibitors (21,22,28,29).

We also found a high correlation between EGFR mutations and overexpression ($p < 0.0001$). Similarly, Suzuki *et al* also confirmed that EGFR overexpression was correlated with EGFR TK domain mutations (30). In addition, many investigators have extensively studied associations between EGFR mutations and the downstream molecules such as pAkt and pMAPK in lung cancer cell lines and revealed that EGFR mutations are almost always accompanied by enhanced signaling of intracellular cascades in preclinical settings (24,31-33). Hosokawa *et al* also showed that EGFR mutations are related to phosphorylated Akt and that EGFR phosphorylation is closely correlated with EGFR protein expression (34). Cappuzzo *et al* observed that gefitinib sensitivity is associated with high EGFR protein expression (35). These studies indicate the possible use of NSCLC patient peripheral blood for the evaluation of TKI efficacy.

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