Detection of *EGFR* Mutation Status in Lung Adenocarcinoma Specimens with Different Proportions of Tumor Cells Using Two Methods of Differential Sensitivity

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Introduction: To evaluate epidermal growth factor receptor (*EGFR*) mutation status in lung adenocarcinoma specimens with different proportions of tumor cells using two methods with different sensitivities.

Methods: *EGFR* mutation status was determined by peptide nucleic acid (PNA) clamping and direct sequencing. The samples consisted of 41 cell blocks of malignant pleural effusions with various proportions of tumor cells, as well as 23 lung biopsy specimens containing more than 20% tumor cells and the corresponding surgically resected tumors.

Results: In the analysis of malignant pleural effusions, *EGFR* mutations were detected only by PNA clamping in four of nine patients who exhibited partial response to EGFR tyrosine kinase inhibitors; all the cell blocks of these four patients contained less than 20% tumor cells. Direct sequencing revealed wild-type *EGFR*, whereas PNA clamping revealed mutant *EGFR*, in one of five patients who exhibited progressive disease in response to EGFR tyrosine kinase inhibitor; the cell block of this patient contained a high proportion of tumor cells. A comparison of biopsy specimens containing sufficient tumor cells and the corresponding surgically resected tumors revealed discordance in the *EGFR* mutation status in four patients based on PNA clamping, whereas no discrepancies were observed by direct sequencing.

Conclusions: Highly sensitive methods, such as PNA clamping, may be superior to direct sequencing for the detection of *EGFR* mutations in diagnostic specimens with a low proportion of tumor cells. Direct sequencing may be more representative when diagnostic specimens with a high proportion of tumor cells are available.

Key Words: Direct sequencing, Epidermal growth factor receptor mutation, Lung adenocarcinoma, PNA clamping, Sensitivity.

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The epidermal growth factor receptor (*EGFR*) gene encodes a widely expressed transmembrane receptor protein that has been implicated in the development and progression of cancer.[1] The observation that non-small cell lung cancer (NSCLC) cells frequently express *EGFR*[2–4] has raised the level of interest in clinical trials of EGFR tyrosine kinase inhibitors (TKIs) such as gefitinib (Iressa; AstraZeneca, Wilmington, DE) and erlotinib (Tarceva; OSI Pharmaceuticals, Melville, NY).[5–11] Somatic mutations in the region of *EGFR* that encodes the tyrosine kinase domain of the receptor (exons 18–21) have been identified in NSCLC, and many studies have reported that NSCLC with these mutations is highly responsive to EGFR TKIs.[6–11] As such, the *EGFR* mutation status of NSCLC is used to help guide treatment decisions and/or to determine eligibility for clinical trials.

Direct sequencing is used routinely for the detection of *EGFR* mutations. However, the sensitivity of direct sequencing is suboptimal for many clinical tumor samples; mutant DNA must comprise ≥25% of the total DNA to be readily detected.[12,13] However, available samples for mutational analysis in lung cancer are often limited to small tissue biopsies or cytologic specimens. Moreover, clinical samples that are used to diagnose lung cancer typically contain a high proportion of normal cells in addition to cancer cells. These considerations continue to drive the development and evaluation of different techniques for detecting *EGFR* mutations. Recently, highly sensitive methods including the scorpion amplified refractory mutation system, mutant-enriched polymerase chain reaction (PCR), LightCycle PCR with Taqman-MGB probes, and peptide nucleic acid (PNA) clamping have been developed.[12,13–21] These assays are more sensitive than direct sequencing and detect mutations against a 100- to 1000-fold higher background level of wild-type alleles. With these methods, *EGFR* mutations are readily detectable in various clinical samples; thus, they could potentially be integrated into routine clinical practice for molecular characterization of NSCLC.

In this study, the *EGFR* mutation statuses of various clinical samples from patients with lung adenocarcinoma were evaluated by two methods with different sensitivities: PNA-mediated real-time PCR clamping and direct sequencing. On the basis of the differences observed between the two

methods in terms of determining *EGFR* mutation status, we assessed which method is most appropriate for a given proportion of tumor cells in various clinical lung adenocarcinoma specimens.

MATERIALS AND METHODS

Materials

Cell Lines

To confirm the sensitivities of PNA clamping and direct sequencing, three different cell lines were used, including one carrying wild-type *EGFR* (A549) and two carrying mutations in *EGFR* (PC9 and H1975). Wild-type cells were mixed with mutant cell lines in varying proportions. All cell lines were established from NSCLCs. The A549 and H1975 cell lines were obtained from American Type of Culture Collection (Rockville, MD). The PC9 cell line was obtained from the Konkuk University Medical Center (Seoul, Korea). PC9 cells carry a Glu746-Ala750 deletion mutation in exon 19 of the *EGFR* gene, and H1975 cells carry two point mutations, T790M and L858R, in exons 20 and 21, respectively.

Cell Blocks of Malignant Pleural Effusions

Cases were reviewed by using a pathological database of patients who had undergone tapping of pleural effusions and who had been diagnosed with lung adenocarcinoma at Chungbuk National University Hospital between January 2006 and December 2010. Pathologically confirmed, ethanol-fixed, and paraffin-embedded cell blocks of pleural effusions obtained from patients with lung adenocarcinoma were included. The percentage of tumor cells in the cell block of each malignant pleural effusion (MPE) was determined independently by two pathologists, and the average was taken. The *EGFR* mutation status was evaluated on the basis of the tumor cell percentage in the cell blocks.

Adequate Lung Biopsy Specimens at Diagnosis and Corresponding Surgically Resected Tumors

To evaluate the most appropriate detection method for *EGFR* mutations in samples containing a sufficient number of tumor cells, a pathological database of lung adenocarcinoma patients who had undergone lung biopsy at initial diagnosis and later underwent surgical resection at Chungbuk National University Hospital between January 2007 and January 2011 was consulted. Formalin-fixed, paraffin-embedded biopsy specimens of lung adenocarcinoma containing more than 20% tumor cells and the corresponding matched surgically resected tumor tissues were used.

DNA Isolation

Cell lines

Cell lines were expanded in RPMI-1640 medium containing 10% (vol/vol) fetal calf serum and were cultured according to standard procedures. Genomic DNA was prepared with a High Pure PCR Template Preparation Kit (Roche Applied Science, Mannheim, Germany) and quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The DNA was stored at −20°C until use.

Tumor Samples

DNA was extracted from five paraffin sections (10 μm) representative of the cell blocks and tumor tissue. Before DNA isolation, the tissue was deparaffinized in xylene and then washed in 70% ethanol. DNA was isolated with a High Pure PCR Template Preparation Kit (Roche Applied Science) according to the manufacturer's protocol. The DNA obtained was eluted in 50 μl of elution buffer, and the concentration and purity of the extracted DNA were assessed by spectroscopy with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The average DNA yield from the tumor samples was 100 ng. The extracted DNA was stored at −20°C until use.

PNA Clamping

The *EGFR* mutation status of extracted DNA from cell lines and tumor samples was determined with the PNAClamp EGFR Mutation Detection Kit (Panagene, Inc., Daejeon, Korea). The assay, based on PNA clamping technology, quickly and accurately detects specific mutations or deletions at known positions of the *EGFR* gene.[15,16] PNA is a synthetic DNA analog in which the phosphodiester backbone is replaced by a peptide-like repeat formed by (2-aminoethyl)-glycine units. Because PNA contains no charged phosphate groups, the binding between the PNA probe and target DNA is stronger than that between a DNA probe and target DNA, because of the lack of electrostatic repulsion. In addition, PNA is resistant to DNases and proteases and is stable across a wide range of pH. The PNAClamp EGFR Mutation Detection Kit is schematically shown in Figure 1. PNA probes and DNA primers are used together in the clamping reaction. Positive signals are detected by intercalation of SYBR Green fluorescent dye. The PNA probe suppresses the amplification of the wild-type sequence, thereby enhancing preferential amplification of the mutant sequences by competitively inhibiting the binding of DNA primer to wild-type DNA. Thus,

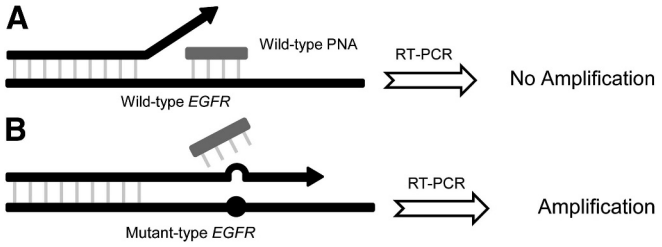

FIGURE 1. The peptide nucleic acid (PNA) clamping system. The PNA oligomer was designed to bind to the bottom strand of the wild-type sequence, spanning mutational hotspots in exons 18–21 of the epidermal growth factor receptor gene. The forward polymerase chain reaction (PCR) primer partially overlapped the PNA binding site. *A*, A PNA/DNA hybrid with a perfect match prevents annealing of the PCR primer and amplification of wild-type DNA. *B*, A PNA/DNA hybrid with a single base pair mismatch does not suppress annealing of the PCR primer or amplification of mutant alleles.