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Detection and comparison of EGFR mutations in matched tumor tissues, cell blocks, pleural effusions, and sera from patients with NSCLC with malignant pleural effusion, by PNA clamping and direct sequencing

Chang Dong Yeo[a], Jin Woo Kim[a], Kwan Hyoung Kim[a], Jick Hwan Ha[b], Chin Kook Rhee[b], Seung Joon Kim[b,*], Young Kyoon Kim[b], Chan Kwon Park[c], Sang Haak Lee[d], Mi Sun Park[e], Hyeon Woo Yim[e]

[a] Department of Internal Medicine, Uijeongbu St. Mary's Hospital, The Catholic University of Korea, Republic of Korea
[b] Department of Internal Medicine, Seoul St. Mary's Hospital, The Catholic University of Korea, Republic of Korea
[c] Department of Internal Medicine, Yeouido St. Mary's Hospital, The Catholic University of Korea, Republic of Korea
[d] Department of Internal Medicine, St. Paul's Hospital, The Catholic University of Korea, Republic of Korea
[e] Department of Biostatistics, Clinical Research Coordinating Center, The Catholic University of Korea, Republic of Korea

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ABSTRACT

Peptide nucleic acid (PNA)-mediated real-time PCR clamping has higher sensitivity than conventional direct sequencing for detecting mutations. Pleural effusion and serum may provide good samples in which to detect epidermal growth factor receptor (EGFR) mutations in non-small cell lung cancer (NSCLC) patients.

We studied 37 NSCLC patients with malignant pleural effusion. EGFR mutations were assessed by PNA clamping and direct sequencing using tumor tissues, cell blocks, pleural effusion, and serum. Concordance between PNA clamping and direct sequencing results, and the diagnostic performance of pleural effusion were investigated.

The κ coefficients for the two methods were 0.68 ($p = 0.0007$), 0.91 ($p < 0.0001$), 0.75 ($p < 0.0001$) and −0.01 ($p = 0.8639$) for tissues, cell blocks, pleural effusion, and serum, respectively. The diagnostic performance of pleural effusion compared with the combination of tumor tissue and cell blocks showed 89% sensitivity, 100% specificity, positive predictive value of 100%, and negative predictive value of 95% by PNA clamping, and 67% sensitivity, 90% specificity, positive predictive value of 75%, and negative predictive value of 86% by directing sequencing. A patient in whom an EGFR mutation was identified in pleural effusion only by PNA clamping showed a significant response to EGFR tyrosine kinase inhibitor (EGFR-TKI) treatment.

In contrast to the limited role of serum samples, pleural effusion had a diagnostic performance for the detection of EGFR mutations in NSCLC that was comparable to that of tumor tissues and cell blocks. The diagnostic performance of PNA clamping was good compared with that of direct sequencing. A more sensitive and accurate detection of EGFR mutations would benefit patients by allowing a better prediction of the response to EGFR-TKI treatment.

1. Introduction

Lung cancer is a leading cause of cancer deaths worldwide. The presence of activating epidermal growth factor receptor (EGFR) mutations is associated with a good response to EGFR tyrosine kinase inhibitor (EGFR-TKI) treatment, making the detection of EGFR mutations clinically important for personalized therapy [1]. Among the variety of methods used to detect EGFR mutations in lung cancer specimens, direct DNA sequencing is the most commonly used and widely available [2,3]. It can exactly detect all mutations. However, it is time-consuming and its sensitivity is suboptimal; mutant DNA must comprise ≥25% of the total amount of DNA for detections to be easily detected [2–4].

Peptide nucleic acid (PNA) is a synthetic DNA analog that binds strongly to its complementary DNA sequence. It has high sensitivity, specificity, and stability as a molecular probe. The PNA probe suppresses the PCR amplification of wild-type sequences, allowing

* Corresponding author at: Division of Pulmonology, Department of Internal Medicine, Seoul St. Mary's Hospital, The Catholic University of Korea, College of Medicine, 222 Banpo-daero, Seocho-gu, Seoul 137-701, Republic of Korea.
Tel.: +82 2 2258 6063; fax: +82 2 599 3589.
E-mail address: cmcksj@catholic.ac.kr (S.J. Kim).

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greater amplification of mutant sequences [5]. The advantages of PNA-clamping PCR are high sensitivity, speed, and simplicity when applied to clinical samples, although this method cannot detect novel mutations [5–7].

Pleural effusion is observed in 8–15% of patients with lung cancer [8]. Approaches for examining molecular biomarkers in body fluids such as effusion or serum may be clinically helpful in predicting the response to EGFR-TKI treatment. The advantages of using pleural fluid are that it is easily accessible, can be sampled by relatively non-invasive methods, and can be repeatedly sampled. Moreover, pleural fluid samples are useful when tumor tissues are not available for mutation analysis. Previous reports have demonstrated the benefits of body fluids for detecting EGFR mutations [9–12]. However, there are insufficient data to evaluate the usefulness of body fluids for determining overall mutation rates and EGFR-TKI response.

In the current study, we analyzed EGFR mutations in matched tumor tissues, cell blocks, pleural effusion samples, and serum samples by both PNA clamping and direct sequencing to investigate the diagnostic performance of PNA clamping and the usefulness of body fluids. To our knowledge, this is the first study to use PNA clamping to detect EGFR mutations in pleural effusion samples (cell-free fluid and cell-block cells) and to compare this technique with direct sequencing.

2. Patients and methods

2.1. Patient Characteristics

We selected 37 consecutive NSCLC patients who presented with malignant pleural effusion at the time of diagnosis and who underwent diagnostic thoracentesis at the Division of Pulmonology of Seoul St. Mary's Hospital (Seoul, Korea) between December 2008 and September 2011. Malignant pleural effusion was confirmed by demonstration of malignant cells on cytological examination or by histologically proven primary lung cancer with the exclusion of other causes of pleural effusion. All subjects provided written informed consent, and the study protocol was approved by the Institutional Review Board of St. Mary's Hospital, The Catholic University of Korea.

2.2. DNA extraction

DNA was extracted from five 5-μm paraffin sections of tumor tissues and cell blocks. Before DNA extraction, the tissues and cell blocks were deparaffinized in xylene and washed in ethanol. Five ml of each pleural fluid and whole blood sample was centrifuged immediately after collection, and about 1 ml of supernatant or serum was used for DNA analysis. DNA was isolated from tumor tissues, cell blocks, pleural effusion, and serum with a High Pure PCR Template Preparation Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. The DNA obtained was eluted in 50 μl of elution buffer, and the concentration and purity of the extracted DNA were assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The amount of DNA used for the study was 200 ng/test (50 ng/exon) for direct sequencing, and 40–80 ng/test (5–10 ng/reaction) for PNA clamping. The extracted DNA was stored at −20 °C until it was used.

2.3. Direct sequencing

EGFR mutations in exons 18, 19, 20, and 21 were detected by PCR-based direct sequencing. PCR amplification was performed with 50 ng of genomic DNA and the following primers (5′ → 3′; forward and reverse, respectively): exon 18, AGGTGACCCTTGTCTCTGTG and CCTGTGCCAGGGACCTTAC; exon 19, CATGTGGCACCATCTCACAA and CCCACACAGCAAAGCAGAA; exon 20, TTCTGGCCACCATGCGAA and GTCTTTGTGTTCCCGGACAT; and exon 21, ACTACTTGGAGGACCGTCG and GGAAAATGCTGGCTGACCTA. PCR cycling was performed with initial denaturation at 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 63 °C for 30 s, and 72 °C for 60 s, with a final extension at 72 °C for 5 min. The amplicons were purified using a HiGene™ PCR Purification Kit (Solgent, Daejeon, Korea). The purified PCR products were sequenced with a BigDye Terminator Kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 3100 DNA analyzer (Applied Biosystems).

2.4. PNA clamping

A PNAClamp™ EGFR Mutation Detection Kit (PANAGENE, Inc., Daejeon, Korea) was used to detect EGFR mutations by real-time PCR, as previously described [5,13]. Twenty-nine EGFR mutation types can be detected with the PNAClamp™ EGFR Mutation Detection Kit. Briefly, all reactions (volume, 20 μl) included template DNA, a primer and PNA probe set, and SYBR Green PCR master mix. Real-time PCR was performed using a CFX96 PCR detection system (Bio-Rad, Philadelphia, PA). PCR cycling consisted of a 5-min hold at 94 °C, followed by 40 cycles of 94 °C for 30 s, 70 °C for 20 s, 63 °C for 30 s, and 72 °C for 30 s. The efficiency was determined by measuring the threshold cycle (Ct) values, which were automatically calculated from PCR amplification plots of fluorescence versus cycle number. ΔCt values were calculated as the Ct value of the standard Ct minus the Ct value of the sample. Higher ΔCt values indicate more efficient amplification of the mutant. A cutoff value of 2.0 was used to indicate the presence of mutant DNA.

2.5. Statistical analyses

Demographic data are presented as the mean ± SD or *n* (%).Concordance between PNA clamping and direct sequencing was determined using Cohen's κ statistic. Sensitivity, specificity, positive predictive value, and negative predictive value were calculated for mutations in pleural effusion compared with those in combined tissue and cell blocks (regarded as the reference standard). These diagnostic parameters were expressed with 95% confidence intervals (CIs). In addition, McNemar's test was used to analyze the agreement between mutations in pleural effusion and combined tissue and cell blocks. Progression-free survival (PFS) was defined as the time from the date on which treatment with EGFR-TKI was started to the date of progression. A two-sided *p*-value <0.05 indicated statistical significance. All statistical analyses were performed using SAS 9.2 software (SAS Institute, Inc., Cary, NC).

3. Results

3.1. Patient characteristics

Table 1 shows the demographic characteristics for the 37 enrolled patients. The mean age of the patients was 70.8 years, and 26 patients (70.3%) were male. The major histological type was adenocarcinoma (89.2%). All 37 patients were diagnosed as primary NSCLC with either biopsy specimens (*n* = 29) or cell block specimens of pleural effusion. Among them, 24 (64.9%) had malignant cells in their effusion specimens and 13 cases (35.1%) were diagnosed as malignant effusion with the exclusion of other causes of pleural effusion.