Research Article

Bronchial brushing cytology is comparable to bronchial biopsy for epidermal growth factor receptor mutation test in non-small cell lung cancer

Joo-Yeon Koo, MD1, Nah-Ihm Kim, MD, PhD1, Taebum Lee, MD1, Yoo-Duk Choi, MD, PhD1

1Department of Pathology, Chonnam National University Medical School, Dong-Gu, Gwangju, Republic of Korea.

ABSTRACT

Objectives: Bronchial brushing (BB) is often used to obtain supplementary samples for diagnosing lung cancer. We examined the possibility of epidermal growth factor receptor (EGFR) testing on BB samples and compared them with bronchial biopsy samples.

Material and Methods: We used 150 BB samples with non-small cell carcinoma submitted to our department within 2 years. Biopsy samples were concurrently submitted for histologic diagnosis. We used the peptide nucleic acid clamping method for EGFR mutation test. Histologic diagnosis identified 137 cases of adenocarcinomas and 13 cases of non-small cell lung carcinoma, not otherwise specified. Each sample was assessed for adequacy and DNA content for EGFR mutation test.

Results: Among BB samples, 28 had exon 19 deletion, 21 had mutations in exon 21, 99 were wild type, and analysis of two failed. The EGFR mutation rate in BB samples was 33.1% (49/148). Among bronchial biopsy samples, 26 had exon 19 deletion, 20 had mutations in exon 21, 92 were wild type, and analysis of 12 failed. The EGFR mutation rate using biopsy sample was 33.8% (46/136). The mutation detection results were nearly identical in both groups of samples (131/138, 94.9%). However, in two cases, an exon 21 mutation was detected in biopsy samples but not in BB samples. In five cases, exon 19 deletion (two cases) and exon 21 mutation (three cases) were detected in BB but not in biopsy samples. The median DNA content was 58.83 ng for BB samples and 48.47 ng for biopsy samples. The failure rate for BB samples was lower than for biopsy samples. Overall, BB samples were comparable to bronchial biopsy samples in terms of DNA quantity and mutation detection results.

Conclusion: We conclude that in case of inadequate biopsy samples, BB samples can be used as a substitute material for EGFR mutation test.

Keywords: Biopsy, Bronchial brushing, Epidermal growth factor receptor, Non-small cell lung cancer

INTRODUCTION

Lung cancer is the most frequent of all forms of cancer and is responsible for approximately 1.38 million deaths each year worldwide. Depending on the size and shape of the tumor cells, lung cancer is divided into non-small cell lung cancer (non-small cell lung carcinoma [NSCLC], 80% of all cases) and small cell lung cancer. The epidermal growth factor receptor (EGFR) is normally found on the surface of epithelial cells and is an important target for anticancer treatment. Mutations in the tyrosine kinase (TK) domain of EGFR in NSCLC patients are associated with clinical response to TK inhibitors (TKIs) such as gefitinib and erlotinib.[1]
Peptide nucleic acid (PNA) is a synthetic DNA analog that binds strongly to its complementary DNA sequence. An artificially designed PNA probe that specific to some DNA sequence inhibits polymerase chain reaction (PCR) amplification of wild-type sequences, but it allows greater amplification of mutant sequences.\textsuperscript{[2-3]}

In the past, cytologic specimen has not been accepted for DNA analysis due to few cellularity.\textsuperscript{[4]} However, in recent years, according to the consensus that preservation of the extracted DNA seems to be important than the actual number cells present in the samples for DNA analysis, cytologic samples are being more frequently used for mutational test, in particular, cytological materials are the only available samples.\textsuperscript{[5-7]} Here, we examined the possibility of EGFR testing on bronchial brushing (BB) samples by compared them to bronchial biopsy samples used for EGFR mutation test.

MATERIAL AND METHODS

BB material from 150 patients with a clinical suspicion of lung cancer was submitted for cytomorphologic diagnosis at the Department of Pathology of Chonnam National University Hospital from January 2017 to December 2018. All BB samples were submitted as liquid-based cytology (LBC) sample. At the same time, bronchial biopsy samples from the same patients were submitted for histologic diagnosis. EGFR mutation analyses were performed on different types of samples (biopsy samples and BB samples) collected by the same person. Informed consent for mutation testing was obtained from all the patients. This study was approved by the Institutional Review Board of our hospital (CNUH 2016-213).

BB samples were preserved in ThinPrep vials at room temperature and were used for DNA extraction within 1 week using a QIAamp DNA Mini kit and the column method. In the biopsy samples, the areas of the tissue slide containing any tumor cells to be of concern were reviewed by a pathologist; then, the suspected tumor cells were scraped with a side-cutting wooden applicator. DNA was extracted from paraffin-embedded samples with a QIAamp FFPE Tissue DNA Mini kit (Qiagen, Valencia, CA, USA) using the column method. Each sample DNA was quantified using a Biospec-nano spectrophotometer (Shimadzu, Kyoto, Japan). All samples were diluted to 10 ng/µl for optimal DNA synthesis in real-time PCR.

A PNA Clamp (PNAClamp) Mutation Detection Kit (Panagene Inc., Daejeon, Korea) was used to detect EGFR mutations by real-time PCR. The reaction mixtures (20 µl) contained template DNA, a primer and PNA probe set, and SYBR Green PCR master mix (all reagents were from the PAN clamp kit). Real-time PCR was performed in a LightCycler (Roche, Zurich, Switzerland) as follows: 5 min at 94°C followed by 40 cycles of denaturation at 94°C for 30 s, PNAC at 70°C for 20 s, primer binding at 63°C for 30 s, and elongation at 72°C for 30 s. The efficacy of PNAC was determined by measuring the threshold cycle (Ct) value. Ct values for the control and mutation assays were obtained from SYBR Green amplification plots. The delta Ct (ΔCt) value was calculated (control Ct–sample Ct), ensuring that the sample and control Ct values were from the test and wild-type control samples, respectively. The cutoff ΔCt was defined as 2 for the exon 19 deletion, and T790M and L858R substitutions. PCR efficacy was determined by measuring the Ct values, which were automatically calculated from PCR amplification plots of fluorescence versus the number of cycles. The delta (Δ)Ct–1 value (control Ct–sample Ct) was calculated to identify the presence of an EGFR mutation. When the ΔCt–1 value was more than 2, the sample was considered as mutant. When the ΔCt–1 value was between 0 and 2, the ΔCt–2 value (sample Ct–non-PNA Ct value) was calculated. For EGFR quantification, we used the corrected ΔCt value, which standardized the variations in sample quality and were calculated as follows: Corrected ΔCt = standard Ct − sample Ct × 25/non-PNA.

DNA quantification comparison was performed using the Fisher’s exact test for categorical variables. All analyses were performed using IBM SPSS software version 19.0 (IBM Co., Armonk, NY, USA). \( P < 0.05 \) indicated statistical significance.

RESULTS

The study included 90 men and 60 women 64 ± 10 years of age (range, 36–70 years); there were 137 cases of adenocarcinomas and 13 cases of NSCLC – not otherwise specified. The diagnosis was based mainly on biopsy samples and additional immunohistochemical staining of these samples. In cases of rare tumor cells on biopsy sample, supplementary cytologic examination was added for pathologic diagnosis.

Among the 150 BB samples, EGFR mutations were detected in 49 (32.7%), including exon 19 deletion in 28 cases and exon 21 mutations in 21 cases. No mutations (wild type) were detected in 99 cases. In the remaining 2 cases, the results were invalid for EGFR mutation test. Among the 150 biopsy samples, EGFR mutations were detected in 46 cases (30.7%), including exon 19 deletion in 26 cases and exon 21 mutations in 20 cases. No mutations (wild type) were detected in 92 cases. In 12 cases, the results were invalid for EGFR mutation test; these included the 2 cases in which the results were also invalid in BB samples and 10 cases in which no mutations (wild type) were detected in BB samples [Table 1]. Therefore, the failure rate of EGFR mutation test was lower in BB cytology samples than in biopsy samples.
With the 12 cases that showed invalid results excluded, the mutation detection results were nearly identical in both groups of samples (131/138, 94.9%) [Table 1]. Among the seven cases that showed discrepancy between the BB and biopsy samples, in five cases, exon 19 deletion (two cases) and exon 21 mutation (three cases) were detected in BB samples, but no mutations (wild type) were found in biopsy samples. In the other two cases, an exon 21 mutation was detected in biopsy samples, but no mutations (wild type) were found in BB samples. These seven cases with discrepancies cases are summarized in Table 2.

The median DNA content was 58.83 ng for 150 BB samples and 48.47 ng for 150 biopsy samples. The DNA content was not significantly different between the two sample groups. Among invalid cases, the median DNA content in 2 BB samples was 3.4 ng and that in 12 biopsy samples was 2.9 ng. With the 12 invalid cases excluded, the median DNA content of BB samples was 59.57 ng and that of biopsy samples was 52.43 ng, with no significant difference between the two groups. For 10 cases, whose biopsy samples were invalid and no mutations (wild type) were detected in BB samples, the median DNA content was 78.84 ng in BB samples but only 2.51 ng in biopsy samples [Table 3].

There was also no statistically significant difference of median DNA content between mutation (exon 19 deletion or L858R mutation) group and no mutation (wild type) group in BB samples or biopsy samples.

**DISCUSSION**

Cytology is a non-invasive diagnostic method. In respiratory disease, BB sample is used for the diagnosis of cancer.[7,8] Initially, after the LBC method was first introduced in gynecologic cytology, the frequency of LBC use tended to increase continuously to gynecology sample, non-gynecology sample, and fine-needle aspiration sample. Because immunostaining and DNA analysis can provide stable results for any samples,[9] the use of LBC is widely recommended for cytologic examination and DNA-based molecular testing.[10,11]

In this study, DNA quantification was a good marker for the feasibility of EGFR mutation test. Regardless of sample type, the DNA content of samples that yielded invalid results in EGFR test was very low. Therefore, DNA quantification is an important step in EGFR testing. Although the method used to extract DNA from tumor cells is the gold standard in EGFR mutation analysis, it is often difficult to obtain a sufficient amount of tumor cells from cytology specimen of NSCLC patient.[6] We hypothesized that EGFR mutation analysis could be possible not only with DNA in the sediment of cytological samples but also with cytology cell-free DNA (ccfDNA) in the supernatant fluids of cytological samples, and ccfDNA may be able to compensate for the lack of adequate DNA in EGFR mutation analysis using cytological materials.[12] In this study, the median DNA content of BB cytologic samples was not lower than that of biopsy samples.

The PNAc technology (Panagene Inc.) recently approved by Korean Food and Drug Administration approved uses PNA-mediated real-time PCR using benzothiazole-2-sulfonyl as the amine protecting group.[13] PNA is an artificially synthesized polymer that has the properties of both nucleic acids and proteins. PNA can bind to a complementary sequence in the DNA, and its binding capacity is stronger than that of complementary DNA because of the lack of electrostatic repulsion. PNAc enables the detection of mutations in specimens containing as few as approximately 1% of mutant alleles. Other advantages of PNAc include simplicity and speed when applied in clinical settings, although it cannot detect novel mutations.[14]

Molecular testing is now a standard step that is being rapidly incorporated in the routine diagnostics of NSCLC. Personalized treatment generally requires the outcomes of multiple gene analysis and needs a rapid and accurate result from various materials including cytology sample.[15] The higher sensitive PCR method and next-generation sequencing use a small amount of material and

**Table 1:** Comparison between bronchial brushing sample and bronchial biopsy sample for EGFR mutation test.

| Bronchial brushing sample | Bronchial biopsy sample |
|---------------------------|-------------------------|
| Exon 19 deletion          | 28 (18.7%)              |
| Identical (26 cases)      | Exon 19 deletion        | 26 (17.3%) |
| Exon 21 mutation          | 21 (14.0%)              |
| Identical (18 cases)      | No mutation (wild type) | 2 (1.3%)  |
| No mutation (wild type)   | 99 (64.7%)              |
| Identical (87 cases)      | Exon 21 mutation        | 18 (12.0%) |
| No mutation (wild type)   | 3 (2.0%)                |
| Exon 21 mutation          | 2 (1.3%)                |
| No mutation (wild type)   | 87 (58.0%)              |
| Invalid                   | 10                      |
| Invalid                   | 2                       |
| Total                     | 150                     |

Identical (131/138: 94.9%)
produce results for detecting the numerous mutations.\(^{[16,17]}\) These new methods have been tested on cytological samples. Further, clinical-based large studies are necessary for its clinical and therapeutic validation.

Our study has some weak points: (1) It was a retrospective study performed in one institution; (2) the content of tumor cells was not calculated in either BB or biopsy samples; and (3) the clinical outcome after treatment was not considered. Additional large-scale, multicenter studies are needed to determine the appropriateness of cytologic samples for EGFR test; such studies should consider intratumoral heterogeneity, abnormal genetic changes in the tumor, various mutation detection tools, and EGFR-TKI treatment outcomes.

Obtaining a sufficient tumor biopsy sample for diagnosis in patients with lung cancer may not always be possible, mainly because of the complications of the procedure for patients, most of whom have emphysema and a major risk of pneumothorax, and because of technical difficulties.\(^{[18,19]}\) There is an increasing demand for larger samples obtained through minimally invasive techniques to perform molecular testing in NSCLC patients to guide therapeutic decision making. Therefore, cytological samples often permit effective initial diagnosis of NSCLC.

### CONCLUSION

Our current results demonstrate that EGFR mutational status determined using BB samples is similar to that provided by testing histologic samples. In cases of inadequate biopsy samples, BB samples may be a good substitute material for EGFR mutation test.

### COMPETING INTERESTS STATEMENT BY ALL AUTHORS

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### AUTHORSHIP STATEMENT BY ALL AUTHORS

Taebum Lee: Contributions to design

Nah-Ihm Kim: Acquisition of data

Joo-Yeon Koo: Drafting the article and revising it critically for important intellectual content

Yoo-Duk Choi: Analysis and interpretation of data and final approval of the version.

| Table 2: Discrepancy cases between bronchial brushing sample and bronchial biopsy sample for EGFR mutation test. |
|---|---|---|---|---|
| Cell type | Mutation result of BB sample | Mutation result of biopsy sample | DNA content of BB sample (ng) | DNA content of biopsy sample (ng) |
| ADC | No mutation (wild type) | Exon 21 mutation | 123.7 | 90.1 |
| ADC | No mutation (wild type) | Exon 21 mutation | 55.2 | 37.2 |
| ADC | Exon 21 mutation | No mutation (wild type) | 21.2 | 12.2 |
| ADC | Exon 21 mutation | No mutation (wild type) | 59.9 | 13.2 |
| ADC | Exon 21 mutation | No mutation (wild type) | 22.7 | 35.7 |
| ADC | Exon 19 deletion | No mutation (wild type) | 121.5 | 112.1 |
| ADC | Exon 19 deletion | No mutation (wild type) | 139.5 | 47.9 |

ADC: Adenocarcinoma, BB: Bronchial brushing

| Table 3: Invalid cases of bronchial brushing sample or bronchial biopsy sample for EGFR mutation test. |
|---|---|---|---|---|
| Cell type | Result of BB sample | Result of biopsy sample | DNA content of BB sample (ng) | DNA content of biopsy sample (ng) |
| ADC | No mutation | Invalid | 74.2 | 2.7 |
| ADC | No mutation | Invalid | 110 | 1.1 |
| ADC | No mutation | Invalid | 47.2 | 3.7 |
| NSCLC, NOS | No mutation | Invalid | 124.1 | 2.2 |
| ADC | No mutation | Invalid | 72.7 | 4.2 |
| ADC | No mutation | Invalid | 33.2 | 0.6 |
| ADC | No mutation | Invalid | 36.9 | 1.2 |
| ADC | No mutation | Invalid | 181 | 2.1 |
| ADC | No mutation | Invalid | 64.2 | 1.7 |
| ADC | No mutation | Invalid | 44.9 | 5.6 |
| ADC | Invalid | Invalid | 6.4 | 6.8 |
| NSCLC, NOS | Invalid | Invalid | 2.4 | 3.2 |

ADC: Adenocarcinoma, NSCLC: Non-small cell carcinoma, NOS: Not otherwise specified
ETHICS STATEMENT BY ALL AUTHORS
This study was conducted with approval from Institutional Review Board (IRB) (CNUH 2016-213).

LIST OF ABBREVIATIONS (In alphabetic order)
BB: Bronchial brushing
ccfDNA: cytology cell-free DNA
EGFR: Epidermal growth factor receptor
LBC: Liquid based cytology
NOS: Not otherwise specified
NSCLC: Non-small cell lung cancer
PCR: Polymerase chain reaction
PNA: Peptide nucleic acid
TK: Tyrosine kinase
TKI: Tyrosine kinase inhibitors

EDITORIAL/PEER-REVIEW STATEMENT
To ensure the integrity and highest quality of CytoJournal publications, the review process of this manuscript was conducted under a double-blind model (authors are blinded for reviewers and vice versa) through automatic online system.

REFERENCES
1. Pao W, Ladanyi M. Epidermal growth factor receptor mutation testing in lung cancer: Searching for the ideal method. Clin Cancer Res 2007;13:4954-5.
2. Yoon SH, Choi YD, Oh IJ, Kim KS, Choi H, Chang J, et al. Peptide nucleic acid clamping versus direct sequencing for the detection of EGFR gene mutation in patients with non-small cell lung cancer. Cancer Res Treat 2015;47:661-9.
3. Kim HJ, Lee KY, Kim YC, Lee SY, Jang TW, et al. Detection and comparison of peptide nucleic acid-mediated real-time polymerase chain reaction clamping and direct gene sequencing for epidermal growth factor receptor mutations in patients with non-small cell lung cancer. Lung Cancer 2012;75:321-5.
4. Da Cunha Santos G, Saieg MA, Geddie W, Leighl N. EGFR gene status in cytological samples of nonsmall cell lung carcinoma: Controversies and opportunities. Cancer Cytopathol 2011;119:80-91.
5. Hagiwara K, Kobayashi K. Importance of the cytological samples for the epidermal growth factor receptor gene mutation test for non-small cell lung cancer. Cancer Sci 2013;104:291-7.
6. Yang JCH, Wu YL, Chan V, Kurnianda J, Nakagawa K, Saigo N, et al. Epidermal growth factor receptor mutation analysis in previously unanalyzed histology samples and cytology samples from the phase III Iressa Pan-ASia Study (IPASS). Lung Cancer 2014;83:174-81.
7. Kawahara A, Fukumitsu C, Taira T, Abe H, Takase Y, Murata K, et al. Epidermal growth factor receptor mutation status in cell-free DNA supernatant of bronchial washings and brushings. Cancer Cytopathol 2015;123:620-8.
8. Aikawa E, Kawahara A, Hattori S, Yamaguchi T, Abe H, Taira T, et al. Comparison of the expression levels of napsin A, thyroid transcription factor-1, and p63 in nonsmall cell lung cancer using cytocentrifuged bronchial brushings. Cancer Cytopathol 2011;119:335-45.
9. Tsai TH, Wu SG, Chang YL, Wu CT, Tsai MF, Wei PF, et al. Efusion immunocytochemistry as an alternative approach for the selection of first-line targeted therapy in advanced lung adenocarcinoma. J Thorac Oncol 2012;7:993-1000.
10. Reynolds JP, Tubbs RR, Minca EC, MacNamara S, Almeida FA, Ma PC, et al. EGFR mutational genotyping of liquid based cytology samples obtained via fine needle aspiration (FNA) at endobronchial ultrasound of non-small cell lung cancer (NSCLC). Lung Cancer 2014;86:158-63.
11. Wu CY, Hou LK, Ren SX, Su B, Chen G. High feasibility of liquid-based cytological samples for detection of egfr mutations in Chinese patients with NSCLC. Asian Pac J Cancer Prev 2014;15:7885-9.
12. Kim HR, Lee SY, Hyun DS, Lee MK, Lee HK, Choi CM, et al. Detection of EGFR mutations in circulating free DNA by PNA-mediated PCR clamping. J Exp Clin Cancer Res 2013;32:50.
13. Kim HJ, Kim WS, Shin KC, Lee GH, Kim MJ, Lee JE, et al. Comparative analysis of peptide nucleic acid (PNA)-mediated real-time PCR clamping and DNA direct sequencing for EGFR mutation detection. Tuberc Respir Dis 2011;70:21-7.
14. Min KW, Kim WS, Jang SJ, Choi YD, Chang S, Jung SH, et al. Comparison of EGFR mutation detection between the tissue and cytology using direct sequencing, pyrosequencing and peptide nucleic acid clamping in lung adenocarcinoma: Korean multicentre study. QJM 2016;109:167-73.
15. Nakajima T, Yasufuku K, Nakagawara A, Kimura H, Yoshino I. Multigene mutation analysis of metastatic lymph nodes in non-small cell lung cancer diagnosed by endobronchial ultrasound-guided transbronchial needle aspiration. Chest 2011;140:1319-24.
16. Sanchez-Font A, Chalela R, Martin-Ontiuyelo C, Alberos-Gonzalez R, Dalmases A, Longaron R, et al. Molecular analysis of peripheral lung adenocarcinoma in brush cytology obtained by EBUS plus fluoroscopy-guided bronchoscopy. Cancer Cytopathol 2018;126:860-71.
17. Mohar B, Jezek SS, Molek KR, Stemberger C, Kurpis M, Kupanovac Z, et al. Detection of an EGFR mutation in cytological specimens of lung adenocarcinoma. Cytojournal 2016;13:444-51.
18. Wang XY, Chen SX, Emerson RE, Wu HH, Cramer HM, Curless K, et al. Molecular testing for EGFR mutations and ALK rearrangements in the cytological specimens from the patients with non-small cell lung cancer. Appl Immunohistochem Mol Morphol 2019;27:119-24.
19. Liu N, Sun RJ, Du J, Dong QZ, Fan CF, Li QC, et al. Comparison of epidermal growth factor receptor gene mutations identified using pleural effusion and primary tumor tissue samples in non-small cell lung cancer. Appl Immunohistochem Mol Morphol 2018;26:44-51.
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