Reliability of Direct Sequencing of EGFR: Comparison between Cytological and Histological Samples from the Same Patient

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Abstract. *The results of a recent study have shown the superiority of treatment with gefitinib or erlotinib in lung tumors positive for epidermal growth factor receptor (EGFR) mutation. As a consequence, the complete diagnosis of lung cancer cannot be limited to histotype classification, but should include a series of molecular biology analyses. In most cases, the diagnosis of lung cancer is performed on cytological specimens; therefore, there is a need to obtain a complete and reliable molecular diagnosis on cytologic specimens. Brushing, transbronchial needle aspiration (TBNA) and broncho alveolar lavage during fibro-bronchoscopy allow the sampling of the lung and the mediastinal lymph node. The aim of this study was to demonstrate that direct sequencing of exons 19 and 21 of EGFR in lung tumors, carried out on the cytological samples obtained through fibro-bronchoscopy, is as reliable as the same analysis carried out on a histological surgical sample obtained from the same individual. We considered 50 patients with a histological diagnosis of lung adenocarcinoma whose cytological samples, obtained by fibro-bronchoscopy and histological samples, obtained by surgical resection were available. A comparison of the sensitivity and reliability of the molecular biology analyses carried out on histological and cytological samples of the same patient was carried out. The combined mutation percentage of exons 19 and 21 of EGFR was 10%. The results of the analyses carried out on cytological samples matched those obtained from the histological samples. The feasibility of EGFR analysis on cytological samples has already been demonstrated in previous studies, however these studies referred to the method of fluorescence in situ hybridization, or did not perform any comparison between histological samples from the same patient; our work, on the other hand, shows that direct sequencing of exons 19 and 21 of the EGFR gene is feasible on fibro-bronchoscopy cytological samples with the same reliability offered by the histological samples obtained from the same patient.*

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Platin-based couplets are a staple of first-line chemotherapy for advanced non-small cell lung cancer (NSCLC) in patients with good performance status (1). However, the results of a recent study have shown the superiority of treatment with gefitinib or erlotinib in tumors positive for epidermal growth factor receptor (*EGFR*) mutation (2). As a consequence, the complete diagnosis of lung cancer cannot be limited to histotype classification, but should include a series of molecular biology analyses (*EGFR, K-Ras*, ALK fusion) that allow to single out the therapeutic target of the new drugs currently available and of those being presently developed (3-6). The diagnostic course of lung cancer staging includes among the first investigations a fibrobronchoscopy which, if correctly performed, can be decisive in most cases. Fibrobronchoscopy with transbronchial needle aspiration (TBNA), with or without ultrasound guiding, allows the sampling of mediastinal lymph nodes in contact with the bronchial tree and thus, in addition to providing a diagnosis, is also useful for staging purposes (7-12). In most cases the diagnosis of lung cancer is performed on cytological specimens and for this reason in order to perform molecular biology analyses on samples, suitable quantities of material is required (13, 14). The aim of this study was to demonstrate that direct sequencing of exons 19 and 21 of *EGFR* gene in lung tumors, carried out on the cytological samples obtained through fibrobronchoscopy with brushing, bronchoalveolar lavage (BAL) or TBNA without ultrasound guiding, is as reliable as the same analysis carried out on a histological surgical sample obtained from the same individual; thus sparing the patient more expensive, invasive and riskier procedures and granting them more rapid access to new therapeutic options.

Patients and Methods

Patients and samples. Fifty patients with a histologic diagnosis of lung adenocarcinoma, whose cytological samples obtained by TBNA, BAL or brushing and histological samples obtained by surgical resection were available, were included in this study. The cytological samples obtained by TBNA or brushing were smeared on slides and immediately fixed with 95˚ ethyl alcohol; some of these slides were used for the cytomorphological and immunocytochemical investigations, whereas others were used to obtain the DNA required for molecular biology analyses. Sampling adequacy was evaluated during bronchoscopy by means of rapid on-site cytological examination (ROSE). The histological samples were obtained from tissue belonging to surgically removed lung tumors. Each sample was subjected to molecular biology techniques for *EGFR* mutation detection. A comparison of the sensitivity and reliability of the molecular biology analyses carried out on histological and cytological samples of the same patient was carried out.

Methods. Direct sequencing of exons 19 and 21 of *EGFR* was performed on the histological and cytological samples under blind conditions: *i.e.* the operator did not know to which patient belonged the samples and whether the patient had been tested positive or negative at previous molecular investigations. As far as the cytological samples were concerned, the cytologist had chosen slides with at least 100 cells of which >70% neoplastic cells, for *EGFR* analysis. Genomic DNA was extracted from tumors and normal lung samples according to standard procedures (15, 16). Genetic analysis of the *EGFR* gene was performed by PCR amplification of exons 19 and 21 with flanking intronic sequences and direct sequencing of the PCR products. Primer: exon 19 (forward 5'-ACCATCTCACAATTG CCAGTTAAC-3'; reverse 5'- GAGGTTCAGAGCCATGGACC-3'), exon 21 (forward 5'-TCACAGCAGGGTCTTCTCTGTTT-3'; reverse 5'-ATGCTGGC TGACCTAAAGCC-3'). Tyrosine Kinase exons were amplified in a 384-well format PCR setup. PCR was performed in a total volume of 10 μl, containing 1xTaqMan buffer, 1.5 mmol/l $MgCl_2$, 800 μmol/l dNTPs, 300 nmol/l each primer, 0.3 units Taq DNA polymerase, and 10 ng genomic DNA. Thermal cycling conditions included 4 min at 95˚C, followed by 35 cycles of 95˚C for 30 s, 60˚C for 30 s, 72˚C for 1 min, and one cycle of 72˚C for 7 min. The PCR products were then purified by Multiscreen 384-PCR filter plate (Millipore Corp, Bedford, CA, USA) and subjected to bi-directional dye terminator sequencing using the same primers used for amplification. Sequencing fragments were detected by capillary electrophoresis using an ABI Prism 3100 DNA analyzer (Applied Biosystems, Foster City, CA, USA). In all cases, samples harboring mutations were reamplified and resequenced using the experimental conditions. Sequence chromatograms were analyzed by Mutation Surveyor 2.2 (SoftGenetics, State College, PH, USA), followed by manual review. Anon radioactive Single-strand conformation polymorphism assay (SSCP) was divised to screen for mutations in exons 19 and 21, as previously described (21), with the following modifications. After completion of the PCR reaction (performed as reported above, in a volume of 30 μl), the product was diluted 1:5 in loading buffer (95% formamide, 2 mmol/l EDTA, pH 8.3). Fifteen microliters of the diluted samples were denatured (5 min at 90˚C), immediately cooled on ice, and loaded onto a non-denaturing polyacrylamide gel. The concentration of acrylamide was 10% for the screening of exon 19, and 12% for the screening of exon 21. Tumor samples were loaded side by side with the corresponding normal lung control tissue. Electrophoresis was carried out for 14 h at 20˚C at 3 W. On complete migration, the gels were subjected to silver staining using a PlusOne Silver Staining Kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Positive cases were reamplified in the same experimental conditions, and subjected again to SSCP to confirm the mutations. The shifted bands were removed from the gel, and the recovered DNA was amplified in duplicate and subjected to direct sequencing as reported earlier.

Results

Patient characteristics and diagnosis are summarized as follows: 34 men and 16 women, age: 64±10 (range 36-70) years. The diagnoses of lung adenocarcinoma based on the cytological samples tallied with those obtained by histological examination in 92% (46/50) of patients; in four patients, the cytological diagnosis was unclassified non small cell lung carcinoma (NSCLC). The combined percentage of mutation of exons 19 and 21 of *EGFR* was 10%: 5 adenocarcinomas out of 50 analyzed, 4 of exon 19 and 1 of exon 21. The results of the analyses carried out on cytological samples matched those obtained from the histological samples in all 5 mutated and in the 45 non-mutated samples, with a positive predictive value of 1 and negative predictive value of 1 (using histology as the reference). The cytological samples of patients with mutations were 2 TBNA, 2 brushing and 1 BAL. Figure 1 shows an electropherogram of *EGFR* analysis in one of the five mutated cases and shows: a segment of exon 19 where the sequencer has revealed a conflict in the reading of sequence. The pattern of cytology is similar to that of histology.

Discussion

Progress in NSCLC chemotherapy now requires not only a histotype diagnosis, but also the in-depth analysis of some genome traits by means of molecular biology (18). This will be even more important in the future because the chemotherapeutic agents presently developed have precise molecular targets and their efficacy can be predicted precisely through this kind of analysis. In most cases, the diagnosis of lung cancer is performed on cytological specimens; therefore, there is a need to obtain a complete and reliable molecular diagnosis on cytological specimens. TBNA, brushing and BAL are techniques used during fibrobronchoscopy that allows cytological (and sometimes also histological) samples to be obtained from bronchus, lung and peribronchial lymph nodes in contact with the airways. The main advantage offered by TBNA is the possibility of diagnosis and staging the N-parameter of the TNM classification with a single procedure during the first fibrobronchoscopy carried out on the patient, with no need for hospitalization and without resorting to more invasive, expensive and riskier procedures such as mediastinoscopy (19, 20). The feasibility of *EGFR* analysis on cytological samples has already been demonstrated in previous studies, however these studies relied on the method of *in situ*

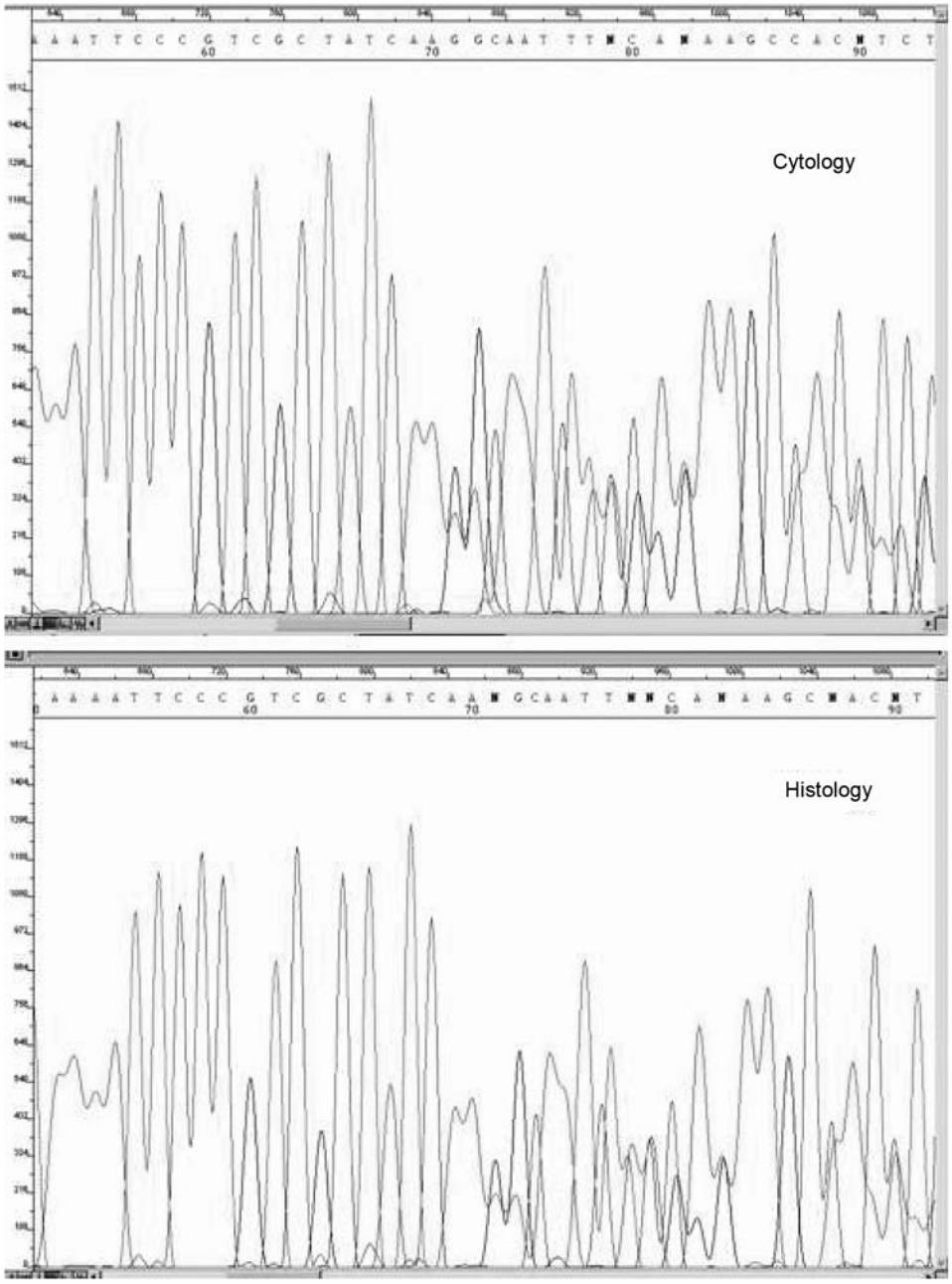

Figure 1. *Electropherogram of EGFR analysis in cytology vs histology in the same patient for a segment of exon 19 where the sequencer has revealed a conflict in the reading of sequence.*

hybridization (FISH), or did not make any comparison between histological samples from the same patient (13, 14, 21-24); this work, on the other hand, shows that direct sequencing of exons 19 and 21 of *EGFR* gene can be also performed on cytological samples with the same reliability as the one offered by the analysis of the histological samples obtained from the same patient. Therefore, we believe that during the diagnostic procedure carried out for suspected NSCLC, it is advisable to obtain a suitable sample in terms of quality and quantity, and the case survey presented in this paper demonstrates that this can be performed by means of fibrobronchoscopy and ROSE. The data here show that direct sequencing of exons 19 and 21 of *EGFR* gene can be performed on cytological samples obtained by fibrobronchoscopy; the analyses performed were equally reliable as those performed on histological samples obtained from the same patients.

Conflict of Interest Statement

The Authors have no connection to any companies or products mentioned in this article, the authors have not any conflict of interest with regards to the contents of this study.

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