A Comparative Study of Cell Block versus Biopsy for Detection of Epidermal Growth Factor Receptor Mutations and Anaplastic Lymphoma Kinase Rearrangement in Adenocarcinoma Lung

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Abstract

Background: Lung cancer is a leading cause of deaths attributed to cancer worldwide. Epidermal growth factor receptor (EGFR) mutation and anaplastic lymphoma kinase (ALK) rearrangement are commonly found in patients of adenocarcinoma lung against which targeted therapy is available. In this era of personalized medicine, it is a rationale to detect these molecular alterations in cases of lung carcinomas.

Aims: The objectives were to compare the diagnostic efficacy of cytological samples for the detection of EGFR and ALK protein expression using immunocytochemistry in nonsmall cell lung carcinoma. Materials and Methods: We compared 22 cell blocks and biopsies for the detection of EGFR and ALK protein expression by immunohistochemistry (IHC). EGFR IHC was performed using EGFR Receptor (E746-A750 del Specific) (6B6) monoclonal antibody and ALK IHC was done using Ventana anti-ALK (D5F3) monoclonal primary antibody. Results: Two cases were found to be positive; 20 cases were negative for EGFR IHC both in biopsies and cell blocks. ALK IHC was positive in one case; negative in 21 cases. The results of IHC were also concordant for biopsies and cell blocks. The sensitivity and specificity were 100% for immunocytochemical detection of ALK and EGFR in cell blocks with respect to biopsies. Conclusion: We conclude that cell blocks can serve as a potential substitute for biopsies for detection of EGFR and ALK protein by immunocytochemistry, whenever patient presents with effusion and biopsy cannot be done or when tissue is not adequate.

Keywords: Anaplastic lymphoma kinase, cell block, epidermal growth factor receptor, lung cancer

INTRODUCTION

Lung cancer is the leading cause of cancer death not only in India but also worldwide. In the past few years, major efforts are being done for the correct diagnosis and treatment of lung cancer. The discovery of molecular markers in nonsmall cell lung carcinoma (NSCLC) has started a new chapter in the diagnosis and treatment of lung carcinoma. The important predictive markers in adenocarcinoma lung are epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK) against which targeted therapy in the form of tyrosine kinase inhibitors are available. This has revolutionized the field of personalized medicine. Mutations in the EGFR family of receptor tyrosine kinases are found in 10%–23% of lung adenocarcinomas.[1-4] The two most common EGFR mutations are small deletions in exon 19 (E746-A750) and the L858R missense mutation in exon 2. The most common aberration of the ALK gene in lung cancer is an inversion event on the short arm of chromosome 2, resulting in the fusion of ALK gene with the echinoderm microtubule-associated protein like 4 (EML4) gene locus. The EML4–ALK translocation is found in 2%–7% of lung adenocarcinomas.[5,6] The preferred methods for detection of EGFR are gene sequencing and polymerase chain reaction (PCR), whereas for ALK, it is fluorescence in situ hybridization (FISH). However, these methods are not available in most of the laboratories. Immunohistochemistry (IHC) is relatively less costly and widely available method. IHC is
being applied on tissue samples and the cytological material in which we can get multiple sections for immunohistochemical studies is cell block. Tissue is always an issue; hence, World Health Organization (WHO) in 2015 has recommended the preservation of cytological material for further molecular studies. Cytological material is more important in advanced stages of the disease, when resection is not possible. This study was designed to evaluate the diagnostic efficacy of cytological samples for the detection of EGFR and ALK protein expression using immunocytochemistry in NSCLC.

**Materials and Methods**

This study was a tertiary hospital- and laboratory-based case series for diagnostic evaluation. Cell blocks were prepared and biopsies were taken from the suspected cases of lung carcinomas. For the preparation of cell blocks, effusion fluids or the residual material from transbronchial needle aspirate/bronchoscopic-guided fine needle aspirates (FNAs)/computed tomography (CT)-guided FNA samples, which were taken for preparing smears for cytological diagnosis, were flushed in a 10 mL disposable centrifuge tube having freshly prepared Nathan alcohol formalin substitute consisting of nine parts of 100% ethanol and one part of 40% formaldehyde and centrifuged at 4,000 rpm for 6 minutes. Supernatant fluid was decanted and the deposits in form of cell pellets were formed. The cell pellets were wrapped in filter paper, placed in a cassette, and was processed in the automatic tissue processor using a 6- to 8-hour processing schedule. The cell blocks were embedded in paraffin and sectioned at 3–4 µm thickness. No additional passes were done to obtain material for cell block preparation. Informed consent was taken from the patients. Pertinent clinical details were documented including the age, sex, presenting clinical features, and history of smoking. Both biopsies and cell blocks were screened for the presence of NSCLC. NSCLC cases were further categorized as squamous cell carcinoma, adenocarcinoma, adenosquamous carcinoma, and undifferentiated carcinoma by applying a panel of immunohistochemistry on biopsies comprising of CK7, thyroid transcription factor 1 (TTF-1), and p63/CK5/6/p40 positivity indicates squamous cell carcinoma. Other IHC markers were also applied wherever is required. EGFR and ALK IHC were performed on biopsies of cases with adenocarcinomatous component and corresponding cell blocks. EGFR IHC was performed using EGFR receptor (E746-A750 del specific) (6B6) XP™ rabbit monoclonal antibody (cell signalling, Danvers, MA) and EGFR receptor (L858R Mutant Specific) (43B2) rabbit monoclonal antibody (cell signalling, Danvers, MA). A positive and negative control was run with every batch. The positive controls used were cases diagnosed to have the E746A750 or L858R mutations by real-time PCR. The negative control was a case that was negative for EGFR mutations by real-time PCR. Before immunostaining, the slides were heated at 56°C for 3 hours in a drying oven. Slides were deparaffinized with xylene, washed with alcohol, and rehydrated in deionized water. Antigen retrieval was performed by using retrieval solution, EDTA at pH 8.0. Endogenous peroxidase activity was blocked by incubating the slides for 5 minutes in 0.03% hydrogen peroxide (Envision/HRP, Dako, Dakopatts, Denmark). After rinsing in wash buffer, the sections were incubated for 1 hour at room temperature with EGFR antibodies both of which are diluted to 1:100 in Tris–HCl buffer antibody diluents (Dako). Slides were rinsed in wash buffer, and then incubated for 30 minutes with peroxide-labeled polymer conjugated to goat anti-rabbit immunoglobulins (Envision/HRP, Dako). The chromogenic reaction was carried out with 3, 3'-diaminobenzidine chromogen solution. Counterstaining was done with hematoxylin.

The IHC for EGFR protein is membranous and/or cytoplasmic. Four grades were employed: Grade Zero: no staining; Grade 1+: light yellow staining (+) with no obvious particulates or yellow staining with obvious particulates (++) in <10% of tumor cells; Grade 2+: yellow staining with obvious particulates (++) in >10% tumor cells or brown staining with obvious particulates (+++ in <10% of tumor cells; and Grade 3+: brown staining with obvious particulates (+++ in >10% tumor cells. Score of 0 was considered negative for EGFR mutation. Score of 1+ or 2+ was considered equivocal for EGFR mutation. Score of 3+ was considered positive for EGFR mutation.

ALK IHC was done using Ventana anti-ALK (D5F3) rabbit monoclonal primary antibody, together with the Optiview DAB IHC detection kit and Optiview Amplification kit on the Benchmark XT stainer. For ALK, a positive and negative control was run with every batch. Appendix was used as a positive control and immunostaining was obtained in the ganglion cells. In each case, the rabbit monoclonal Ig served as the negative control. A sample for considered positive when strong granular cytoplasmic staining was present in tumor cells (any percentage of positive tumor cells) and negative when strong granular cytoplasmic staining was absent in tumor cells. EGFR and ALK expression was compared for biopsies and cell blocks.

**Statistical analysis**

Continuous data were summarized as mean ± SD, whereas discrete (categorical) in no. and %. Categorical groups were compared by Chi-square ($\chi^2$) test. The diagnostic accuracy (sensitivity and specificity) of EGFR and ALK protein expression in cell block was compared with the respective biopsy (gold standard). A two-tailed ($\alpha = 0.05$) $P$ value <0.05 was considered statistically significant. Analyses were performed on SPSS software (Windows version 17.0).

**Results**

In this study, 57 cell blocks were prepared among which 55 were prepared from aspirates from intrathoracic (IT) mass and 2 were prepared from bronchioalveolar lavage (BAL) fluid. Among these, 36 cell blocks were malignant, 5 of
which were small cell carcinoma, 30 were NSCLC, and 1 case was of Ewing’s sarcoma. Among 30 cases of NSCLC, 8 were squamous cell carcinoma, 18 were adenocarcinomas, 2 were undifferentiated carcinoma, 1 was adenosquamous carcinoma, and 1 was adenocarcinoma with neuroendocrine differentiation. There were 8 cases which were nonmalignant and 13 cell blocks were inadequate (these were given a diagnosis of malignancy on histology). The main reason for inadequacy was scant neoplastic cells, or hemorrhagic background or when cell block contains normal cells only. The study group comprises of 22 cases of NSCLC, which have adenocarcinomatous component. The clinical profile of the patients is given in Table 1.

The age range of adenocarcinoma patients at presentation was 27–70 years with a mean age of 55.1 years. The male to female ratio was 2.1:1. In order to determine the cell block efficacy to detect EGFR and ALK in NSCLC, the expression of IHC markers were compared for biopsy (gold standard) and cell block on 22 samples that have adenocarcinomatous component.

On biopsy, 20 (90.9%) cases were negative and 2 (9.1%) cases were positive for EGFR. Both the cases showed positivity for mutation-specific antibody for exon 19 (E746-A750) deletion. On cell block, similar results were obtained. We observed high correlation between results of biopsies and cell blocks for EGFR detection by IHC with a sensitivity of 100%. Results of IHC expression of ALK on biopsies and cell blocks also showed high correlation with 1 case positive for ALK both on biopsy and cell block and other 21 cases were negative for ALK antibody expression both on biopsies and cell blocks [Figures 1–3]. The sensitivity of ALK detection by IHC on cell block is 100% compared with biopsies.

**DISCUSSION**

This study was done to analyze the role of cell blocks in detecting mutant EGFR and EML4-ALK fusion protein in NSCLC and to determine the sensitivity and specificity of cell block versus formalin fixed paraffin embedded (FFPE) biopsies in the detection of these mutant proteins using IHC. In total, 55 samples of aspirates, where biopsies were also obtained from IT masses and 2 BAL fluids, were collected; and cell blocks were prepared. Twenty-two samples were identified as adenocarcinoma or nonsquamous morphology. These cases were further subjected to test EGFR and ALK by IHC.

Among 22 cases tested for mutant EGFR protein expression, 2 cases (9%) were positive for EGFR mutation. Both the cases showed exon 19 (E746-A750) deletions. The results are similar on biopsy and cell block in terms of positivity and negativity. Although EGFR detection by monoclonal specific antibody is not approved by Food and Drug Administration (FDA), it is being used in clinical situations and being evaluated for comparative results. Kato et al. compared the results of IHC and direct sequencing for EGFR and found a sensitivity of 82% and 75% and specificity of 100% and 97% in detecting exon 19 deletion and exon 21 point mutation, respectively.[8] Similarly, Nakamura et al. in a study observed a sensitivity of 92% and specificity of 100% in detecting exon 19 deletion

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**Table 1: The clinical profile of patients (n=22)**

| Parameters | n (%) |
|------------|-------|
| Sex        |       |
| Female     | 7 (31.8) |
| Male       | 15 (68.2) |
| Age (years) |       |
| <40        | 2 (9.1) |
| >40        | 20 (90.9) |
| Smoking habit |       |
| No         | 9 (40.9) |
| Yes        | 13 (59.1) |
| Complaints |       |
| Breathlessness | 21 (94.4) |
| Pain in chest | 10 (45.4) |
| HEMOPTYSIS | 7 (31.8) |
| Cough      | 4 (18.8) |

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**Figure 1:** (a and b) Biopsy from intrathoracic mass with positivity for CK7 (c) and TTF-1 (d) diagnosed as adenocarcinoma, (e and f) negative for epidermal growth factor receptor protein expression for clone specific immunohistochemistry, (g) negative for anaplastic lymphoma kinase protein expression using DSF3 clone, (h and i) cell block with clusters of tumor cells, (j) negative staining for anaplastic lymphoma kinase protein on cell block, (k and l) Negative staining with clone-specific epidermal growth factor receptor immunohistochemistry on cell block (a and h: haematoxylin and eosin x100, ×100, b and i: haematoxylin and eosin x100, c-g and j-l: DAB, ×100)
when IHC results were compared with PCR. IHC as method for testing EGFR mutation expression is cost effective, fast, and is available in many laboratories. The gold standard for detection of EGFR mutations is direct sequencing. However, this is expensive, needs high expertise, and requires least 20% of all DNA sample. DNA-based molecular techniques, for example, real-time PCR results, can be affected by degraded and poor nucleic acid quality obtained from FFPE material. IHC is, however, limited by being able to detect only the two commonest mutations in EGFR. If negative, the molecular methods are required to check for other mutations.

Among 22 cases which were tested for ALK IHC, only one case (4.54%) was positive on cell block. Results were concordant with the biopsy results for positive and all negative cases. The sensitivity was 100% and specificity was 100% with respect to biopsy. However, FISH is an FDA approved method for ALK detection and IHC is also a preferred method with a reported sensitivity and specificity of 67%–100% and 93%–100%, respectively (using FISH as the standard procedure). FISH is a costlier technique than IHC. Moreover, its interpretation also needs expertise, which is not a problem with IHC.

The most important advantage of cell blocks over conventional smears is that they can provide multiple sections that are suitable for further IHC and molecular studies. The method is simple and can be readily adaptable in routine hospital laboratory. Moreover, in many setups, the guided FNA procedures are not performed by pathologists, so spreading and drying artifacts is also an issue in direct smears, which the pathologists receive for diagnosis. In a study conducted by Pawar et al., the sensitivity and specificity of cell blocks in detecting lung malignancies were came out to be 96% and 92.59%, respectively, and cell block yielded six (12%) more cases as malignant, which were not diagnosed by conventional smears. But many a times, cell blocks require excesses material for the formation of pellet, which is not always feasible.

There are certain limitations of the current study, which includes the number of samples that are less. Although 57 cell blocks had been prepared, but only 22 cases were diagnosed as adenocarcinoma lung limiting the sample size. Thus, a larger elaborated study is needed to further support the results of the study.

The method used for detection of EML4–ALK fusion protein is IHC, which has replaced FISH in most centers; however, the use of EGFR mutant clones has currently not been accepted as a gold standard and does not cover the entire range of mutations. The study has used naïve pretreatment samples for analysis. No follow-ups or detection of posttreatment/chemotherapy changes have been recorded.
**Conclusion**

Molecular testing for EGFR and ALK is an essential part of the NSCLC diagnostic workup as targeted therapies are available against these predictive markers. Cell blocks are potential alternative for detection of these markers by IHC, whenever tissue sample is not available or inadequate. Significantly, the WHO 2015 classification of lung tumors has included cytology and small biopsies together in a clubbed classification.

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**Conflicts of interest**

There are no conflicts of interest.

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