Research Article

Adequacy of pleural fluid cytology for comprehensive molecular analysis of lung adenocarcinoma: Experience of a large health-care system

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ABSTRACT

Objectives: Pleural fluid evaluation is an effective modality for identifying actionable genetic mutations to guide therapy in lung carcinoma.Clinicians requesting molecular studies often send large volumes of fluid to be processed that is not possible or cost effective and is hence not standard of practice in most cytopathology laboratories. We wanted to establish the characteristics of an adequate specimen that would yield reliable results with current molecular testing platforms.

Material and Methods: A review of 500 malignant pleural effusions, from pulmonary and non-pulmonary sources, was undertaken over a 4-year period. Of these 44 cases (from 42 patients) that were positive for primary lung adenocarcinoma were included in the study. Molecular analysis was performed on 42 specimens. A complete next generation sequencing (NGS) panel was performed on 36 specimens. Individual testing for estimated glomerular filtration rate, KRAS, anaplastic lymphoma kinase, and ROS1 was performed on six specimens. The number of malignant cells and proportion of tumor to non-tumor nucleated cells (T:NT) on cell blocks was recorded as <20%, 20–50% and >50%.

Results: The minimum volume on which a complete NGS panel could be performed was 20 ml with cell count of 1000 and T:NT proportion of 20–50%. The minimum number of tumor cells required for successful molecular analysis for T:NT proportion of <20%, 20–50%, and >50% was 300, 250, and 170 cells, respectively.

Conclusion: We concluded that tumor cell proportion, rather than specimen volume, is of prime importance for determining the efficacy of pleural fluid for molecular studies. Evaluation of both absolute and relative numbers of tumor cells is critical for assessing the adequacy and predicting successful yield for molecular analysis.

Keywords: Pleural effusion, Lung adenocarcinoma, Molecular analysis

INTRODUCTION

About 15% patients with lung cancer present with pleural effusion,[1] which is classified as stage IV disease. The 5-year survival of patients with malignant pleural effusion (MPE) is very poor.[2] However, achievements in molecular diagnostics and precision medicine have brought about a significant impact in improving overall disease-free survival rates. Pleural fluid examination is a minimally invasive procedure in the diagnosis and work up of patients with malignant effusions.[3,4] Pleural effusion can be the primary manifestation of an occult or metastatic tumor in a small proportion of patients, or can be an early sign of disease recurrence.[5] The performance and diagnostic utility of this procedure correlates with the tumor volume in the lung and with the histologic subtype. Also compared with
needle biopsy, pleural fluid has a more robust diagnostic yield as the cell population is representative of a larger surface area. Diagnosis is based on cytomorphology, in conjunction with ancillary studies including immunohistochemistry and molecular studies, the results of which have direct therapeutic implications for the patient.

Pleural fluid evaluation is also an effective modality for identifying actionable genetic mutations to guide therapy. Up to 20% of lung adenocarcinomas contain activating mutations in epidermal growth factor receptor (EGFR), which can be effectively targeted by specific tyrosine kinase inhibitors like erlotinib. Similarly, up to 5% of lung adenocarcinomas harbor chromosomal rearrangements of the anaplastic lymphoma kinase (ALK) gene, with resultant overexpression of the ALK protein that can be effectively managed with ALK inhibitors like crizotinib. Although antibodies for immunohistochemical detection of these genetic alterations are available, they suffer from lack of sensitivity and specificity. Hence, the guidelines from National Comprehensive Cancer Network, the College of American Pathologists (CAP) and the International Association for the Study of Lung Cancer strongly recommend molecular detection of actionable EGFR mutations and ALK rearrangements in patients with non-small cell lung carcinomas (NSCLC).[8,9] This detection not only leads to initiation of specific therapy but also predicts an inferior response to immune checkpoint inhibitor therapies. The first case of EGFR mutation in NSCLC was detected by polymerase chain reaction (PCR) in a pleural effusion specimen by Huang et al. in 2005.[10] This was followed by other reports of therapy-responsive EGFR mutations.[11-13] In addition, the molecular detection of BRAF V600E mutation, MET amplification, NTRK1 gene fusions, and ERBB2 exon 20 insertion mutations has also been shown to be actionable genetic alterations, and their detection in pleural effusion fluid can also lead to specific and effective therapies. Thus, pleural fluid is an ideal specimen for formulating targeted therapies for individual patients.

We wanted to establish the characteristics of an adequate specimen that would yield reliable results with current molecular testing platforms. The main variables studied for this purpose were the specimen volume, and total as well as relative tumor cellularity, as assessed on a cell block preparation. We also wanted to determine a cutoff for a minimum volume of pleural fluid that would be diagnostically meaningful, and to study if increasing the volume of the specimen over 100 ml increases the diagnostic yield significantly.

MATERIAL AND METHODS

This retrospective study (slide and chart review) was conducted after approval by the Institutional Review Board. A review of 500 MPEs, from pulmonary and non-pulmonary sources, was undertaken over a 4-year period from January 2015 to December 2018.

Cell block preparation

50 ml of pleural fluid was transferred into a conical centrifuge tube and was centrifuged for 5 min at 2200 to 2500 RPM. The supernatant was discarded and the sediment was vortexed in equal amount of albumin-95% ethanol. This was again centrifuged for 5 min at 2500 RPM. The supernatant was discarded and the sediment carefully scooped with a spatula onto a piece of filter paper. The filter paper with the sediment was then placed in a tissue cassette and processed similar to a biopsy specimen in the histology laboratory.[14]

Cell block slides from 41 cases of primary lung adenocarcinoma were reviewed by two pathologists. Total number of malignant cells and background non-tumor nucleated cells in one section of the cell block was counted manually and was expressed as a ratio (tumor to non-tumor cells).

Molecular testing was performed by next generation sequencing (NGS) at Foundation Medicine on formalin fixed paraffin embedded cell block material (n = 35). This test detects genetic alterations in 324 genes directly or indirectly responsible for various stages of carcinogenesis. Actionable biomarkers particularly useful for guiding NSCLC therapy included in this panel are EGFR exon 19 deletions, EGFR exon 21 L858R mutation, EGFR exon 20 T790M mutation, ALK rearrangements, and BRAF V600E mutation. Some of the older cases (n = 6) underwent limited molecular testing. Testing for EGFR and KRAS mutations was performed by PCR amplification, followed by DNA sequencing. Testing for ALK and ROS1 rearrangements was performed by fluorescence in situ hybridization (FISH).

RESULTS

Five hundred cases of MPEs were reviewed that included predominantly metastatic carcinomas from various primaries, and few cases of sarcoma, lymphoma, melanoma, and mesothelioma. Of these 500 positive cases, 44 cases (from 42 patients) were positive for primary lung adenocarcinoma. Cell block slides were available for 41 of these 44 cases. The tissue of origin was determined using an appropriate immunocytochemistry panel that included CK7, CK20, p40, TTF-1, and Napsin A to confirm pulmonary origin. For cellular effusions with mixed morphotypes of cells, additional panels of immunostains were performed on serial cell block sections using a modified version of the SCIP approach (subtractive coordinate immunoreactivity pattern).[15,16]

Molecular analysis was performed on all 44 cases; however, only 41 of these cases that had a concurrent cell block were included in the study. A complete NGS was performed on 35 out of 41 specimens. Two of these 35 specimens were deemed inadequate due to low cellularity and did not yield conclusive results. Thus, a complete NGS result was available for 33 of 35 specimens (94.3%). Individual molecular testing was performed on six specimens from 2015 before NGS
testing was widely available or requested by the oncologists. The individual testing included PCR for detection of point mutations in EGFR and KRAS genes, and FISH for detection of rearrangements of ALK and ROS1 genes.

The minimum volume submitted was 10 ml and the maximum volume was 120 ml. The submitted volumes of the two inadequate specimens were 10 ml and 20 ml. The minimum volume of fluid that was diagnostically adequate, irrespective of the proportion of tumor cells, was 30 ml.

The minimum total cellularity, counted as number of tumor and non-tumor cells in the entire cell block, was 55 cells, and the maximum total cellularity was estimated to be over 10,000 cells. The total cellularity of the two inadequate specimens was 55 cells and 300 cells. We divided our specimens into four groups based on the total cellularity: 1–100 cells, 101–500 cells, 501–1000 cells, and more than 1000 cells.

The proportion of tumor cells in the cell block versus other benign non tumor nucleated cells was assessed as tumor to non-tumor ratio (T:NT). The specimens were divided into three groups for analysis based on the proportion of tumor cells being <20%, between 20% and 50%, and more than 50% of total nucleated cells.

Of the two inadequate specimens, the one with a total cellularity of 55 cells had a T:NT proportion of more than 50%, while the specimen with a total cellularity of 300 cells had <20% tumor cells.

The minimum number of malignant cells recommended for molecular testing, irrespective of the tumor cell proportion, is 500 cells. However, a significant proportion of our specimens (n = 18) had a total cellularity between 101 and 500 cells, but these were subjected to molecular analyses, anyway. Of these 18 specimens, only one specimen was inadequate that had a proportion of tumor cells of <20%. Although our specimen numbers were small, the proportion of tumor cells, rather than the total cellularity, seems to be more important for specimen adequacy. The distribution of total cellularity and tumor cell proportion across the specimens is shown in Table 1. Correlation of volume with cellularity is shown in Table 2.

**DISCUSSION**

The role of pleural fluid cytology in the diagnosis and staging of primary lung adenocarcinoma cannot be underestimated. It is a relatively non-invasive procedure which reduces healthcare costs, guides tumor staging and allows for early and targeted therapeutic interventions for individual patients, which is the sine qua non of “personalized” or “precision” medicine. Our study represents an institutional experience of studying the adequacy and other characteristics of pleural effusion fluids for the molecular diagnostics in lung adenocarcinoma.

A few studies have been performed to investigate the adequate volume of pleural fluid for a morphologic diagnosis of malignancy. Thomas et al. determined that a minimum volume of 25 ml is required for a positive diagnosis, and that volumes over 50 ml do not significantly increase the diagnostic yield. This volume was utilized for morphologic, histochemical, and immunohistochemical assessment. However, molecular studies were not performed on these specimens.

Similar studies by Ong et al. revealed that the diagnostic yield of pleural fluid increased with examination of multiple specimens, and with the concurrent examination of a pleural biopsy specimen. However, this study did not specifically address the ideal minimum volume for examination. Similar to the study by Thomas et al., molecular studies were not performed.

In the most recent collaborative guidelines issued by the CAP, Roy-Chowdhuri et al. performed an analysis of seven studies that attempted to report on the ideal volume of pleural fluid to avoid a false negative diagnosis. However, similar to the studies by Thomas et al., and Ong et al., these studies emphasized on the morphologic diagnosis of malignancy, without taking into consideration ancillary molecular testing. In one of these studies, a volume as low as 10 ml was considered adequate for a solely morphologic documentation of malignant cells. Two of the studies in their analysis concluded that the positive yield of a fluid did
not significantly increase with collection volumes exceeding 50–75 ml.[21, 22] Thus, though the recent CAP guidelines opine that “the proceduralist should send as much fluid volume as reasonably attainable,” they could not definitively recommend a maximum volume for examination. A minimum volume of 50–100 ml was suggested, but not recommended.

MPEs have been used in several studies to assess oncogenic driver mutations in pulmonary adenocarcinoma. Complete NGS was performed on 33 specimens. Multiple studies have performed oncogene analysis in pleural effusions; however, they used PCR based platforms for somatic mutation profiling.[1, 2] However, these studies did not determine the adequacy criteria of the submitted pleural fluid specimen for molecular testing. We performed molecular testing for detection of point mutations in EGFR and KRAS genes, and of rearrangements of ALK and ROS1 genes in six cases from 2015. However, targeted NGS testing works as a unifying platform for the detection of multiple genetic alterations at the same time and reveals several more potentially actionable mutations for therapeutic targeting.[23, 24]

The volume of pleural fluid submitted by clinicians ranged from a minimum of 10 ml to a maximum of 120 ml. The minimum cellularity (referring to one histologic section of cellblock) was 55 cells, and the maximum cellularity was over 10,000. All our specimens with a volume of over 30 ml, a cellularity of over 100 cells per cell block section, and a tumor cell proportion of over 20%, were adequate.

MPEs tend to comprise varying amounts of benign reactive cells, namely, mesothelial cells, histiocytes, and leukocytes. If the proportion of these cells is significantly more than that of malignant cells, molecular methods are likely to fail or give erroneous results as a result of intermixing of benign and malignant DNA. Thus, we propose that counting the tumor and non-tumor cells in one section of the cellblock and estimating a proportion is of importance to avoid a false negative result on NGS platforms. Cell blocks from a tumor cell proportion of <20% should not be forwarded for molecular studies and a repeat specimen can be requested.

We examined the major possible determinants influencing the specimen adequacy for molecular studies, including volume, overall cellularity, and tumor percentage. MPE volume does not have a direct correlation with total cellularity or tumor cell content. Furthermore, the effusion volume of cases that were deemed inadequate for molecular studies was not significantly different from the volume of those cases that were considered adequate and underwent full molecular profiling.

CONCLUSION

We conclude that of the three major determinants, effusion volume is the least important. In paucicellular specimens lacking adequate absolute numbers of tumor cells, or in cellular specimens with a predominance of non-tumor cells, increasing the submitted volume of effusion fluid to more than 100 ml will not lead to an increase in molecular diagnostic yield.

COMPETING INTERESTS STATEMENT BY ALL AUTHORS

The authors declare that they have no competing interests.

AUTHORSHIP STATEMENT BY ALL AUTHORS

SDD collected and analyzed data and prepared and revised the manuscript. KC helped collect and analyze data. SS helped collect data. BC helped prepare the manuscript. PK, SK, and CG helped review and edit the manuscript. KD conceptualized the project, and helped collecting and analyzing data, and final reviewing and editing the manuscript. All authors have read and approved the final manuscript.

ETHICS STATEMENT BY ALL AUTHORS

This study was conducted with approval from the Institutional Review Board of the institution associated with this study. Authors take responsibility to maintain relevant documentation in this respect.

LIST OF ABBREVIATIONS (In alphabetic order)

ALK: Anaplastic lymphoma kinase
BRAF: Rapidly accelerated fibrosarcoma-B
CAP: College of American Pathologists
EGFR: Epidermal growth factor receptor
ERBB2: Avian erythroblastosis oncogene-B
FISH: Fluorescence in situ hybridization
KRAS: Kirsten rat sarcoma virus
MPE: Malignant pleural effusion
NGS: Next generation sequencing
NSCLC: Non-small cell lung carcinoma
PCR: Polymerase chain reaction
RPM: Rotations per minute
T:NT: Tumor : non-tumor.

EDITORIAL/PEER-REVIEW STATEMENT

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REFERENCES

1. Naito T, Satoh H, Ishikawa H, Yamashita YT, Kamma H, Takahashi H, et al. Pleural effusion as a significant prognostic factor in non-small cell lung cancer. Anticancer Res 1997;17:4743-6.
2. Goldstraw P, Crowley J, Chansky K, Giroux DJ, Groome PA,
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Rami-Porta R, et al. The IASLC lung cancer staging project: Proposals for the revision of the TNM stage groupings in the forthcoming (seventh) edition of the TNM classification of malignant tumours. J Thorac Oncol 2007;2:706-14.

3. Loddenkemper R, Boutin C. Thoracoscopy: Present diagnostic and therapeutic indications. Eur Respir J 1999;6:1544-55.

4. Fenton KN, Richardson JD. Diagnosis and management of malignant pleural effusions. Am J Surg 1995;170:69-74.

5. Sundling KE, Cibas ES. Ancillary studies in pleural, pericardial, and peritoneal effusion cytology. Cancer Cytopathol 2018;126 Suppl 8:590-8.

6. Frist B, Kahan AV, Koss LG. Comparison of the diagnostic values of biopsies of the pleura and cytologic evaluation of pleural fluids. Am J Clin Pathol 1979;72:48-51.

7. Shidham VB, Atkinson BF, editors. Approach to diagnostic cytopathology of effusions. In: Cytopathologic Diagnosis of Serous Fluids. 1st ed., Ch. 3. Amsterdam: Elsevier, WB Saunders Company; 2007. p. 31-42.

8. The National Comprehensive Cancer Network. Non-Small Cell Lung Cancer. Fort Washington, PA: NCCN Clinical Practice Guidelines in Oncology; 2015.

9. Lindeman NI, Cagle PT, Beasley MB, Dacic S, Giacccone G, et al. Molecular testing guideline for selection of lung cancer patients for EGFR and ALK tyrosine kinase inhibitors: Guideline from the college of American pathologists, international association for the study of lung cancer, and association for molecular pathology. Arch Pathol Lab Med 2013;137:828-60.

10. Huang MJ, Lim KH, Tzen CY, Hsu HS, Yen Y, Huang BS. EGFR mutations in malignant pleural effusion of non-small cell lung cancer: A case report. Lung Cancer 2005;49:413-5.

11. Soh J, Toyoooka S, Aoe K, Asano H, Ichihara S, Katayama H, et al. Usefulness of EGFR mutation screening in pleural fluid to predict the clinical outcome of gefitinib treated patients with lung cancer. Int J Cancer 2006;119:2353-8.

12. Tsai TH, Wu SG, Chang YL, Wu CT, Tsai MF, Wei PF, et al. Effusion immunochemistry as an alternative approach for the selection of first-line targeted therapy in advanced lung adenocarcinoma. J Thorac Oncol 2012;7:993-1000.

13. Soh J, Toyoooka S, Ichihara S, Suehisa H, Kobayashi N, Ito S, et al. EGFR volume of pleural fluid required for accurate volume of pleural fluid required for accurate predicts tumor responsiveness and resistance to gefitinib. Lung Cancer 2007;56:445-8.

14. Shidham VB. CellBlockistry: Chemistry and art of cell-block making—a detailed review of various historical options with recent advances. Cytojournal 2019;16:12.

15. Shidham VB, Atkinson BF, editors. Immunochemistry of effusion fluids: Introduction to the SCIP approach. In: Cytopathologic Diagnosis of Serous Fluids. 1st ed., Ch. 5. Amsterdam: Elsevier, WB Saunders Company; 2007. p. 55-78.

16. Shidham VB. Cell-blocks and other ancillary studies (including molecular genetic tests and proteomics). Cytojournal 2021;18:4.

17. Thomas SC, Davidson LR, McKeen ME. An investigation of adequate volume for the diagnosis of malignancy in pleural fluids. Cytopathology 2011;22:179-83.

18. Ong KC, Indumathi V, Poh WT, Ong YY. The diagnostic yield of pleural fluid cytology in malignant pleural effusions. Singapore Med J 2000;41:19-23.

19. Roy-Chowdhuri S, Dacic S, Ghofrani M, Illei PB, Layfield LJ, Lee C, et al. Collection and handling of thoracic small biopsy and cytology specimens for ancillary studies: Guideline from the college of American pathologists in collaboration with the American college of chest physicians, association for molecular pathology, American society of cytopathology, American thoracic society, pulmonary pathology society, papanicolaou society of cytopathology, society of interventional radiology, and society of thoracic radiology. Arch Pathol Lab Med 2020; DOI: 10.5858/arpa.2020-0119-CP.

20. Buckley O, Benfayed W, Geoghegan T, Persaud T, Jeffers M, Khosa F, et al. Thoracoscopy for potential malignancy: Does volume matter? Hong Kong J Radiol 2008;11:72-5.

21. Rooper LM, Ali SZ, Olson MT. A minimal fluid volume of 75 mL is needed to ensure adequacy in a pleural effusion: A retrospective analysis of 2540 cases. Cancer Cytopathol 2014;122:657-65.

22. Wu H, Khosla R, Rohatgi PK, Chauhan SS, Paal E, Chen W. The minimum volume of pleural fluid required to diagnose malignant pleural effusion: A retrospective study. Lung India 2017;34:34-7.

23. Yeo CD, Kim JW, Kim KH, Ha JH, Rhee CK, Kim SJ, et al. 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. Lung Cancer 2013;81:207-12.

24. Liu D, Lu Y, Hu Z, Wu N, Nie X, Xia Y, et al. Malignant pleural effusion supernatants are substitutes for metastatic pleural tumor tissues in EGFR mutation test in patients with advanced lung adenocarcinoma. PLoS One 2014;9:e89946.

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