ABSTRACT

Introduction: Endobronchial ultrasound-guided transbronchial needle aspiration (EBUS TBNA) is an important means of obtaining a tissue for advanced lung cancer. Optimizing the EBUS TBNA needling technique is important to maintain procedural simplicity and maximize sample quality for emerging molecular diagnostics.

Methods: We prospectively explored three versus 10 agitations of the needle in sequential passes into the lymph node using separate needles. Resulting Diff-Quik cytology smears were quantitatively assessed using microscopic (tumor cellularity, abundance scores, erythrocyte contamination) and DNA yields. Microscopy was reported by two cytopathologists, and an inter-rater assessment was made by four additional cytopathologists.

Results: In 86 patients confirmed as having malignant disease by EBUS TBNA (45 males, 41 females), a mean of 5.3 smears were made per patient with a total of 459 smears scored by pathologists and 168 paired smears extracted for DNA. There was no significant difference between three versus 10 agitations for smear cellularity ($p = 0.44$), DNA yield ($p = 0.84$), or DNA integrity ($p = 0.20$), but there was significantly less contamination by erythrocytes from three agitations (chi-square $p = 0.008$). There was significantly more DNA in the first pass into the node using three agitations than with other passes and with...
10 agitations (pass x agitations interaction, p = 0.031). Reviewing pathologists correctly classified smears as more than or equal to 25% cellularity 86.3% of the time (κ = 0.63 [95% confidence interval: 0.55–0.71]).

Conclusions: Three agitations are noninferior to 10 agitations for overall abundance of malignant cells and DNA content on smears. A smear with adequate DNA for panel sequencing could almost always be made with the first needle pass using three agitations.

Keywords: Cytology smears; Molecular testing; Lung cancer; EBUS TBNA

Introduction

Molecular assessment of advanced lung cancer samples from endobronchial ultrasound-guided transbronchial needle aspiration (EBUS TBNA) is now standard of care.1,2 Although tissue diagnosis sensitivity is still high for EBUS TBNA, including single-gene molecular testing, there remains a baseline false-negative testing rate for molecular analysis owing to poor sample quality or tissue malignant cell content.3–11

Published guidelines state that up to three additional needle passes can improve the chances of obtaining adequate material for molecular testing.1,12 A range of other needling methodologies, including needle size, use of suction, and the stylet, have been reported,1 yet the problem of insufficient tissue for molecular testing continues.

One aspect that has not been studied in any detail is the total number of agitations of the needle within the lymph node for each pass. Typical numbers of agitations in clinical practice would be 10 to 20.1,13,14 In cytology lymph node for each pass. Typical numbers of agitations the total number of agitations of the needle within the continues.

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The rationale for this is to minimize tissue injury and cellular disruption. In our pilot study,15 we noted that simply taking more samples by increasing passes of a needle did not necessarily result in greater DNA yield; some cases had good DNA amounts with one pass and others had poor yield with five passes. Another study exploring the number of EBUS TBNA needle agitations (10 versus 20) to diagnose sarcoidosis found an equivalence in tissue diagnosis when using 10 needle agitations.17

We hypothesized that there could be at least equivalent results with as few as three agitations versus the more often used 10 agitations of the needle within the node. A simpler procedure with less trauma to the node through fewer agitations would be desirable. A secondary end point was to explore which parts of the pushed-out needle content gave the most abundant material. Traditionally, the first drops of the needle material are used to make the Diff-Quik smears, whereas we previously reported that the last drops of the needle content gave abundant smears.16,18 The last drops out of the needle may contain more malignant cells because more cells enter the needle with the very initial passage of the needle into the node.

Diff-Quik smears are an excellent source of diagnostic material for molecular analysis.19 Smears were therefore the source of study comparisons but also reveal the feasibility of the methodology changes for smear use in clinical practice. Quantitative, paired analyses were undertaken using pathologist reporting of smears for malignant cell yields together with DNA yields obtained from these smears. Inter-rater pathologist agreement was undertaken for two reasons—first, the reporting reference pathologists were not blinded to the number of needle agitations, and, second, because additional data on this topic are useful in building future recommendations on the basis of these results.

Materials and Methods

Patients

The study was performed at three tertiary referral hospitals, which are as follows: Royal Brisbane and Women’s Hospital, Sunshine Coast University Hospital, and Gold Coast University Hospital. Patients were those presenting with a high pretest likelihood of a malignant mediastinal or hilar lymph node. Exclusion criteria were patients unsafe for a bronchoscopic procedure and those unable to give informed consent. Institutional review board from the Royal Brisbane and Women’s Hospital granted approval for the study (HREC/17/QRBW/301), ratified by The University of Queensland (2018/HE001615), and all patients gave written informed consent.

Bronchoscopic Procedures

Procedures were performed either under conscious sedation or general anesthetic. Olympus 21-gauge Vizishot 1, NA-201SX-4021 needles were used.

In addition to the study samples as described subsequently, all patients had Papanicolaou (PAP)-stained slides collected after rapid onsite evaluation (ROSE) positivity, and components of each needle pass contributed to a saline pot for subsequent formalin fixation, paraffin embedding, and cell-block creation. A maximum total number of five passes were made to ensure sufficient material for the cell block for diagnosis and
standard-of-care mutation testing. Cell blocks were not analyzed as part of the study cellularity descriptions, only the Diff-Quik smears, and the creation of these smears was as follows: two separate needles were used, marked for three or 10 agitations, and individual labeled smears were made from these needles. A randomized order was used for first needle agitation number (three or 10). All procedures had ROSE. Needle movement combined the fanning and slow-pull techniques. Suction was used unless the first aspirate was excessively bloody. For the secondary end point, two smears were made: the “first drops” smears were from the first material appearing on reinsertion of the stylet into the needle. Then, almost all the remainder of the material was pushed into the cell-block pot (only used for standard-of-care testing) and the very “last drops” were then extruded onto a second smear.

**Microscopy Assessment**

Diff-Quik smears were scored as previously described. Two experienced pathologists (M.S. and L.N.) reported the percentage cellularity and the overall estimated abundance (number) of malignant cells. Percentage was estimated using at least 10 high-power fields and averaging the respective percentage values and reported as (0, <25%, 25%–50%, 50%–75%, and >75%), recorded as 0, 1, 2, 3, and 4 cellularity scores. Quartiles of abundance of malignant cells were reported ranging from 0 (no malignant cells), <1+ (less than approximately 100 cells), 1+ (up to 1000 cells), 2+ (up to 2000 cells), 3+ (up to 4000 cells), to 4+ (>approximately 4000 cells).

Interrater agreement of the slide reporting system was undertaken by four different expert pathologists/cytologists (D.G., L.W., K.C., and S.S.; Supplementary Table 1). They initially trained on a set of 10 open-label slides scored by the reference pathologists. They then reported on a subset of 100 smears, each independently reporting percent malignant cells and abundance. They were blinded to agitation number and reference pathologists’ scores.

Erythrocyte contamination of smears was categorized subjectively as negligible—mild, moderate, or extensive. This analysis was performed on only the first passes of the needle into the lymph node to reduce the confounding effect of subsequent passes.

**Diff-Quik Slide DNA Extraction**

See Supplementary Material for slide scanning and extraction methods, as previously reported. Samples for extraction were selected sequentially, selected as pairs where possible (including two pairs if possible in a case), except where smear preparation was deemed unsatisfactory. In some cases, if a third pass was performed, an additional smear was selected.

**Statistics**

In our previous study, we found a SD of 1970 ng for smear DNA analysis when comparing two data sets (smears and matched cell blocks), where the absolute difference was 1000 ng. If there is truly no difference in DNA yield between three and 10 agitations, then 134 samples are required to be 90% sure that the lower limit of a one-sided 95% confidence interval (CI) was above the noninferiority limit of -1000. Each biopsy yielded multiple smears to be assessed corresponding to multiple passes, randomized to involve three or 10 agitations within the lymph node. Thus, all analyses of smears were based on repeated measures linear models with tissue sample as the random effect. Percent cells, abundance, DNA content, and integrity were analyzed on a normal scale, whereas DNA yield was analyzed on a log-normal scale, and these results presented as geometric means and 95% CIs. Results were also analyzed by needle pass (entry of the needle from the bronchus) into the lymph node, whether first entry, second, third, or fourth passes. This was done to give potentially the most unaffected results, because subsequent pass yields might be affected by earlier passes disrupting nodal tissue. An interaction model was developed to combine results of DNA yield for the pass into the node (pass 1 versus pass 2) and the number of agitations (three versus 10).

Interrater agreement of pathologists’ assessment of percentage and overall abundance of malignant cells was displayed using heat maps of reference ratings versus comparator ratings. Agreement was assessed using weighted Kappas with linear weights to penalize for larger discrepancies. Asymmetry of disagreements was assessed using Bowker’s test.

**Results**

A total of 101 patients were consented for the study; of these, 15 patients had benign results on EBUS TBNA, and these were not studied further. Table 1 details the remaining 86 patients diagnosed with having malignancy which formed the cohort for analysis. Pathologists reviewed 454 Diff-Quik smears and a subset of 168 smears including 80 pairs of three versus 10 agitations were used for DNA extraction and analysis.

**Malignant Cell Content of Smears**

Figure 1 illustrates differences in malignant tumor cell yield between three and 10 agitations of the node and between the first and last drops from the needle. Figure 1A shows results for cellularity and Figure 1B for abundance. Overall, there was no significant difference
observed for cellularity or abundance of malignant cells between three versus 10 agitations \((p = 0.29\) and 0.17, respectively). Nevertheless, the last drop out the needle deposited on the slide had higher cellularity \((p = 0.0138)\) and higher abundance score \((p = 0.0108)\) than the first drop (Fig. 1). Smears derived after 10 needle agitations contained moderate or large contamination by erythrocytes significantly more often than slides from three agitations (Pearson’s chi-square \(p = 0.008;\) Table 2).

**DNA Content of Smears**

Median DNA yield was 348 ng (range: 48–3650 ng), with only one slide yielding less than 50 ng and only 17 (10%) yielding less than 100 ng DNA (Supplementary Fig. 1). Significantly greater DNA yield was obtained for small cell carcinoma compared with other histologic types \((p = 0.0009)\), with no significant differences between any of the other diagnosis types (Supplementary Fig. 2).

There were no significant differences in DNA yield from smears derived from three versus 10 agitations \((p = 0.44)\) or between first and last drop slides \((p = 0.32)\) (Fig. 2, Supplementary Fig. 1 and Supplementary Table 2). When analyzing all needle passes into the lymph node, the difference between the first and last drop smears seems larger with 10 agitations than with three agitations, but the interaction is not significant \((p = 0.36;\) Fig. 2A). There was also no statistical difference in DNA yield for three versus 10 agitations analyzing only the first pass into the node \((p = 0.27;\) Fig. 2B). When comparing first needle passes with subsequent passes (first versus second pass), first passes with three agitations had significantly more DNA yield than second passes with three agitations \((p = 0.0310;\) Fig. 2C). An interaction model was developed which revealed that there was significantly more DNA in the first entry into the node using three agitations, but the interaction is not significant \((p = 0.36;\) Supplementary Fig. 3A). There was also no significant difference in the DNA integrity between three versus 10 agitations \((p = 0.20)\) or between the first and last drops of aspirate \((p = 0.35)\), and the interaction was not significant \((p = 0.14)\) (Supplementary Fig. 3A). Furthermore, to minimize the potential impact of subsequent needle passes on DNA quality, we directly compared DNA integrity between three versus 10 agitations from the first pass of the needle only (Supplementary Fig. 3B), which revealed no significant difference. Overall, 14 smears (seven from three agitations and seven from 10 agitations) extracted for DNA had no malignant cells on the pathologist smear review. These smears had DNA yields ranging from 93 ng to 255 ng.

| Table 1. Demographic Data and Number of Diff-Quik Slides for 86 Patients Confirmed as Malignancy at the EBUS TBNA Procedure |
|-------------------|---|
| Variable                       | Number |
| Sex               |   |
| Male             | 45  |
| Female           | 41  |
| Age              |   |
| Mean (SD)        | 66.0 (8.9) |
| Median           | 67  |
| Range            | 43–86 |
| Final tissue diagnosis by EBUS TBNA |   |
| NSCLC            | 21  |
| Adenocarcinoma   | 19  |
| Squamous cell carcinoma | 12  |
| Small cell carcinoma | 24  |
| Metastatic other sites | 10  |
| Node sampled     |   |
| 4L               | 13  |
| 4R               | 14  |
| 7                | 32  |
| 10R/L            | 3   |
| 11/12/13 R       | 15  |
| 11L              | 6   |
| Hilar mass       | 3   |
| Number of slides per patient |   |
| Mean (SD)        | 5.3 (2.8) |
| Number of slides per pass |   |
| 2 slides/pass    | 59 patients |
| 1 slide/pass     | 27 patients |
| Statistical comparisons |   |
| Number of agitations comparison (3 vs. 10) |   |
| Paired slides    | 229 |
| Total slides     | 459 |
| First drop vs. last drop comparison |   |
| Paired slides    | 186 |
| Total slides     | 372 |
| DNA yield and integrity |   |
| Paired slides    | 80  |
| Total slides     | 168 |

EBUS TBNA, endobronchial ultrasound-guided transbronchial needle aspiration; L, left; R, right.

**Practical Findings**

Regarding three agitations alone, 136 of 227 (60%) smears had either more than 25% cellularity or more than 1000 cells on abundance, thresholds which would typically be used for smear selection. Nevertheless, from the objective assessment of DNA yield, if smears with no malignant cells are excluded (as would occur at ROSE), then 100% and 88% of the three agitation smears yielded more than 50 ng and more than 100 ng of DNA, respectively. Moreover, there was no difference between three and 10 agitations in the number of cases that yielded various DNA threshold levels (>200, 500, 1000, 2000 ng; Supplementary Table 2) which might affect suitability of slides for different types of molecular testing (e.g., panel testing, whole exome/genome sequencing).
Inter-Rater Agreement of Smear Scoring by Pathologists

The implementability of the smear slide scoring was tested by assessing the inter-rate agreement between four additional cytopathologists. The distribution of the consensus scores for the percentage of malignant cells (Fig. 3) and for the overall abundance of malignant cells (Fig. 4) for 100 slides as reported by the index pathologists (Supplementary Table 3) was compared with the scores reported independently by the four invited pathologists. Figures 3A and 4A show the actual slide score comparisons; Figures 3B and 4B show the percentage of concordance and discordance in each score for malignant cells against the reference score.

We calculated the linear weighted kappa to assess agreement, penalizing disagreement by the absolute difference in scores. A $\kappa$ of 0 reflects lack of agreement, and $\kappa$ of 1.0 reflects perfect agreement. For the percentage of malignant cell scores (Fig. 3) $\kappa$ of 0.57 (95% CI: 0.51–0.63) and for abundance of malignant cell scores (Fig. 4) $\kappa$ of 0.53 (95% CI: 0.48–0.59), indicating moderately strong agreement. The disagreement was asymmetric (Bowker’s test $p < 0.0001$ for both percent and abundance scores).

The invited “comparison” reviewers more often overestimated than underestimated, relative to the reference reviewers, and the categories with lower reproducibility being in the middle categories of 25% to 50% and 50% to 75%. We therefore evaluated the ability of the four invited reviewers to classify the slides in a more simplistic stratification on the basis of the slides harboring less than 25% or more than or equal to 25% malignant cells on the smears versus the reference reviewers (Fig. 3C). We undertook this additional analysis because this cutoff is likely to be a useful indicator of whether the slide meets criteria to be used for gene panel molecular testing. The misclassification rate was 21.8% (95% CI: 18.0%–26.1%), with a $\kappa$ of 0.63 (95% CI: 0.55–0.71) across the entire set for this threshold of 25% cellularity (Fig. 3C). Therefore, there was an improvement in performance by limiting the reporting by the reviewing pathologists to just two categories.

Results of Standard-of-Care Cell Blocks and PAP Smears

From 86 malignant cases, cell blocks contained diagnostic malignant cells in 78 cases; the remaining eighth cases were diagnosed by PAP smear. Formal scoring of the cell-block sections was undertaken using the same abundance scoring system as was used for the Diff-Quik smears in 67 cases. Abundance scores of 0 (nil), 1, 2, 3,
and 4 (highly abundant) were observed in 9%, 15%, 31%, 24%, and 21% of cases, respectively. This overall distribution of abundance of malignant cells in the cell blocks was similar to that observed in the smears.

Discussion

Two international societies\(^1,14\) have called for more research to delineate the utility of a range of technical aspects of the EBUS TBNA procedure. The effect of number of needle agitations within the node has been unknown until now. Needle agitations back and forth within the lymph node bring cellular material into the lumen of the needle, helped by either only capillary action or application of suction.\(^13\) Merely taking more agitations would not necessarily help if capillary action is the dominant mechanism. Indeed, in other sites, "capillary sampling" with cytology needle aspirates has been reported, whereby just the needle with no suction is equivalent to aspirates with suction.\(^15,21,22\)

In line with this, we reveal for the first time that needle technique affects the cytologic quality of the smears and the malignant cell and DNA yield. We revealed the following:

1. Three agitations are noninferior to 10 agitations for overall abundance of malignant cells and the subsequent DNA content.
2. More malignant cells were present in the smears (by microscopy) from the last drops of the needle.
3. The best combination of technical factors for higher tumor content is three agitations with the first pass of the needle into the node.
4. There is no real benefit to using a higher number of needle agitations to obtain higher DNA yields.

Collectively, we conclude that proceduralists can expect that a smear made from the first needle pass, using three agitations, and using the last drop from the needle will yield more cellularity on the smear for most effective pathologist interpretation of cancer in the procedure room, including the highest DNA yield for subsequent molecular testing.

Proceduralists can therefore be confident of having adequate smear samples for molecular testing very
quickly within the EBUS TBNA procedure. Overall, across multiple needle passes, using three agitations per pass would also shorten and simplify the procedure. Other benefits include significantly fewer instances of blood-contaminated smears to improve smear interpretation. EBUS TBNA is a very safe procedure, but adverse events occur, in which hemorrhage is the most often reported (0.7%). Thus, potentially fewer agitations might reduce this risk even further.

Dhooria et al. reported needle agitations in lymph nodes in sarcoidosis. From 131 confirmed cases, there was no difference in the diagnostic yield of 10 agitations (52 of 65, 80.0%) and 20 agitations (57 of 68, 83.8%). Our study has extended these results by going much lower with agitation number.

Diff-Quik smears are an excellent source of DNA, but formalin-fixed, paraffin-embedded cell-block extractions are also most often used. We could not analyze the impact of agitations on cell-block yield; however, we believe that the smear results can reasonably be extrapolated to the cell-block pot because we analyzed both parts of the needle aspirate content (the first and last drops) and found adequate cellularity in both, that is throughout the needle content.

The microscopy-based scoring of cellularity is important to determine whether sufficient cells are present.
obtained for diagnosis and molecular testing. Scoring is, despite best practice, partly subjective and finding agreement is therefore important. Good agreement from invited pathologists using the scoring system supported the results of our reference pathologists on the unblinded smears. This was enhanced when a two-tiered classification on the basis of a threshold of 25% malignant cellularity was used, reflecting a putative cutoff for success in detecting actionable mutations in panel sequencing. The agreement compared favorably to previous reports of pathologist inter-rater agreements in pulmonary pathology.24–26 Natali et al.27 and Nakajima et al.28 reported good agreement between pathologist and pulmonologist on ROSE smears using a similar three-class scoring of malignant cell content, low (<10%), medium (10%–50%), and high (>50%) (κ = 0.78).

DNA yield is also an important surrogate for predicting sequencing success. A recent report evaluated cytology preparations for DNA yield and genomic sequencing in 207 samples, including EBUS TBNA.29 Targeted gene panel sequencing was possible in more than 70% of cases. We previously revealed successful sequencing from DNA smears,11 and as little as 10 ng is required for some sequencing platforms using smears.24,30 Here, 88% and 66% of smears yielded more than 100 ng and more than 200 ng DNA, respectively, more realistic limits to allow for excellent DNA quality control before sequencing.

DNA results and smear cellularity were higher in small cell carcinoma versus other histologic types. These data are new to our knowledge. This not only reveals why some adenocarcinoma samples have poor yield for molecular testing31 but also reveals the future potential to extend molecular testing on small cell carcinoma smears.32

In conclusion, proceduralists can anticipate that the first pass of the needle using three agitations will give the best DNA yield on the smear. The last drops from the needle gives more smear cellularity. Cytopathologic analysis of the slide based on malignant cellularity and overall abundance is reproducible among pathologists and may represent a quick and effective means of identifying slides for DNA extraction and molecular testing.

CRediT Authorship Contribution

David Fielding: Conceptualization, Methodology, Investigation, Resources, Writing—Original Draft, Writing—Review and Editing, Project administration, Funding acquisition.

Andrew J. Dalley: Formal analysis, Resources, Writing—Review and Editing.

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Figure 4. Inter-rater agreement for the scoring of the malignant cell abundance scores on Diff-Quik slides. Heat maps display the results for all 100 slides with comparisons made across 400 reviews (100 slides × four invited reviewers) versus the reference pathologist scores. The diagonal boxes illustrate the agreement for each of the quartiles scored, and the off-diagonals the discordances. (A) The actual slide score comparisons. (B) The percentage of concordance and discordance in each score for malignant cells against the reference score.
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Supplementary Data
Note: To access the supplementary material accompanying this article, visit the online version of the JTO Clinical and Research Reports at www.jtocrr.org and at https://doi.org/10.1016/j.jtocrr.2022.100403.

Acknowledgments
This research was funded by grants from The 2017 Priority-Driven Collaborative Cancer Research Scheme, funded by Cancer Australia, Grant number 1147067; the Cancer Council of Queensland, Grant number 1147067; the Australian New Zealand Interventional Pulmonology Group grants (supported by Olympus Australia) 2018; and the Royal Brisbane and Women’s Hospital Foundation Smart Grants. The authors thank the patients who consented to be involved in this study and all nursing staff at The Royal Brisbane & Women’s Hospital, Gold Coast University Hospital, and Sunshine Coast University Hospital Bronchoscopy departments.

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