Destaining of Diff-Quik stained cytologic smears is not necessary for the detection of anaplastic lymphoma kinase gene rearrangement in lung adenocarcinoma by fluorescence in situ hybridization

ABSTRACT

Background: Anaplastic lymphoma kinase (ALK) gene rearrangement analysis by fluorescence in situ hybridization (FISH) is one of the standard molecular tests for targeted therapy of lung adenocarcinoma. However, insufficient cell block cellularity may impede molecular testing. A recent study showed that Diff-Quik (DQ) stained cytology smear is suitable for ALK by FISH.

Aims: The aim of our study was to observe the impact of destaining intervals on the quality of FISH signals and determine if DQ smears without destaining would allow FISH analysis.

Materials and Methods: Thirty-five DQ smears from 27 cases of lung adenocarcinoma were analyzed for ALK gene rearrangement by FISH. Twenty three DQ smears were destained for different intervals, including 30 s (13 cases), 1 min (6 cases), or 2 min (4 cases). Twelve DQ smears were not subjected to destaining. For further validation, FISH signals in 8 smears and 6 cell blocks were compared with the paired destained DQ smears. The signal quality was semi-quantified and analyzed with Chi-squared test.

Results: Of the total 27 selected cases, three (11%) were positive for ALK gene rearrangement, whereas 24 (89%) were negative. FISH signal was satisfactory in all DQ smears. There was no significant difference in the quality of signal among smears with different destaining intervals ($P = 0.55$) or between smears with and without destaining ($P = 0.41$). DQ smears without destaining showed identical FISH results and similar or better signals as compared with paired destained smears and cell blocks in all cases.

Conclusions: Duration of destaining intervals does not impact the quality of FISH signal on DQ smears. Destaining of DQ smears is not necessary for ALK by FISH.

Key words: Anaplastic lymphoma kinase (ALK); cytology smear; Diff-Quik (DQ); fluorescence in situ hybridization (FISH); lung adenocarcinoma

Introduction

ALK gene rearrangement analysis by fluorescence in situ hybridization (FISH) is one of the standard molecular tests for targeted therapy of lung adenocarcinoma. ALK rearrangements define a molecular subgroup of lung adenocarcinoma that is...
susceptible to targeted kinase inhibition with crizotinib. In about 5% of lung adenocarcinoma, ALK gene is rearranged with echinoderm microtubule-associated protein-like 4 (EML4) gene forming EML4-ALK fusion gene, which encodes a cytoplasmic chimeric protein with constitutive kinase activity. Multiple distinct EML4-ALK chimeric variants have been identified, representing breakpoints within various EML4 exons, all of which have transforming activity. EML4-ALK is more prevalent in patients who have never smoked or who have a history of light smoking. Other rarer fusion partners for ALK [such as kinesin family member 5B (KIF5B) and TRK-fused gene (TFG)] have also been reported in lung adenocarcinoma.

FISH is so far the standard method to detect ALK rearrangement. FISH analysis is performed with dual color break apart from ALK probes with one probe hybridizing to the 3′ end of the gene, and the other one hybridizing to the 5′ end, and able to detect ALK gene rearrangement with different gene fusion partners.

The College of American Pathologists, International Association for the Study of Lung Cancer, and Association for Molecular Pathology (CAP/IASLC/AMP) molecular testing guideline recommends performance of ALK by FISH on resection specimen, biopsy, or cytology cell blocks. Majority (79%) of lung cancer cases present as a metastatic disease at the time of initial diagnosis. Endobronchial ultrasound (EBUS)-guided fine needle aspiration (FNA) is routinely performed for work-up of lung cancer with suspicious hilar lymph node metastasis for both diagnosis and staging. Cytopology cellblock is thereby the only available material recommended for molecular studies in majority cases of lung adenocarcinoma. However, in a high percentage of cases, FNA cellblocks are either acellular (up to 37%) or have insufficient number of tumor cells for performing molecular studies. Utilization of cytology smears has become a very important option for molecular tests to avoid repeat procedures, particularly DQ smears, which are the most available and reliable smears usually assessed with on-site evaluation. Betz et al. recently demonstrated that destained DQ smears could be can be effectively used for ALK rearrangement analysis by FISH.

The aim of our study was to observe the impact of destaining intervals on quality of the FISH signals and to determine if foregoing destaining of DQ-stained smears would allow ALK FISH analysis.

Materials and Methods

Thirty-five DQ-stained smears from 27 cases of lung adenocarcinoma were included in the study, which was approved by the Institutional Review Board of our institute. The smears were obtained with EBUS-guided FNA. Cellblocks from six of the 27 cases were selected for comparison. The ALK status was known in these six cases from prior FISH analysis with cellblocks. Three of them had a wild type ALK gene, and the other three had rearranged ALK gene.

ALK by FISH was basically performed as per the manufacturer’s instructions. Briefly, the coverslips were removed by immersing the slides in xylene for 1 h up to 2 days (check periodically), and then in 100% ethanol for 2-3 min for the removal of xylene. The DQ-stained smears in our study represented the patient samples that had been processed in our laboratory on different days for ALK gene rearrangement test. We assumed that short destaining interval should work for ALK FISH because DQ stain does not have autofluorescence. We initially tried 2 min of destaining interval (four cases), and progressively shortened the destaining intervals [1 min (6 cases), 30 s (13 cases), non-destaining (12 cases)] once satisfactory results were achieved with the prior interval. Because ALK signals in all (100%) cases tested for 2 min and 1 min of destaining intervals were satisfactory, we did not test more cases for them. For destained cases, slides were dipped continuously in 0.5% HCl/50% ethanol for different intervals as mentioned above, followed by a wash with running tap water for 1 min and air drying. The air-dried smears were pretreated with the Vysis Paraffin Pretreatment IV and Post-Hybridization Wash Buffer Kit (Abbott Molecular Inc. Des Plains, IL). Hybridization was performed with Vysis ALK Break Apart FISH Probe Kit [Abbott Molecular Inc. Des Plains, Illinois (IL), U.S.A.] and post-hybridization washing was done with Vysis Paraffin Pretreatment IV and Post-Hybridization Wash Buffer Kit (Abbott Molecular Inc. Des Plains, IL, U.S.A.). ALK by FISH on the cellblocks was performed on 4-micron paraffin sections with the same reagents. Scoring for both smears and cellblocks was performed according to the standard criteria following the manufacturer’s instructions. Fifty tumor nuclei were scored for each case. Orange and green signals that were fused (yellow); touched, or separated by a distance <2 signal diameters apart were considered as negative for ALK rearrangement. Orange and green signals apart for 2 signal diameters or a single orange signal without a corresponding green signal (5′ deletion) were considered

### Table 1: ALK FISH signal quality of Diff-Quik stained cytologic smears with different destaining time

| Destaining Time (minute) | Case number | Poor | Fair | Fair/Good | Good | Excellent |
|--------------------------|-------------|------|------|-----------|------|-----------|
| 2                        | 4           | 0    | 0    | 1         | 3    | 0         |
| 1                        | 6           | 0    | 1    | 3         | 1    | 1         |
| 0.5                      | 13          | 0    | 3    | 5         | 3    | 2         |
| 0                        | 12          | 0    | 2    | 2         | 4    | 4         |
positive for ALK rearrangement. Cases with 15% positive cells were considered positive for ALK rearrangement. In cases with 10-50% positive cells, an additional 50 cells were scored by a different person.

The quality of signal was semi-quantified as follows. “Excellent” indicated that the signal was very clear and bright without autofluorescence or haziness. “Good” implied that the signal was clear and bright with some haziness or autofluorescence. “Fair” meant that the signal was generally clear (sometimes not in all areas) and readable with autofluorescence and/or haziness. “Fair/Good” meant that the signal was not evenly distributed with mixed good and fair areas. “Poor” meant that the signal was not readable. The differences of signal quality among smears with different destaining time, and between 23 smears with destaining and 12 smears without destaining were analyzed with Chi-squared test. FISH signals of DQ smears without destaining were also compared with paired destaining smears in eight cases. To validate ALK FISH analysis on DQ smears, DQ smears were compared with the paired cellblocks in six cases for FISH signals and results.

Results

Of the total 27 selected cases, three (11%) were positive for ALK gene rearrangement, whereas 24 (89%) were negative. None of the cases were inadequate for the analysis. The ALK FISH results for different destaining time were summarized in Table 1. The signal quality of all the 35 smears tested with different conditions (0 min, 0.5 min, 1 min, and 2 min of destaining) was at least generally clear and readable with some autofluorescence and/or haziness (“Fair”). No smear showed poor or no signal. Statistical analysis with Chi-squared test did not show significant difference in the quality of signal among the groups with different destaining time ($P = 0.55$). “Good” or “Excellent” signal was achieved in 66.7% (8/12) of smears without destaining, and in 38.5% (5/13), 33.3% (2/6) and 75% (3/4) of smears with 30 s, 1 min, and 2 min of destaining respectively. There was also no statistically significant difference in signal quality between cases without destaining (12 smears) and those with destaining (23 smears) ($P = 0.41$) [Table 2]. Particularly, the signals of smears without destaining achieved similar quality as those of the destained ones [Figure 1].

To reduce the variation among the cases, we next compared the signals in DQ smears from same cases with and without destaining in eight cases [Table 3]. These included the three cases with ALK gene rearrangement and five cases with wild-type ALK genes. Excellent concordance in FISH results and the quality of signals were achieved in all the cases (100%). In seven of eight cases (87.5%), the quality of signal was either similar (four cases) or better (three cases) in smears without destaining than that of destained ones. Only in one case (case 1), the signal of destained smear was better (“Excellent”) compared to that of the smear without destaining (“Good”).

To validate the results, we compared the ALK rearrangement results in six cases between smears and available cellblocks [Table 3]. The results were consistent among them for all cases with either wild-type (case 3-5) or rearranged ALK (case 6-8). Also, the signal quality in smears was similar to or better than the cellblocks in all six cases.

Discussion

With the advent of targeted therapy, small biopsies, and cytologic specimens of primary and metastatic disease

| Table 2: ALK FISH signal quality of Diff-Quik stained cytologic smears with and without destaining |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Destaining      | Case no. | Poor | Fair | Fair/Good | Good | Excellent |
|-----------------|----------|------|------|-----------|------|-----------|
| Yes             | 23       | 0    | 4    | 9         | 7    | 3         |
| No              | 12       | 0    | 2    | 2         | 4    | 4         |

| Table 3: Comparison of ALK FISH results in cell blocks and Diff-Quik smears with or without destaining |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Case no.        | Cell block      | Diff-Quik smear | Result consistency |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| 1               | ND               | Good            | WT              | Excellent       | WT              | Yes             |
| 2               | ND               | Excellent       | WT              | Excellent       | WT              | Yes             |
| 3               | Fair             | Fair            | WT              | WT              | Fair           | Yes             |
| 4               | Fair             | Excellent       | WT              | WT              | Fair           | Yes             |
| 5               | Good/Fair        | Good/Fair       | WT              | Good            | WT              | Yes             |
| 6               | Good/Fair        | Good/Fair       | RA              | RA              | FAIR           | Yes             |
| 7               | Good             | Good            | RA              | Good            | RA              | Yes             |
| 8               | Good             | Good/Fair       | RA              | Good/Fair       | RA              | Yes             |

*Destaining for 30 s, WT: Wild type ALK gene, RA: Rearranged ALK gene, ND: Not done

Figure 1: ALK FISH on Diff-Quik stained cytology smears with destaining for 30 s (a) or without destaining (b). Both of them show bright FISH signal, clean background, and 5′ FISH signal (green) deletion
are being increasingly used for molecular studies. Lack of sufficient material in a subset of cases may impede molecular testing, resulting in repeat procedures to obtain more diagnostic material. Direct smears of lung adenocarcinoma have been shown to provide adequate material for epidermal growth factor receptor (EGFR) and Kirsten rat sarcoma viral oncogene homolog (KRAS) mutation testing.\[^{17-20}\] Recently, in a pioneer study with DQ smear Betz et al. showed that after destaining the DQ-stained slides for 1 h, the ALK break apart probe set could be hybridized to the tumor cell-enriched area for a successful FISH analysis.\[^{16}\] However, sacrifice of smears used for molecular testing represents a potential disadvantage of this approach. This can be obviated by restaining of smear after FISH, thereby allowing for preservation of the slide for inclusion in the diagnostic archive.

In the current study, we showed that the results were not affected by different destaining time. More importantly, we demonstrated that DQ-stained slides can be directly used for ALK by FISH study and destaining of these slides was not necessary. Satisfactory results were obtained in all of our cases and the quality of signal was at least as good as that of destained slides. FISH results on DQ smears when compared with the paired cellblocks with diagnostic tissue showed the same outcome, as also reported in the previous study.\[^{16}\] Removing destaining step from the protocol significantly improves the efficiency of the test.

Compared to cellblocks, DQ smears for ALK FISH and other molecular studies have several advantages. First, at the time of onsite adequacy assessment of FNA, DQ-stained smear can be examined to ensure that tumor cells are sufficient for not only diagnostic evaluation but also for molecular studies. In contrast, the cellularity of cellblock and Papanicolaou (PAP)-stained smear is unknown during the procedure, and may not yield diagnostic material for performing molecular tests.\[^{14}\] Second, DQ smears allow for analysis of whole nuclei of the tumor cells, thereby obviating the likelihood of prone signal loss while performing FISH. In contrast, the presence of truncated nuclei in the cellblock section may result in probe signal loss. Additionally, we have shown in our study that destaining of DQ smear is not necessary for ALK analysis by FISH. Concordance in ALK FISH results between paired destained DQ slides and DQ slides without destaining was 100%. Hence, after ALK analysis by FISH, the slides can be returned to files for archival preservation of diagnostic material. The need for restaining of these slides as suggested by Betz et al. is completely circumvented.\[^{16}\]

Traditionally fresh-formalin fixed paraffin embedded block have been preferred for Vysis FISH analysis because of Food and Drug Administration (FDA) approval. Attempts at improving the cellblock yield for molecular studies for lung adenocarcinoma by obtaining two additional FNA passes have not significantly improved the cellblock diagnostic yield (45% to 48%).\[^{13}\] Repeat FNA procedures in the hope of getting better cellblocks for molecular studies do not guarantee procurement of diagnostic material. Several studies, including ours, have demonstrated that cytology smears can be effectively used for ALK FISH analysis.\[^{16,21-23}\] In the current study, we successfully performed ALK by FISH on cytology smears in all the 27 (100%) cases. A high success rate of 97% was also attained in the previous report with DQ smears.\[^{16}\] Similar success rates of ALK FISH have also been achieved with PAP stained ThinPrep smears\[^{21,22}\] and conventional smears (74/94, 88.8%).\[^{23}\]

**Conclusion**

ALK by FISH can be directly performed on DQ cytology smears with shorter or no destaining. The signal quality of non-destained DQ smears is at least as good as the destained slides and the sections of cellblocks. Elimination of the unnecessary destaining step from the procedure improves the efficiency of the test by saving time and cost.

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**Conflicts of interest**

There are no conflicts of interest.

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