Characterizing Atypical BCL6 Signal Patterns Detected by Digital Fluorescence In Situ Hybridization (FISH) Analysis

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Dear Editor,

The BCL6 gene encodes a 706-amino acid sequence-specific repressor transcription factor [1]. BCL6 has been identified as commonly rearranged in 20–40% of diffuse large B cell lymphomas (DLBCL) [2, 3]. BCL6 can be rearranged with the immunoglobulin (Ig) gene loci or non-Ig gene partners [2]. Break-apart (BAP) FISH probes are widely used for clinical testing of genes with multiple translocation partners [4]. The expected signal pattern of a rearranged sample using a BAP FISH probe is a single red and single green signal for derivative chromosomes and a single fusion signal for the normal homolog (1F/1R/1G). However, because of the complexity of genomic changes that can occur in cancer, signal patterns other than 1F/1R/1G, that is, atypical or unusual signal patterns, are also observed [5]. We describe the validation of a locus specific identifier (LSI)-BCL6 FISH assay using a digital analysis system, as well as our experience with unusual signal patterns observed using this assay. We demonstrate that unusual FISH signal patterns can be associated with copy number alterations in addition to rearrangements involving the BCL6 locus at 3q27. This study was granted exempt status by the University of Utah Institutional Review Board (IRB_00084247).

We evaluated 34 formalin-fixed, paraffin-embedded tissue samples to validate the LSI-BCL6 FISH probe. The procedures for preparing, scanning, and analyzing the hematoxylin and eosin (H&E) and FISH slides traditionally and digitally have been described previously [6]. FISH cutoffs for the total rearranged nuclei were calculated using the correlation curve from regression analysis of traditional versus digital FISH. Samples with ≥24% total rearranged nuclei were considered positive for BCL6 rearrangement; samples with ≤15% total rearranged nuclei were considered negative for BCL6 rearrangement. Those between 16% and 23% were interpreted at the discretion of the attending pathologist. If a sample appeared to have an elevated number of unusual BCL6 signal patterns, it was considered positive for BCL6 rearrangement. Four additional samples were identified as having unusual signal patterns after completion of the validation. Two of these had sufficient sample left to perform additional molecular testing. The OncoScan genome-wide molecular inversion probe (MIP) array (ThermoFisher Scientific, Waltham, MA, USA), which contains over 220K probes, was used to identify chromosomal gains or losses, as previously de-
scribed [7]. Next generation sequencing (NGS) was used to determine the sequence of the 3q27 loci.

Regression analysis showed a strong correlation ($R^2 = 0.9$) between the LSI-BCL6 assay by traditional and GenASIs (digital) FISH (Applied Spectral Imaging, Carlsbad, CA, USA). The digital FISH system accurately classified 12/12 BCL6 rearrangement-positive samples (100%) and 22/22 BCL6 rearrangement-negative samples (100%). Unusual signal patterns were defined as nF/nR and nF/nG (Table 1).

Sample 1U, which had elevated 1F/1G and 2F/1G FISH signal patterns, was found to have a copy number gain by MIP array and a rearrangement by NGS (Fig. 1). Sample 3U, which had elevated levels of 1F/1G, was shown to have a BCL6 rearrangement by NGS. NGS identified the BCL6 translocation partner in sample 1U and 3U as IGH and interleukin receptor 21 (IL21R), respectively.

The strong correlation found between traditional and digital FISH analysis methods for BCL6 rearrangements using the LSI-BCL6 fusion probe is similar to our previous study [6]. Research has identified unusual signal patterns using BAP FISH probes. Vargas et al [5] described atypical/unusual signal patterns identified using an EWSR1 BAP probe, as well as issues concerning their identification. We could identify losses of both the 3' and 5' ends of the BCL6 gene. Despite their low frequency (2%), trying to determine how to interpret these unusual signal patterns was important.

NGS confirmed BCL6 translocations in samples 1U and 3U. Sample 1U had elevated FISH signal patterns, suggesting a de-

### Table 1. Distribution of unusual signal patterns in samples tested for BCL6 rearrangement

| Unusual signal patterns | 1F/1G | 1F/1R | 2F/1G | 2F/1R |
|------------------------|-------|-------|-------|-------|
| Sample 1U              | 18.5† | 2.9   | 14.9  | 0.3   |
| Sample 2U              | 2.2   | 10.6  | 0.4   | 9.2   |
| Sample 3U              | 7.3   | 1.0   | 1.0   | 0.5   |
| Sample 4U              | 2.2   | 27.6  | 0.2   | 26.7  |
| Average                | 7.6   | 10.5  | 4.1   | 9.2   |
| SD                     | 7.7   | 12.1  | 7.2   | 12.4  |

|                 | 1F/1G | 1F/1R | 2F/1G | 2F/1R |
|-----------------|-------|-------|-------|-------|
| Negative Average| 1.6   | 1.2   | 1.1   | 0.8   |
| SD              | 1.5   | 1.1   | 1.1   | 1.0   |

|                 | 1F/1G | 1F/1R | 2F/1G | 2F/1R |
|-----------------|-------|-------|-------|-------|
| Positive Average| 9.9   | 11.1  | 2.9   | 1.8   |
| SD              | 8.6   | 6.9   | 1.6   | 2.2   |

*The distribution of unusual signal patterns in the four unusual samples is shown and compared with the average distribution in the 3q27-negative and -positive groups. The averages were derived from the validation samples (12 positive and 22 negative 3q27-rearranged samples).

†Values are expressed as a percentage of the total number of countable signal patterns identified in a sample.

Abbreviations: F, fused signal; G, green signal; R, red signal.

Fig. 1. Results from the combined testing of sample 1U. (A) Next generation sequencing identified a BCL6-IGH fusion.
letion; however, the MIP array indicated a gain. This probably reflects clonal heterogeneity as FISH results correspond to individual cells, whereas the MIP array result is based on DNA extracted from all cells within the tissue. Identification of the causative genetic mechanism may not always be possible; however, as unusual FISH signal patterns are often associated with a copy number gain and rearrangement, combined utilization of FISH, NGS, and microarray technologies will be helpful in most cases. Our findings highlight the importance of follow-up investigation by NGS and microarray for abnormal signal patterns in initial FISH screening for better clinical management.

Authors’ Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article are reported.

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