Elucidating a false-negative *MYC* break-apart fluorescence in situ hybridization probe study by next-generation sequencing in a patient with high-grade B-cell lymphoma with *IGH/MYC* and *IGH/BCL2* rearrangements

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Abstract The identification of *MYC* rearrangements in several mature B-cell neoplasms is critical for diagnostic and prognostic purposes. Commercially available fluorescence in situ hybridization (FISH) probe sets, including *IGH/MYC* dual-color dual-fusion (D-FISH) and *MYC* break-apart probes (BAPs), serve as the primary methodology utilized to detect *MYC* rearrangements. However, performing either *IGH/MYC* D-FISH or *MYC* BAP FISH studies in isolation has been reported to result in false-negative results because of the complex nature of 8q24 rearrangements involving the *MYC* gene region. We report a 60-yr-old male with newly diagnosed high-grade B-cell lymphoma with a negative *MYC* BAP study, but with positive *BCL2* and *BCL6* BAP studies. Per our current laboratory algorithm to concurrently interrogate the *MYC* gene region with both *MYC* BAP and *IGH/MYC* D-FISH probe sets, we performed *IGH/MYC* D-FISH studies and detected an *IGH/MYC* fusion. To further characterize the discrepant *MYC* results obtained by FISH, a next-generation sequencing strategy, mate-pair sequencing (MPseq), was performed and revealed a small insertion (~200 kb) of the *IGH* locus downstream from the *MYC* gene that was undetectable by *MYC* BAP studies. This case highlights the importance of utilizing both *IGH/MYC* D-FISH and *MYC* BAP sets to detect potential cryptic *MYC* rearrangements and also demonstrates the power of MPseq to characterize complex structural rearrangements and copy-number abnormalities unappreciable by FISH.

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Ontology term: B-cell lymphoma

INTRODUCTION

High-grade B-cell lymphoma (HGBL) with *MYC* and *BCL2* and/or *BCL6* rearrangements (previously referred to as double- or triple-hit lymphoma) is a highly aggressive mature B-cell neoplasm most often observed in elderly patients (Kluin et al. 2017; Li et al. 2012, 2016;

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Snuderl et al. 2010; Wang et al. 2015). The detection of *MYC* rearrangements in HGBL and other mature B-cell lymphomas has traditionally relied upon fluorescence in situ hybridization (FISH) strategies (dual-color dual-fusion [D-FISH] and/or break-apart probes [BAP]) that are typically performed on formalin-fixed paraffin-embedded (FFPE) tissue specimens. Although the most common *MYC* translocation partner is the immunoglobulin heavy chain (*IGH*) gene, which can usually be detected by *IGH/MYC* D-FISH studies, the *MYC* BAP has an added value for the detection of *IGK/MYC*, *IGL/MYC*, and non-*IG/MYC* rearrangements (Muñoz-Mármol et al. 2013; Sesques and Johnson 2017). Importantly, several studies have demonstrated that performing either *IGH/MYC* D-FISH or *MYC* BAP studies in isolation can result in false-negative results because of the broad range of 8q24 breakpoints that occur in close proximity to the *MYC* gene region (May et al. 2010; Sun et al. 2012; Muñoz-Mármol et al. 2013; King et al. 2018).

Until recently, the characterization of cryptic *IGH/MYC* fusions has been limited to commercially available FISH probe sets (May et al. 2010; Sun et al. 2012; Muñoz-Mármol et al. 2013). Utilizing mate-pair sequencing (MPseq), a next-generation sequencing (NGS) technology that can detect both balanced and unbalanced structural and copy-number abnormalities, our group previously characterized four unique *IGH/MYC* rearrangement mechanisms observed in mature B-cell neoplasms (Drucker et al. 2014; Johnson et al. 2018; King et al. 2018; Smadbeck et al. 2018). Expanding on our previous report, we describe a 60-yr-old male with newly diagnosed HGBL with negative *MYC* BAP studies and a novel, cryptic *IGH/MYC* rearrangement characterized by MPseq.

RESULTS

Hematopathology Evaluation

The patient is a 60-yr-old male with a reported long-standing history of low-grade follicular lymphoma. Stained slides and FFPE blocks of a left iliac lymph node were submitted for tumor characterization. The lymph node architecture was effaced by a diffuse infiltrate of neoplastic large cells with plasmablastic cytologic features. There was extensive necrosis, and viable-appearing tumor cells were often perivascular in distribution within these areas.

By flow-cytometric immunophenotyping, ~61% of the total analyzed events and 93% of the gated lymphoid events consisted of CD19 (dim) and cytoplasmic CD22-positive B cells that had partial coexpression of CD10 and were immunoglobulin light-chain indeterminate. These B cells were negative for CD20, CD5, CD23, CD34, cytoplasmic CD79a, and terminal deoxynucleotidyl transferase (TdT).

Immunoperoxidase studies were performed using antibodies directed against the following antigens: CD3, CD10, CD20, CD21, CD138, BCL2, BCL6, κ and λ immunoglobulin light chains, MUM1, MYC, and PAX5. The large, neoplastic lymphocytes with plasmablastic cytologic features were CD138 and MUM1-positive, showed expression of CD10 (partial), MYC (90% positive), and BCL2 (100% positive), and contained monotypic cytoplasmic κ immunoglobulin light chains. The B cells were negative for all other antigens for which testing was performed, including CD20, PAX5, and BCL6. No CD21-staining follicular dendritic cell networks were seen.

Genomic Analyses

All genomic studies were performed on a freshly submitted left iliac lymph node specimen. FISH performed on the FFPE lymph node specimen was negative for *MYC* rearrangement by *MYC* BAP studies (Fig. 1A) and positive for *BCL2* and *BCL6* rearrangements in 100% of interphase nuclei analyzed (abnormal cutoffs: ≥7%, ≥9%, and ≥6%, respectively) (Fig. 1C,D).
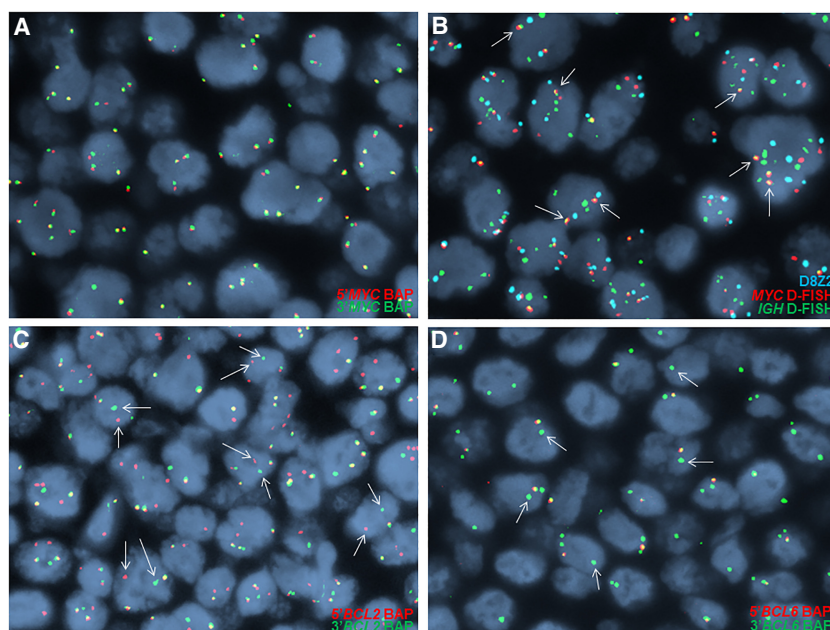
Figure 1. Representative fluorescence in situ hybridization (FISH) results for *MYC* BAP, *IGH/MYC* D-FISH, *BCL2* BAP, and *BCL6* BAP studies performed on a paraffin-embedded left iliac lymph node specimen. (*A*) The *MYC* BAP was negative for *MYC* rearrangement, although additional intact *MYC* fusion signals were observed in 100% of cells analyzed. (*B*) The *IGH/MYC* D-FISH revealed multiple fusions (arrows) representing a cryptic *IGH/MYC* rearrangement. (*C*) An apparently balanced *BCL2* rearrangement indicated by split red and green signals (arrows). (*D*) An unbalanced *BCL6* rearrangement indicated by loss of the 5′ *BCL6* BAP (red) with retention of the 3′ *BCL6* BAP, indicated by a single green signal (arrow).

Per our current laboratory algorithm to concurrently interrogate the *MYC* gene region with both *MYC* BAP and *IGH/MYC* D-FISH probe sets, we performed *IGH/MYC* D-FISH and identified an *IGH/MYC* fusion (abnormal cutoff ≥ 5) (Fig. 1B). Although the *BCL2* rearrangement appeared to be balanced (indicated by split red and green signals (Fig. 1C), the *BCL6* rearrangement was interpreted as unbalanced as indicated by loss of the 5′ *BCL6* BAP signal (red) (Fig. 1D).

To further characterize the discrepant *MYC* BAP (negative) and *IGH/MYC* D-FISH (positive) results, MPseq was performed and revealed an insertional rearrangement involving Chromosomes 8 and 14, resulting in a small segment (~200 kb) of the *IGH* locus from Chromosome 14 inserted downstream from the *MYC* gene on Chromosome 8 (Fig. 2A). Sanger sequencing was subsequently performed and confirmed the *IGH/MYC* rearrangement (Table 1). In addition, a t(14;18)(q32.33;q21.33) was identified by MPseq with breakpoints involving the *IGH* locus on Chromosome 14 and exon 3 of the *BCL2* gene (NM_000633) on Chromosome 18 (Fig. 2B). Last, a segment of 3q27.3 harboring an intact *BCL6* gene was inserted into the 3p24.3 chromosomal region, along with a heterozygous 3q27.3 deletion that spanned the 5′ *BCL6* BAP footprint (Fig. 2C).

DISCUSSION

Both fresh and paraffin-embedded inguinal lymph node tissue involved by lymphoma from a 60-yr-old male patient were evaluated by hematopathology and laboratory genomics and a diagnosis of HGBL with *MYC*, *BCL2*, and *BCL6* rearrangements was rendered. However,
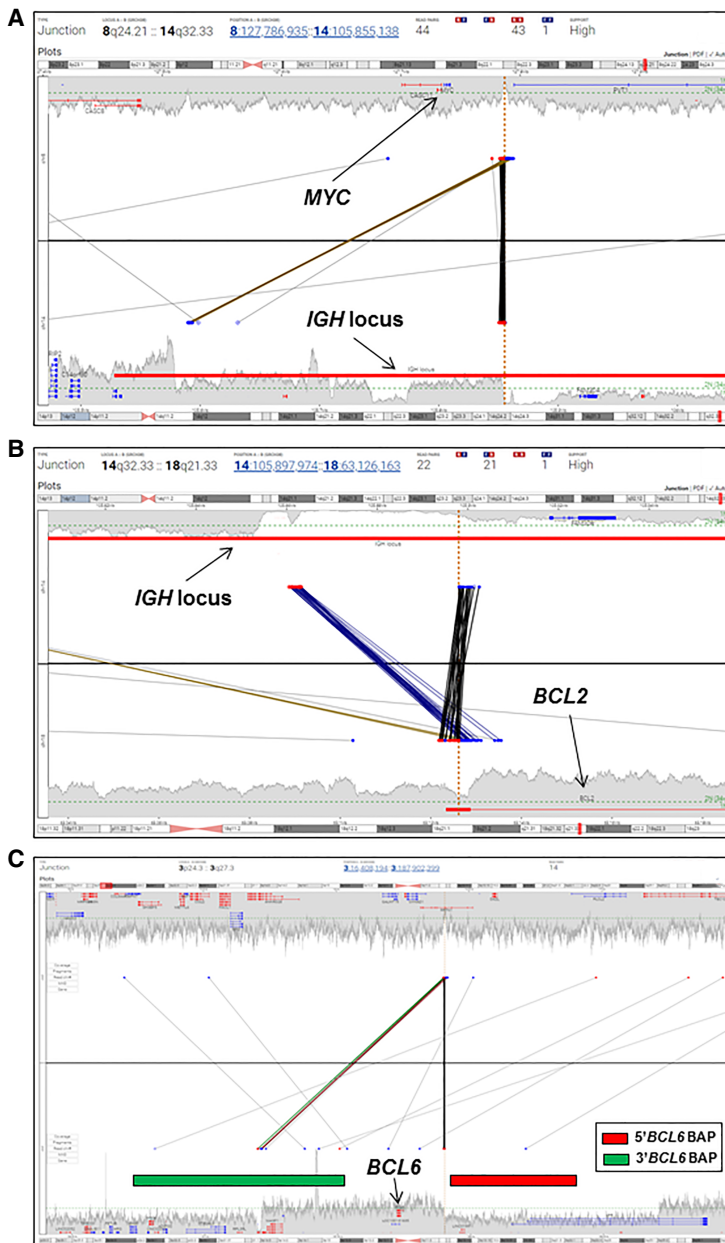
Figure 2. Mate-pair sequencing (MPseq) results. (*A*) Junction plot demonstrating a small segment (~200 kb) of the *IGH* locus from Chromosome 14 inserted downstream from the *MYC* gene on Chromosome 8. (*B*) Junction plot demonstrating a rearrangement between the *IGH* locus on Chromosome 14 and exon 3 of the *BCL2* gene (NM_000633) on Chromosome 18. (*C*) Junction plot demonstrating a 3q27.3 insertional event into the 3p24.3 chromosomal region. In addition, a 3q27.3 deletion spanning the 5′ *BCL6* BAP footprint was observed. Importantly, this insertional event does not disrupt or juxtapose *BCL6* near immunoglobulin heavy or light chains.

additional evaluation by MPseq indicated *IGH/MYC* and *IGH/BCL2* rearrangements, in addition to a complex Chromosome 3 rearrangement of unclear clinical significance that did not disrupt or juxtapose *BCL6* near immunoglobulin heavy or light chains (Fig. 3).

Table 1. Mate-pair (MPseq) and Sanger sequencing results for the *IGH/MYC* rearrangement

MPseq event/ position	Sanger breakpoints [hg38]	MPseq breakpoints [hg38]	Gene/locus	Primer sequence
FF/position A	Chr 8:127,788,254	Chr 8:127,788,231	No gene	GCAACCCCGTCTCCACTAAA
FF/position B	Chr 14:105,589,660	Chr 14:105,589,646	*IGH*	ACTGGGTGAGCTTAGGTGGA
RR/position A	Chr 8:127,787,036	Chr 14:127,786,935	No gene	ACGGAAGTTTGCAAGCAAGA
RR/position B	Chr 14:105,855,473	Chr 14:105,855,138	*IGH*	AGATGGTCTGCTTCAGTGGC

Understanding the limitations of each *MYC* FISH probe set is critical as clinical cytogenetic laboratories routinely screen for *MYC* rearrangements in mature B-cell neoplasms with *IGH/MYC* D-FISH and/or *MYC* BAP sets. In our current case, MPseq accurately detected an insertion of the *IGH* locus downstream from the *MYC* gene region and was subsequently confirmed by Sanger sequencing. Although the *IGH/MYC* D-FISH probe set detected the *IGH/MYC* fusion, the *MYC* BAP demonstrated a false-negative result because the ~200-kb *IGH* insertion did not disrupt the 5′ *MYC* and 3′ *MYC* BAP sufficiently to be appreciated by interphase cell analysis. Additional limitations of FISH are also highlighted by the *BCL6* FISH result. Although the abnormal signal pattern represented an unbalanced 3q27.3 rearrangement, MPseq clearly demonstrated that the *BCL6* gene was not disrupted or juxtaposed near immunoglobulin heavy or light chains. This finding suggests that the 3q27.3 rearrangement does not result in *BCL6* overexpression as indicated by negative BCL6 IHC staining.

In conclusion, we fully characterized a novel *IGH/MYC* fusion by MPseq in a 60-yr-old male with HGBL that went undetected using a commercial *MYC* BAP set. Although MPseq also revealed the expected *IGH/BCL2* rearrangement that was detected by FISH, a deletion spanning the 5′ *BCL6* BAP footprint was observed with unclear clinical
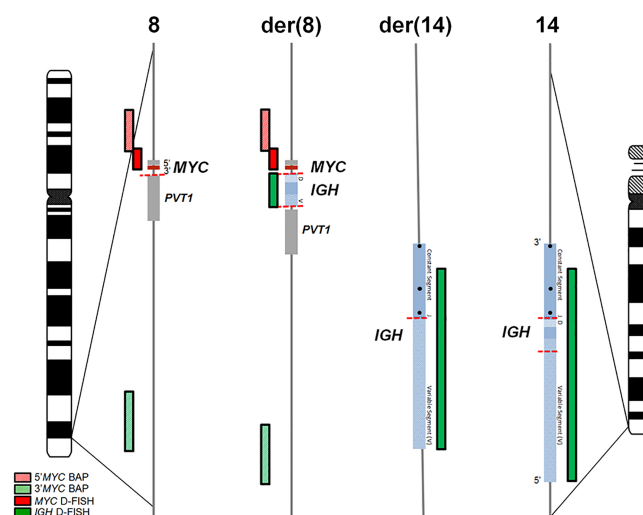

Figure 3. A focused view of the *IGH* and *MYC* gene regions on the derivative copies of Chromosomes 8 and 14. Horizontal dashed red lines indicate the breakpoints on derivative Chromosomes 8 and 14, and the subsequent location of the *MYC* BAP and *IGH/MYC* D-FISH footprints. The insertion of a small segment (~200 kb) from the *IGH* locus downstream from *MYC* on Chromosome 8 produces a single fusion signal by *IGH/MYC* D-FISH studies. The minimal size of the insertional event precluded the detection of this rearrangement by *MYC* BAP studies.

significance. Adding to the literature, this case provides additional evidence that all *MYC* rearrangements cannot be detected using a single commercially available *MYC* FISH probe set. Furthermore, this case highlights the power of MPseq to resolve structural and copy-number abnormalities beyond the capabilities of FISH. This observation may be more relevant in certain hematologic malignancies, including B- and T-cell lymphomas in which rearrangements driven by a positional effect are more common.

METHODS

Fluorescence In Situ Hybridization (FISH)

Fluorescence in situ hybridization (FISH) was performed on a FFPE left iliac lymph node specimen using commercially available (Abbott Molecular) D-FISH (*IGH/MYC*) and break-apart (BAP) (*MYC*, *BCL2* and *BCL6*) probe sets. One hundred interphase nuclei were analyzed for each probe set. The FFPE specimen was subjected to standard FISH pretreatment, hybridization, and fluorescence microscopy according to specimen-specific protocols. FISH analysis was independently evaluated by two qualified clinical cytogenetic technologists and interpreted by a board-certified (ABMGG) clinical cytogeneticist.

Mate-Pair Sequencing (MPseq)

DNA was extracted from a fixed cell pellet (left iliac lymph node specimen) and 1 µg was utilized for mate-pair sequencing library preparation and processed using the Illumina Nextera Mate Pair library kit (Illumina). Library preparation consisted of tagmentation to simultaneously shear and biotinylate the genomic DNA, strand displacement to fill any gaps left by the tagmentation step, and overnight circularization (16–20 h) to produce stabile 2–5 kb DNA fragments. AMPure purification (Beckman Coulter) was performed after the tagmentation and strand displacement steps (0.56× and 0.4×, respectively) to ensure only the longest fragments are selected to complete library preparation. After overnight circularization, non-circularized DNA was digested with exonuclease prior to mechanical shearing of the circularized fragments with a Covaris LE220 System (Covaris). The resulting biotinylated DNA fragments were bound to Dynabeads M-280 Streptavidin (Thermo Fisher Scientific) and subsequently processed through end repair, a-tailing, ligation of 7-bp Illumina adapters (a component of the TruSeq DNA library prep kit), and PCR using the PCR Primer Cocktail (Illumina) and KAPA HiFi HotStart Ready Mix PCR Kit (KAPA Biosystems). A 0.67× AMPure purification was performed to complete library preparation. MPseq libraries were multiplexed at two samples per lane to be sequenced on the Illumina HiSeq 2500 in rapid run mode. On both ends of each mate-pair fragment, 101 base pairs were sequenced to a bridged coverage of 43× and a base coverage of ∼6×. Data were aligned to the reference genome (GRCh38) using BIMAv3, and abnormalities were identified and visualized using SVAtools, an in-house developed bioinformatics tool. Additional information regarding MPseq technology and bioinformatics tools have been previously described (Drucker et al. 2014; Johnson et al. 2018; Smadbeck et al. 2018).

Sanger Sequencing

Reference DNA sequences spanning the minimal 5′ and maximal 3′ positions of MPseq approximate breakpoints were used for primer design using Primer3Plus. End point PCR was performed on patient DNA with a 50% 2X Paq5000 Hotstart PCR Master Mix (Agilent) using touchdown PCR. Results were visualized on a 2% agarose gel in a UV light box, and amplicon sizes were estimated. Selected amplicons were purified with Exo-SAP-IT PCR Product Cleanup Reagent (Thermo Fisher Scientific) and Sanger sequencing was performed on a

3730*xl* DNA Analyzer (Thermo Fisher Scientific). The resulting sequences were analyzed using Sequencher DNA Sequence Analysis Software (Gene Codes Corporation) and mapped to the GRCh38 genome using the BLAT function in the UCSC genome browser to determine precise breakpoints in this rearrangement.

ADDITIONAL INFORMATION

Data Deposition and Access

The variants were submitted to ClinVar (http://www.ncbi.nlm.nih.gov/clinvar/) and can be found under accession numbers SCV000902268.1 and SCV000902269.1.

Ethics Statement

The results described in this manuscript were derived from clinical rather than research testing. Because patient identifiers were removed for the purposes of this manuscript, consent (oral or written) was not required and Mayo Clinic does not require IRB approval for the publication of single case reports.

Author Contributions

B.A.P., J.F.P., and L.B.B. drafted and edited the manuscript, analyzed data, and generated figures. S.A.S. analyzed data, generated figures, and reviewed and edited the manuscript. G.V., J.B.S., P.T.G., and R.P.K. reviewed and edited the manuscript. W.R.M. collected and analyzed data and reviewed and edited the manuscript

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