

## ARTICLE OPEN



## LYMPHOMA

# Non-IG::MYC in diffuse large B-cell lymphoma confers variable genomic configurations and MYC transactivation potential

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*MYC* translocation occurs in 8–14% of diffuse large B-cell lymphoma (DLBCL), and may concur with *BCL2* and/or *BCL6* translocation, known as double-hit (DH) or triple-hit (TH). DLBCL-*MYC/BCL2*-DH/TH are largely germinal centre B-cell like subtype, but show variable clinical outcome, with *IG::MYC* fusion significantly associated with inferior survival. While DLBCL-*MYC/BCL6*-DH are variable in their cell-of-origin subtypes and clinical outcome. Intriguingly, only 40-50% of DLBCL with *MYC* translocation show high *MYC* protein expression (>70%). We studied 186 DLBCLs with *MYC* translocation including 32 *MYC/BCL2/BCL6*-TH, 75 *MYC/BCL2*-DH and 26 *MYC/BCL6*-DH. FISH revealed a *MYC/BCL6* fusion in 59% of DLBCL-*MYC/BCL2/BCL6*-TH and 27% of DLBCL-*MYC/BCL6*-DH. Targeted NGS showed a similar mutation profile and LymphGen genetic subtype between DLBCL-*MYC/BCL2/BCL6*-TH and DLBCL-*MYC/BCL2*-DH, but variable LymphGen subtypes among DLBCL-*MYC/BCL6*-DH. *MYC* protein expression is uniformly high in DLBCL with *IG::MYC*, but variable in those with non-*IG::MYC* including *MYC/BCL6*-fusion. Translocation breakpoint analyses of 8 cases by TLC-based NGS showed no obvious genomic configuration that enables *MYC* transactivation in 3 of the 4 cases with non-*IG::MYC*, while a typical promoter substitution or *IGH* super enhancer juxtaposition in the remaining cases. The findings potentially explain variable *MYC* expression in DLBCL with *MYC* translocation, and also bear practical implications in its routine assessment.

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## INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is a group of heterogeneous aggressive B-cell lymphoma with variable cell-of-origin (COO), genetic changes, molecular mechanisms and clinical outcomes. Based on COO, DLBCL can be broadly classified into activated B-cell like (ABC) and germinal centre B-cell like (GCB) subtype, with a subset of the latter further identified as molecular high grade (MHG)/double-hit signature (DHITsig) due to their enriched *MYC* expression and centromere signatures [1, 2]. Based on genetic alterations, DLBCL can be subdivided into distinct subgroups using LymphGen algorithm or other: MCD (*MYD88*<sup>L265P</sup> and *CD79B* mutations), N1 (*NOTCH1* mutation), A53 (aneuploidy with *TP53* inactivation), BN2 (*BCL6* translocation and *NOTCH2*

mutation), ST2 (*SGK1* and *TET2* mutated) and EZB (*EZH2* mutation and *BCL2* translocation), with the latter subgroup further divided into EZB-MYC+ and EZB-MYC- according to *MYC* signature [3, 4]. There is a broad correlation between COO molecular subtypes and genetic subgroups. ABC-DLBCL largely comprises of MCD, N1 and A53, while GCB-DLBCL is primarily composed of EZB and ST2, with BN2 seen in both ABC and GCB-DLBCL. These subgroups are further underpinned by their distinct molecular mechanisms and different clinical outcomes.

Despite the steady progress in molecular characterization and sub-classification of DLBCL, few of these advances are applied in a routine clinical setting. For routine diagnosis and prognostication of DLBCL, only *MYC*, *BCL2* and *BCL6* translocations are investigated

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along with international prognostic index. *MYC* translocation occurs in 8–14% of DLBCL. This translocation can occur together with *BCL2* and/or *BCL6* translocation, known as double-hit (DH) or triple-hit (TH). Among *MYC* translocation positive DLBCL, ~9% are *MYC/BCL2/BCL6*-TH, ~40% and ~18% are *MYC/BCL2*-DH and *MYC/BCL6*-DH respectively [5–7]. Most of cases with *MYC/BCL2*-DH/TH are GCB subtype or EZB-*MYC*+ [3, 5]. In contrast, those with *MYC/BCL6*-DH are rather heterogeneous in their molecular subtypes, with 30% each being GCB or ABC subtype respectively, 15% due to MHG, and the remaining cases unclassifiable [5]. These cases showed a mutation profile remarkably different from those with *MYC/BCL2*-DH/TH, but do not exhibit any prominent signatures although a proportion of these cases are associated with *NOTCH2* mutation, thus BN2 subtype [5]. For these reasons, the 5th edition of the World Health Organization Classification of Haematolymphoid Tumours (WHO-HAEM5) excludes the cases with concomitant *MYC* and *BCL6* rearrangements (without *BCL2* rearrangement) from the DH entity and renames the entity as diffuse large B-cell lymphoma/high-grade B-cell lymphoma with *MYC* and *BCL2* rearrangements (DLBCL/HGBL-*MYC/BCL2*) to recognise their variable morphology [8].

The clinical outcome of DLBCL/HGBL-*MYC/BCL2*-DH is also heterogeneous. Cases with *IGH::MYC* are significantly associated with worse progression-free survival (PFS) and overall survival (OS), particularly within the first two years of diagnosis, while those with non-*IGH::MYC* showed no significant difference in both PFS and OS from DLBCL without *MYC* translocation [7, 9]. The molecular mechanisms underlying the different clinical impacts by *MYC* translocation partner are unclear. In addition, *MYC* protein expression varies considerably in DLBCL with *MYC* translocation, ranging from negative to 100% positivity in lymphoma cells [10–12]. In DLBCL with *IGH::MYC*, the breakpoint commonly occurs in region spanning the 5'UTR and intron 1 of the *MYC* gene and the switch region of the *IGH* locus respectively, thus placing the *MYC* gene in close proximity of the highly active *IGH* super enhancer, causing *MYC* constitutive over-expression [13]. Moreover, DLBCL with *IGH::MYC* often acquire *MYC* mutations that impair *MYC* protein degradation, consequently sustaining its expression and function [5]. However, the impact of non-*IGH* partner on *MYC* expression is unclear. Among the known non-*IGH* partners of *MYC* translocation including *BCL6*, *ZCCHC7* and *RFTN1*, *BCL6* is the most frequent [13, 14]. It also remains unclear how often non-*IGH::MYC* translocation involves *BCL6* as a partner, and how non-*IGH::MYC* impacts on *MYC* activation given their clear difference in clinical impact from the *IGH::MYC* translocation. To investigate these, we studied 186 cases of DLBCL with *MYC* translocation including 32 *MYC/BCL2/BCL6*-TH, 75 *MYC/BCL2*-DH and 26 *MYC/BCL6*-DH by combined analyses of *MYC* translocation partner and *MYC* protein expression, mutation profiling and breakpoint analysis of *MYC* translocation in selected cases to understand their transactivation potential.

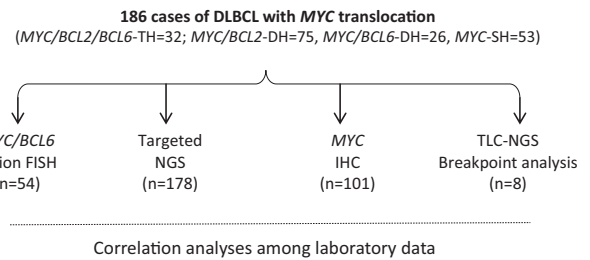
## MATERIALS AND METHODS

The study was performed in accordance with local ethical guidelines for the research use of tissue materials with the approval of the ethics committees of the involved institutions (05-Q1604-10, 04-Q1205-125, 10-H0504-79).

A total of 186 cases of DLBCL with *MYC* translocation were retrieved from surgical files of Addenbrookes Hospital, University of Cambridge and HMDS, St James' University Hospital, Leeds, UK. These cases comprised of 32 *MYC/BCL2/BCL6*-TH, 75 *MYC/BCL2*-DH, 26 cases with *MYC/BCL6*-DH, and 53 cases *MYC*-single hit (SH) (Fig. 1).

### Interphase fluorescence in situ hybridisation (FISH)

Chromosome translocation status at the *MYC*, *BCL2* and *BCL6* locus was available from routine haematopathological diagnosis or previous studies [5]. Further interphase FISH with *MYC/BCL6* (Cytocell), *MYC/IGH* (Abbott), *MYC/IGK* and *MYC/IGL* (Cytocell) dual fusion probes were performed on FFPE tissue slides where indicated in the present study.



**Fig. 1** Summary of DLBCL with *MYC* translocation and experiments carried out.

### Immunohistochemistry

*MYC* (Abcam clone Y69) and *BCL6* (Leica Clone LN22) immunohistochemistry were performed where possible in all cases where tissue materials remained available using the Bond-III system (Leica Biosystems) with the Bond Polymer Refine Detection Kit as the same condition of routine histopathological diagnosis. This was carried out centrally in the Cambridge lab and the staining intensity (weak, moderate, strong) and percentage in tumour cells (>70% or <70%) were scored [11].

### DNA extraction and quality assessment

Histology was reviewed and areas containing confluent lymphoma cells (>40%) in each specimen were microdissected on consecutive tissue sections. DNA was extracted using the QIAamp DNA Micro Kit (QIAGEN, Crawley, UK), quantified with a Qubit® Fluorometer (Life Technologies, UK) and assessed for quality by PCR [5, 15].

### Mutation analysis by targeted sequencing

The mutation data in 125 cases were from a previous study, in which a panel of B-cell lymphoma associated genes ( $n = 70$ ) were sequenced using HaloPlexHS target enrichment and Illumina HiSeq4000 platform, with a well-validated in house variant calling pipeline [5]. In 53 cases, mutation data were similarly obtained but using TWIST capture target enrichment of a much larger gene panel ( $n = 191$ ) (Table S1) [16].

LymphGen genetic subtypes were assigned where possible according to Wright et al [3].

### Targeted locus capture next generation sequencing (TLC-NGS)

TLC-NGS was essentially carried out as previously described [17]. FFPE tissue sections were deparaffinised, followed by a 30 min pretreatment step at 90 °C, digestion with NlaIII restriction enzyme and ligation with T4 DNA ligase. The sample was incubated at 80 °C overnight to reverse crosslinking and then subjected to DNA purification. A total of 100 ng DNA was fragmented and used for NGS library preparation, hybridization with capture probes using Roche HyperCap reagents according to the manufacturer's instructions. Paired-end sequencing was performed using an Illumina Novaseq 6000. TLC-NGS reads were mapped to the human genome (hg19) using BWA-MEM (version: 0.7.17-r1188; settings: -SP -k12 -A2 -B3) in paired-end mode, and gene rearrangements were identified using PLIER (Proximity-Ligation based Identification of Rearrangements) according to previously validated pipeline [17].

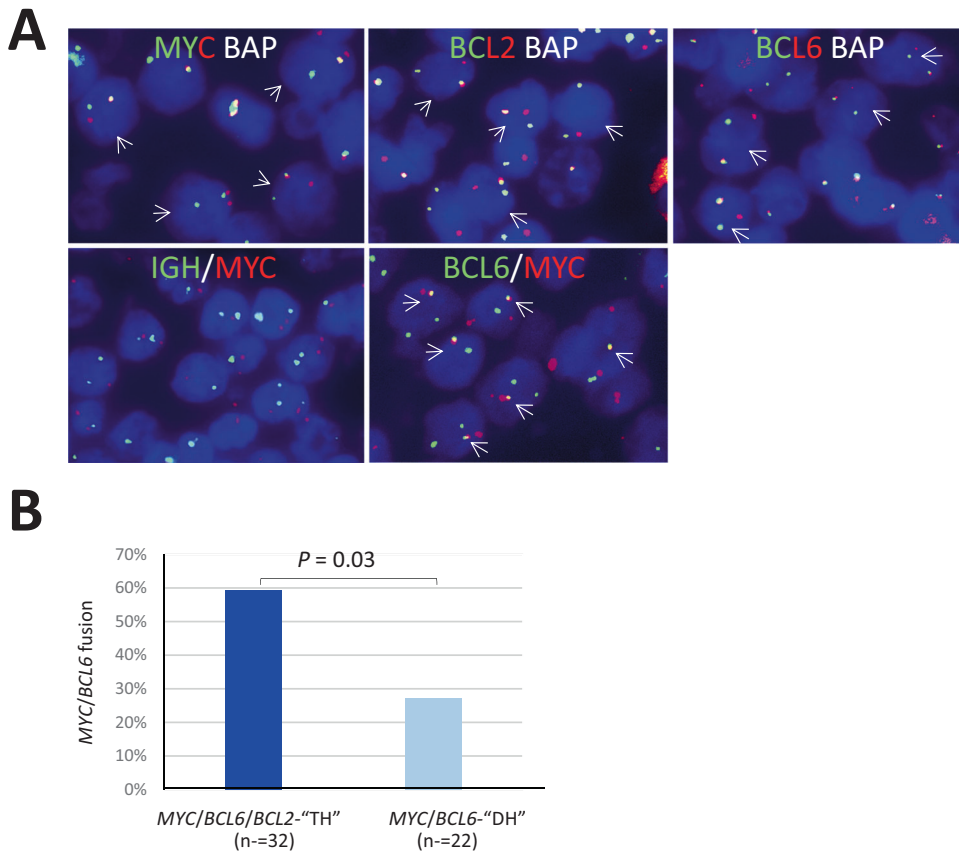
### Statistical analysis

Associations among *MYC* translocation, translocation partner and *MYC* protein expression were analysed using the Fisher's exact test. All quoted *P* values are two-sided.

## RESULTS

### *BCL6* frequently involves *MYC* translocation in DLBCL with *MYC/BCL6/BCL2*-TH or *MYC/BCL6*-DH

Interphase FISH with the *BCL6/MYC* fusion probe was performed in 54 cases of DLBCL with *MYC/BCL2/BCL6*-TH ( $n = 32$ ) or *MYC/BCL6*-DH ( $n = 22$ ). Among these cases, 25 (46.3%) had evidence of genomic fusion between the *MYC* and *BCL6* loci by FISH (Fig. 2A), and the frequency of *MYC/BCL6* fusion was significantly higher in the *MYC/BCL2/BCL6*-TH (19/32 = 59%) than the *MYC/BCL6*-DH (6/22 = 27%) group (Fig. 2B).



**Fig. 2** *MYC/BCL6* fusion accounts for a high proportion of DLBCL with *MYC* and *BCL6* translocation. **A** Example of interphase FISH in a case with a triple hit (TH), in which the *MYC* and *BCL6* translocation detected by their breakapart probes (BAP) are due to *MYC/BCL6* fusion. **B** The frequency of *MYC/BCL6* fusion is significantly higher in cases with *MYC/BCL6/BCL2*-“TH” than those with *MYC/BCL6* “double hit” (DH).

Among the 25 cases with FISH evidence of *MYC/BCL6* fusion, 24 had complete data on *IG/MYC* fusion by interphase FISH with the *MYC/IGH* fusion probe, and additional *MYC/IGK(L)* fusion probe if no evidence of *MYC/IGH* fusion. Six of these cases had an *IGH::MYC* fusion, and this is consistent with previous observation of a three way translocation involving the *MYC*, *BCL6* and *IGH* loci by cytogenetic studies [18].

#### Genetic features of DLBCL with *MYC/BCL6* fusion

Mutation profiling by targeted NGS was carried out in 178 cases, and 135 of these cases were successfully subtyped using the LymphGen algorithm [3].

Overall, the mutation profile of the *MYC/BCL2/BCL6*-TH group is very similar to that of the *MYC/BCL2*-DH group (Fig. 3A), characterised by frequent mutations in follicular lymphoma associated genes (*BCL2*, *CREBBP*, *KMT2D*, *EZH2*, *TNFRSF14*). Our previous study shows that most cases with *MYC/BCL2/BCL6*-TH, like those with *MYC/BCL2*-DH, are GCB, with a subset being MHG [5]. In support of this, the present study further demonstrated that both *MYC/BCL2/BCL6*-TH (22/24 = 92%) and *MYC/BCL2*-DH (65/67 = 97%) groups were predominantly the EZB-MYC+ genetic subtype. Within the *MYC/BCL2/BCL6*-TH group, there were no apparent differences in the mutation profile and LymphGen genetic subtype between *MYC/BCL6* fusion positive and negative cases (Fig. 3A).

In contrast, the mutation profile of DLBCL-*MYC/BCL6*-DH was of less characteristic, but clearly differed from that of the *MYC/BCL2/BCL6*-TH or *MYC/BCL2*-DH group (Fig. 3A). The *MYC/BCL6*-DH cases vary in their COO subtype as shown in our previous study [5]. The present study further demonstrated that these cases varied in their LymphGen genetic subtypes although more frequently being the BN2 subtype or unclassifiable (Fig. 3B). Within the *MYC/BCL6*-

DH group, there were also no apparent differences in the mutation profile and genetic subtype between *MYC/BCL6* fusion positive and negative cases albeit based on few cases.

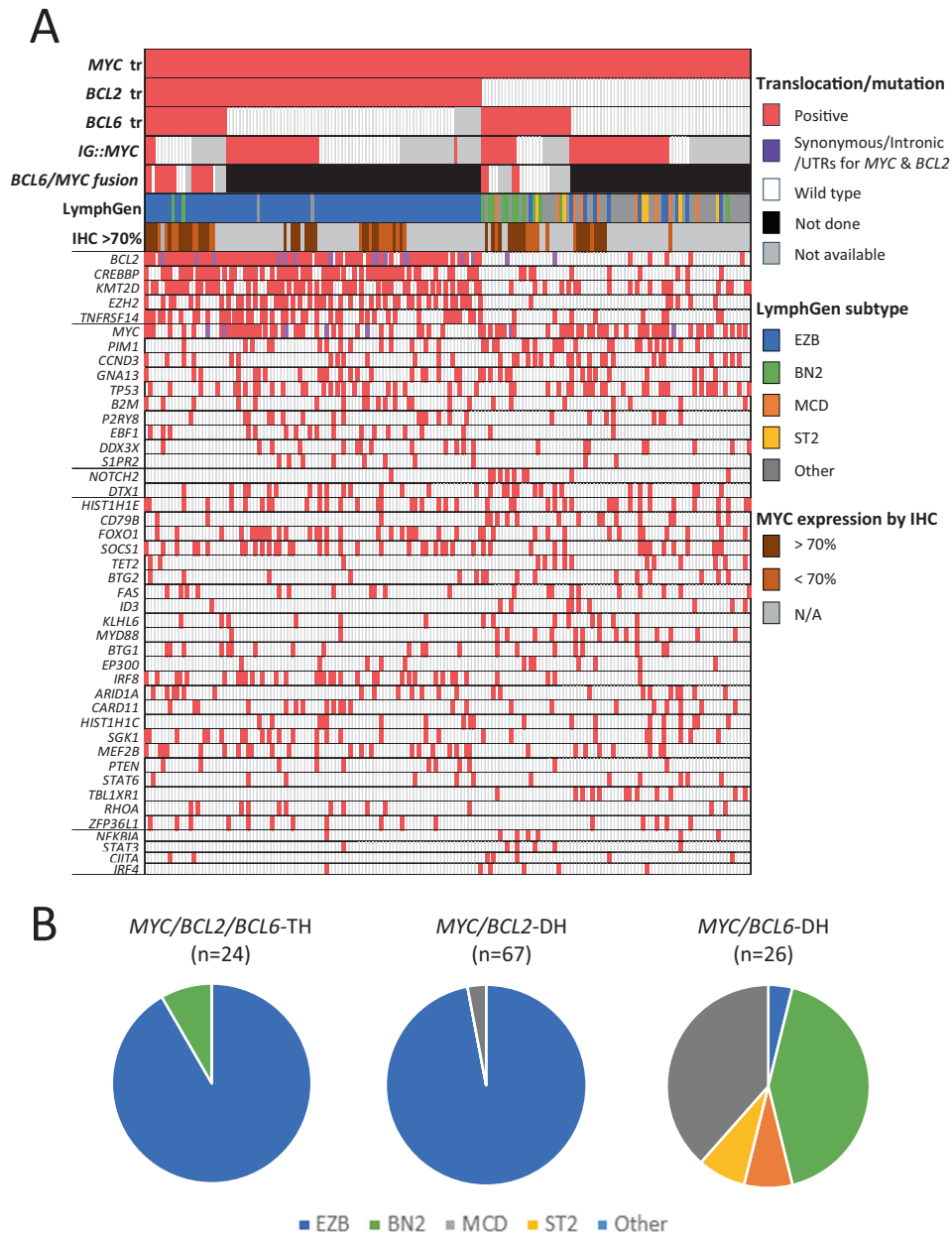
#### *MYC* protein expression is uniformly high in cases with *IG::MYC* but varies in those with non-*IG::MYC*

Given that *MYC* translocation is thought to dysregulate its transcription control, we compared *MYC* protein expression according to *MYC* translocation partner. High *MYC* expression was defined when the protein is expressed in 70% of lymphoma cells with moderate to strong staining by immunohistochemistry as such high *MYC* protein expression has been previously shown to identify high risk cases [11].

High *MYC* protein expression was seen in each of the 20 cases of DLBCL with *IG::MYC* translocation investigated (Fig. 4). Among DLBCL with non-*IG::MYC* translocation including those with *MYC/BCL6* fusion, *MYC* expression was variable, with only up to 50% cases showing a high *MYC* protein expression (Fig. 4B). There was no difference in the proportion of cases with high *MYC* protein expression between the *MYC/BCL6* fusion positive and negative groups (Fig. 4). These findings suggest that non-*IG::MYC* translocations may have variable effects on *MYC* transcription control and not every non-*IG::MYC* translocation can cause constitutive *MYC* expression.

#### Breakpoint analysis of *MYC* translocation reveal insights explaining variable *MYC* expression

To investigate why *MYC* protein expression was variable in cases with non-*IG::MYC* translocation, we performed TLC-NGS and breakpoint analyses in 8 cases, including 4 with non-*IG::MYC* (3 with *MYC/BCL6* fusion) and 4 with *IGH::MYC* respectively. In each case, TLC-NGS investigation confirmed the findings of FISH



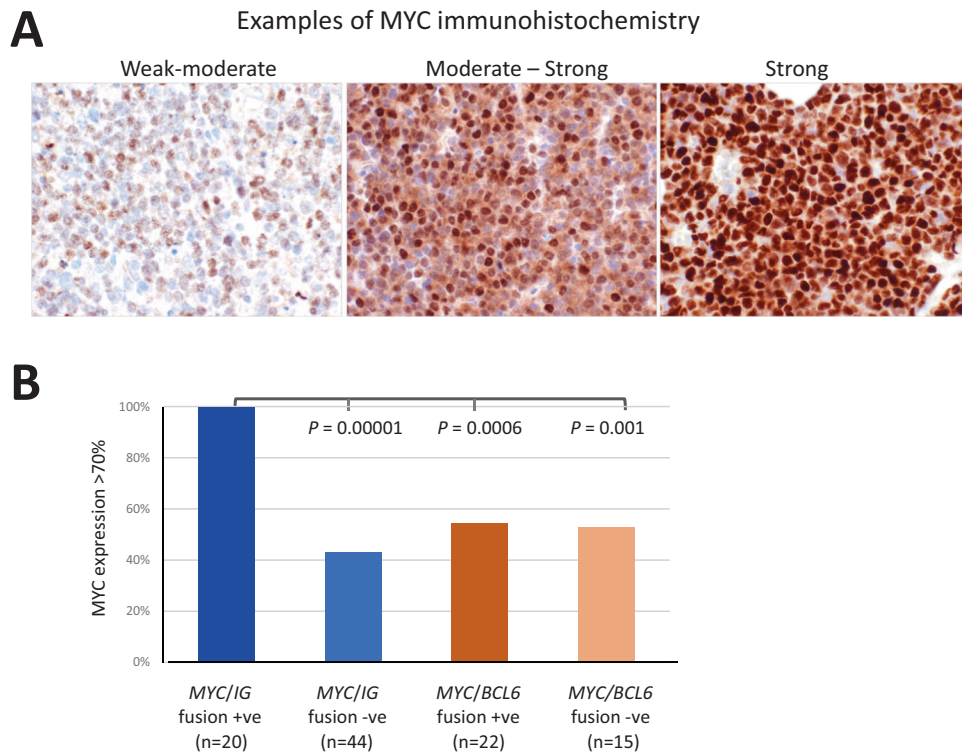
**Fig. 3** Mutation profile (A) and LymphGen genetic subtype (B) according to translocation status. tr translocation, IHC immunohistochemistry, TH triple hits, DH double hits.

analyses, and importantly unravelled the breakpoints and orientation of the involved genes, thus helping to understand their transcriptional potential (Table 1).

Among the three cases with *MYC/BCL6* fusion, two (DLBCL-134, DLBCL-173) involved direct juxtaposition between the *MYC* and *BCL6* loci, with the breakpoints occurring downstream or at the 3'UTR of the *MYC* gene, but upstream or within the intron 1 of the *BCL6* gene (Fig. 5). In both cases, the rearranged *MYC* and *BCL6* genes were in an opposite orientation, thus no structural changes in the 5' region of *MYC* transcriptional control albeit uncertain on any potential effect of the super enhancers downstream of the *MYC* and also at the 5' region of the *BCL6* gene [19, 20]. In both cases, the *MYC* protein expression was weak in <40% lymphoma cells. In the remaining case with *MYC/BCL6* fusion (DLBCL-123), an insertion of a segment of chromosome 3 sequence neighbouring to the *BCL6* locus together with a segment of the *IGH* switch region occurred within the intron 1 of the *MYC* gene (Fig. 6).

Although the precise breakpoints of the inserted *IGH* sequence could not be accurately defined, the involved region spanned the switch super enhancer, which could potentially drive *MYC* expression. In keeping with this, *MYC* protein was strongly expressed in most lymphoma cells in this case (Fig. 6).

Among the 5 cases without *MYC/BCL6* fusion by FISH, TLC-NGS analyses confirmed the FISH observations in each case, and further identified their translocation partners (Table 1). Two cases showed a novel *MYC* translocation: one fused with *TOX* at 8q12 in an opposite orientation (DLBCL-136), the other fused with *HNRNPA1* at 12q13 in the same orientation (DLBCL-154) (Figs. 5, 6). In both cases, the *MYC* breakpoint occurred either upstream (in the case with *TOX*) or in the intron 1 (in the case with *HNRNPA1*) of the *MYC* gene. In the case of *TOX/MYC* fusion, *MYC* transcription was unlikely driven directly by the *TOX* gene as the translocated *TOX* was in opposite orientation with *MYC* and loose its 5' transcriptional regulatory region, but *MYC* protein expression was



**Fig. 4** MYC protein expression and its correlation with MYC translocation partner. **A** Examples of MYC immunohistochemistry and grading; **(B)** high MYC protein expression is invariably seen in DLBCL with *IG::MYC*, but only in up to 50% cases with non-*IG::MYC* translocation..

**Table 1.** Detection of chromosome translocation by TLC-NGS.

Targets* Case	<i>BCL2</i>	<i>BCL6</i>	<i>MYC</i>	<i>IGH</i>
DLBCL-134	<i>IGH</i>	<i>MYC</i>	<i>BCL6</i>	<i>BCL2</i>
DLBCL-173	<i>IGH</i>	<i>MYC</i>	<i>BCL6</i>	<i>BCL2</i>
DLBCL-136	<i>IGH</i>	chr16 ( <i>CIITA</i> , intron 1)	chr8 ( <i>TOX</i> , intron 1)	<i>BCL2</i>
DLBCL-123		<i>IGH</i> , <i>MYC</i>	<i>BCL6</i> , <i>IGH</i>	<i>BCL6</i> , <i>MYC</i>
DLBCL-154	<i>IGH</i> , <i>BCL6</i>	<i>BCL2</i> , <i>IGH</i> ; chr3 (no genes annotated)	chr12 ( <i>HNRNPA1</i> , intron 1)	<i>BCL2</i> , <i>BCL6</i>
LO318	<i>IGH</i> , chr17 (~55 Mb)	chr4 (~40 Mb)	<i>IGH</i>	<i>BCL2</i> , <i>MYC</i>
DLBCL-96		<i>IGH</i>	<i>IGH</i>	<i>BCL6</i> , <i>MYC</i>
DLBCL-178		chr13 ( <i>LCP1</i> , intron 1)	chr14 (~69 Mb)	

\*Various targets captured by the TLC-NGS design, while the fusion partners identified are shown in the corresponding cell in each case.

moderately high. Interestingly, both TLC-NGS and interphase FISH in this case showed increased copies of both the rearranged (3–6 copies by interphase FISH) and non-rearranged (2 copies by interphase FISH) *MYC* alleles, in keeping with the variable staining extensity among lymphoma cells (Figs. 5, S1). In the case with *HNRNPA1::MYC* fusion, *MYC* was in the same orientation with *HNRNPA1*, and placed under the transcription control of *HNRNPA1*. *HNRNPA1* encodes a heterogeneous nuclear ribonucleoprotein that is ubiquitously expressed, and strong *MYC* protein expression was uniformly seen in lymphoma cells of this case (Fig. 6).

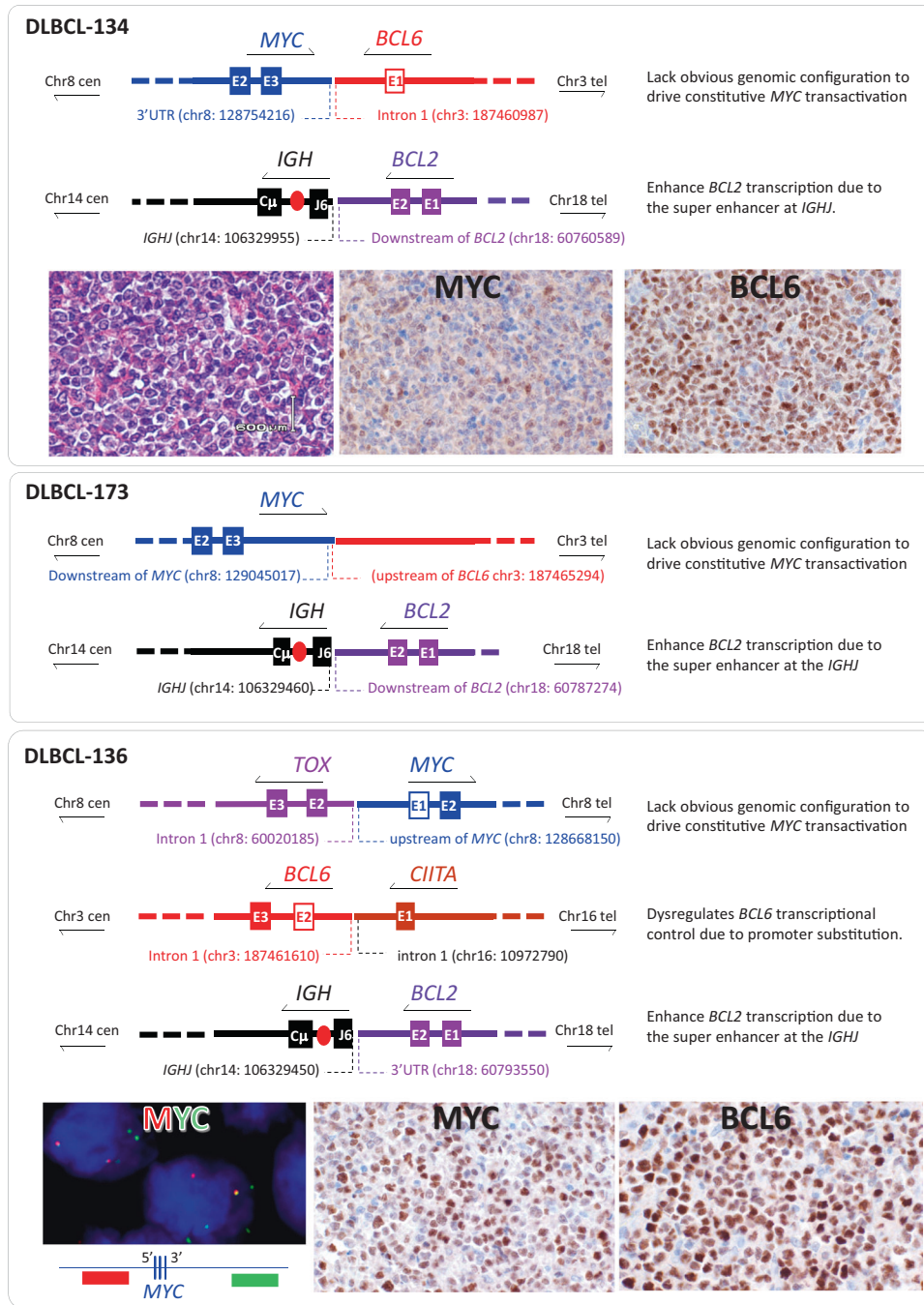
In the remaining three cases (LO318, DLBCL-96, DLBCL-178) without *MYC/BCL6* fusion, *MYC* translocation was associated with *IGH* (Table 1).

Apart from the above novel *MYC* translocations, TLC-NGS also identified previously known *LCP1::BCL6* (DLBCL-178, Fig. 7) and *CIITA::BCL6* fusion each in one case (DLBCL-136, Fig. 5). In both cases, the genomic fusion was in the same orientation and the

breakpoint was in the intron 1 of both *BCL6* gene and its partner gene, and these genomic configurations are typical of *BCL6* promoter substitution by its translocation which causes enhanced *BCL6* expression (Figs. 5, 7). In the case with *HNRNPA1::MYC* fusion (DLBCL-154), TLC-NGS revealed additionally a complex fusion among *BCL6*, *IGH* and *BCL2* (Fig. 6), with the *IGH* segment (from the joining to the switch region) in between the *BCL6* and *BCL2* gene on derivative chromosome 3. In this case, the presence of *IGH* super enhancers (at both joining and switch region) most likely drive constitutive *BCL6* and *BCL2* transactivation, hence the strong expression of both proteins in lymphoma cells (Fig. 6).

## DISCUSSION

The present study reports several significant novel findings, and they include: (1) *MYC* and *BCL6* translocation in a significant proportion of DLBCL, particularly those with *MYC/BCL2/BCL6*-TH,



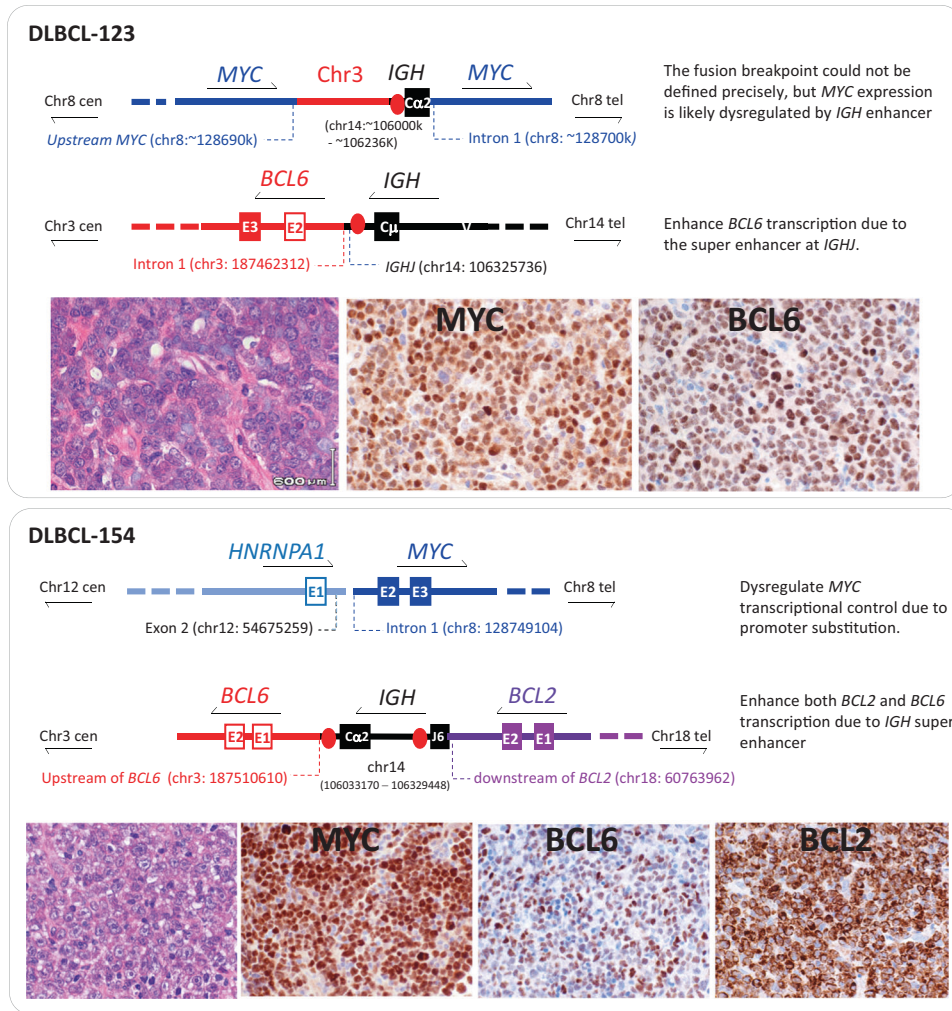
**Fig. 5** *MYC* translocation with *BCL6* or other partners lacks genomic configuration that activates *MYC* transcription. Genomic breakpoint sequencing analyses was performed by targeted locus capture-based next generation sequencing (TLC-NGS) with sequence annotations based on human genome (hg19). E: exon (fill box: coding exon; non-filled box: non-coding exon). Cen: centromere; Tel: telomere.

are due to a direct juxtaposition between the *MYC* and *BCL6* loci, rather than being an independent event; (2) *MYC* protein expression is uniformly high in DLBCL with *IG::MYC*, but varies in those with non-*IG::MYC*, including *BCL6/MYC* fusion; (3) *MYC* translocation with non-*IG* partner may not always acquire a genomic configuration that enables *MYC* constitutive transactivation, resulting in high *MYC* expression. These findings provide molecular insights, which explain several perplexing features of DLBCL with *MYC* translocation, and also bear practical implications in routine prognostic assessment.

*MYC* and *BCL6* translocation detected by interphase FISH with their respective break-apart probes was commonly referred as

independent oncogenic events, thus recorded as DH or TH when additional *BCL2* translocation is present. Remarkably, 59% of the so-called *MYC/BCL2/BCL6*-TH and 27% of *MYC/BCL6*-DH DLBCL are actually due to a direct genomic fusion between the *MYC* and *BCL6* loci. The finding is not totally unexpected as *MYC* is one of the many promiscuous translocation partners of *BCL6*, and *t(3;8)(q27;q24)/BCL6::MYC* and *t(3;8;14)(q27;q24;q32)/IGH::BCL6/MYC* have been previously reported [18, 21].

A major molecular mechanism underpinning the oncogenic potential of *MYC* translocation is its transactivation due to juxtaposition to a super enhancer, such as those at the *IGH* joining and switch region or promoter substitution. The *IGH* super



**Fig. 6** MYC translocation with IGH or novel partner confers genomic configuration that activates MYC transcription. Genomic breakpoint sequencing analyses was performed by targeted locus capture-based next generation sequencing (TLC-NGS) with sequence annotations based on human genome (hg19). E: exon (fill box: coding exon; non-filled box: non-coding exon). Cen: centromere; Tel: telomere.

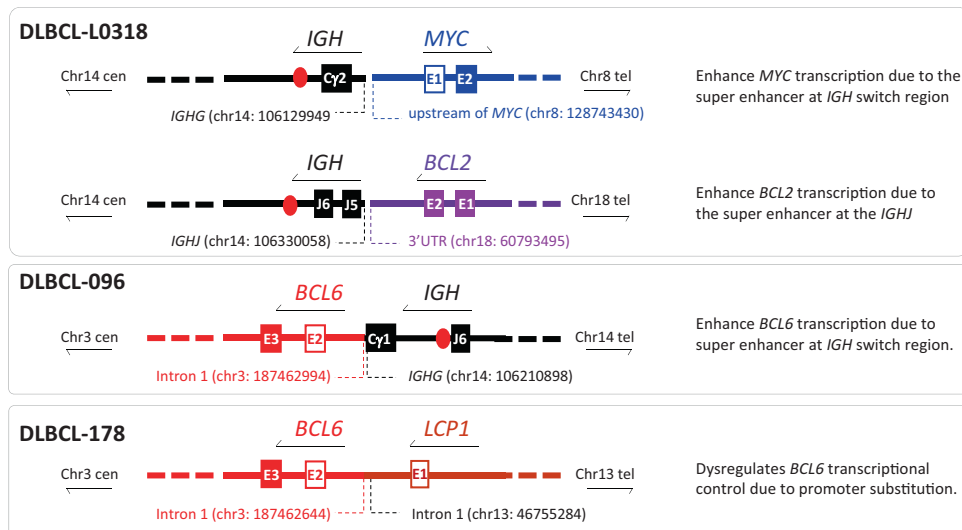
enhancers are expected to be highly active in all mature B-cells as they express high levels of immunoglobulin. Such super-enhancer mediated transcriptional activation, unlike promoter substitution, is independent of the genomic orientation of the MYC and IG genes and to a certain extent also of the “linear” distance between the two genes [22], thus explaining the uniform high MYC protein expression seen in DLBCL with IGH::MYC, and also Burkitt lymphoma.

Among the 4 cases of DLBCL with non-IGH::MYC investigated by TLC-NGS, 3 showed MYC gene in an opposite orientation with its translocation partner (BCL6, TOX), without affecting the MYC promoter region. The moderate variable MYC expression in the case with TOX::MYC (DLBCL-136) is most likely the result of MYC gene amplification (Fig. 5, Fig. S1). Otherwise, there was no evidence of constitutive MYC expression in these cases. There were potential super enhancers downstream of the MYC gene and in the translocated BCL6 region [19, 20], the potential impact on these super enhancers by these translocations is unclear. As the transactivation potential of super enhancers depend on cell type and differentiation stage and is regulated by a range of factors, such as genetic/epigenetic modifications and transcriptional factor binding [20, 23, 24], different translocations may give rise to variable potentials of MYC transactivation, from low levels of dysregulation to utmost constitutive activation. Nonetheless, lack of high MYC expression in these cases suggests these

translocations do not cause MYC constitutive transactivation. This speculation is in keeping with the previous observation that a proportion of DLBCL with MYC translocation lack high MYC mRNA and protein expression [11, 12]. In contrast, the remaining case (DLBCL-154) with HNRNPA1::MYC is a typical promoter substitution, and shows strong uniform MYC expression as expected since HNRNPA1, encoding for an RNA binding protein, is ubiquitously expressed (Fig. 6).

The above findings potentially explain why IGH::MYC, but not non-IGH::MYC confers significantly inferior survival in patients with DLBCL-MYC/BCL2-DH [7], and also why ~25% of DLBCL with MYC translocation, including those with a MYC/BCL2-DH, are conventional GCB, but not MHG subtype [1]. Our observations also highlight the heterogeneous MYC expression in DLBCL with non-IGH::MYC translocation. Of note, 44% of DLBCL with non-IGH::MYC/BCL2-DH lacked high MYC protein expression above 70% (Fig. 3A). It remains to be investigated whether there is any potential difference in clinical outcome between non-IGH::MYC translocation positive DLBCL with high and low MYC protein expression, and whether those with high MYC expression are similar to cases with IGH::MYC in their clinical outcome. To address this pivotal question, a large cohort of genetic subtype matched DLBCL with MYC translocation, such as those with MYC/BCL2-DH, is required.

In DLBCL, MYC and BCL6 translocation are most likely acquired due to relentless exposure to somatic hypermutation and class



**Fig. 7** *MYC* translocation with *IGH* or novel partner confers genomic configuration that activates *MYC* transcription. Genomic breakpoint sequencing analyses was performed by targeted locus capture-based next generation sequencing (TLC-NGS) with sequence annotations based on human genome (hg19). E: exon (fill box: coding exon; non-filled box: non-coding exon). Cen: centromere; Tel: telomere.

switch activities during B-cell expansion in germinal centres, and are likely a secondary event [13, 25]. This is particularly evident in cases with *BCL2* translocation, which is the primary genetic event, occurring as a consequence of erroneous VDJ recombination at the pre-B stage of B-cell development in the bone marrow. The secondary structural changes may not be always a driver event, similar to the point mutations in many well-known lymphoma genes acquired due to somatic hypermutation activities [26]. In view of this and the above discussion, it is pertinent to question whether every non-*IG::MYC* translocation in DLBCL is an activation event, albeit to be attested in future studies.

In routine clinical practice, interphase FISH is used for detection of *MYC*, *BCL2* and *BCL6* translocation, together with their translocation partners, although commonly only including *IGH*. Among *MYC* translocation positive DLBCL, *IG::MYC* accounts for ~55% of cases [7, 9]. The full spectrum of non-*IG* partners of *MYC* translocation remains to be characterised although *BCL6* may account for a majority. A major challenge to delineate whether a non-*IG/MYC* translocation is a constitutive activation event, thus clinically important, is to characterise its genomic configuration, search for evidence that enables *MYC* constitutive transactivation. This cannot be resolved by interphase FISH even when the translocation partner is known, but requires breakpoint analyses such as by TLC-NGS which is not yet available in a routine clinical setting. In the absence of any knowledge of genomic configuration of the translocation, the pathogenic potential and the prognostic value of non-*IG/MYC* translocation need to be interpreted in conjunction with *MYC* protein expression.

Our findings also raise the debate whether all DLBCL should be investigated for *MYC* translocation with regard to risk stratification in routine histopathological diagnosis by interphase FISH or first screened by *MYC* immunohistochemistry (where necessary immunohistochemistry with an alternative antibody to rule out potential false negative due to mutation impairing the antibody binding site [12]), and only cases with *MYC* protein expression above a certain level (to be determined) selected for further FISH analyses. Further breakpoint analysis of non-*IG/MYC* translocation and their correlation with the level of *MYC* protein expression in a large cohort should help to resolve these practical issues. Nonetheless, it is important to routinely investigate whether *MYC* translocation is associated with *IG* (both heavy and light chain) loci and *MYC* protein expression as both have been shown to be associated with adverse clinical outcome.

In summary, a significant proportion of DLBCL with both *MYC* and *BCL6* translocations are due to direct juxtaposition between the two genomic loci. *MYC* translocation involving non-*IG* loci including *BCL6* varies in their genomic configurations, and may not often gain genomic configuration that can cause constitute *MYC* transactivation, leading to its enhanced protein expression. The prognostic value of *MYC* translocation needs to be interpreted in conjunction with its translocation partner and *MYC* protein expression level.

#### DATA AVAILABILITY

All core data generated or analysed during this study are included in this published article, and additional raw data are available from the corresponding author on reasonable request.

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## AUTHOR CONTRIBUTIONS

FISH, targeted NGS, data collection and analyses: CZ, SB, FC, DJ, MMT, ZC, YL, JM, MQD; TLC-NGS: ES, JFS, HF, MQD; FISH and pathology: LRB, HL, HED, ES, MQD; Clinical data: NS, SKN, MK, MPP; Study design, case contribution, coordination and research funding: MQD, RT, DRW, AJD, CB, PWMJ; Manuscript writing and preparation: MQD with contributions from all authors. All authors read and approved the final manuscript.

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## COMPETING INTERESTS

ES, JFS and HF are employees of Cergentis, which owns patents on the TLC-NGS method. The authors declare no conflict of interest.

## ADDITIONAL INFORMATION

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