Distinct genetic changes reveal evolutionary history and heterogeneous molecular grade of DLBCL with *MYC*/*BCL2* double-hit

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ABSTRACT

Using a Burkitt lymphoma-like gene expression signature, we recently defined a high-risk molecular high-grade (MHG) group mainly within germinal centre B-cell like diffuse large B-cell lymphomas (GCB-DLBCL), which was enriched for *MYC*/*BCL2* double-hit (*MYC*/*BCL2*-DH). The genetic basis underlying MHG-DLBCL and their aggressive clinical behaviour remain unknown. We investigated 697 cases of DLBCL, particularly those with *MYC*/*BCL2*-DH (n=62) by targeted sequencing and gene expression profiling. We showed that DLBCL with *MYC*/*BCL2*-DH, and those with *BCL2* translocation, harbour the characteristic mutation signatures that are associated with follicular lymphoma and its high-grade transformation. We identified frequent *MYC* hotspot mutations that affect the phosphorylation site (T58) and its adjacent amino acids, which are important for MYC protein degradation. These *MYC* mutations were seen in a subset of cases with *MYC* translocation, but predominantly in those of MHG. The mutations were more frequent in double-hit lymphomas with IG as the *MYC* translocation partner, and were associated with higher MYC protein expression and poor patient survival. DLBCL with *MYC*/*BCL2*-DH and those with *BCL2* translocation alone are most likely derived from follicular lymphoma or its precursor lesion, and acquisition of *MYC* pathogenic mutations may augment MYC function, resulting in aggressive clinical behaviour.

INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is the most common lymphoma in adults, accounting for around 75% of aggressive lymphomas. The current standard treatment for DLBCL is immunochemotherapy, typically R-CHOP (rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone), with 60% of patients living 10 years or more.[1] Patients who fail R-CHOP treatment respond poorly to currently available alternative therapies, and mortality is highest within the first two years after diagnosis.[1] The ability to risk-stratify patients with a low probability of cure following R-CHOP at diagnosis, and to enter them into clinical trials investigating alternative therapies is a significant unmet clinical need. A number of biomarkers have been investigated, but only *MYC* translocation and to a lesser extent, cell of origin (COO), are used routinely or in a clinical trial.[1]

MYC translocation occurs in ~10% of DLBCL, and is frequently (21-83%) accompanied by an additional *BCL2* and/or *BCL6* translocation, known as double-hit (DH) or triple-hit (TH).[2-15] *MYC/BCL2*-DH DLBCL are generally aggressive and respond poorly to currently available therapies, with the majority of patients dying within two years of diagnosis, although a minority of cases experience a long term survival.[15,16] The clinical outcomes for *MYC*/*BCL6*-DH DLBCL are less clear owing to the small number of cases investigated and their heterogeneous COO.[14,17,18] Patients with a single *MYC* translocation (*MYC*-SH) show variable clinical courses. A subset of these cases has *TP53* mutations and displays a worse survival, similar to that of *MYC/BCL2*-DH.[18,19] There remains a need to clarify the prognostic stratification of *MYC* translocation-bearing DLBCL.

Based on COO classification, DLBCL has been divided into two broad subgroups: activated B-cell like (ABC) and germinal centre B-cell like (GCB) subtypes, with the ABC-DLBCL showing enhanced NF-κB activation and generally a worse prognosis.[20,21] Although the COO

classification provides broad biologically distinct categories, there is an apparent heterogeneity in clinical outcome within each subtype. Further sub-classification of these broad molecular subtypes has been investigated by several recent studies based on clustered genetic changes and/or gene expression signatures.[22-26] Among these recent advances, Sha et al and Ennishi et al have defined a clinically and biologically distinct high-risk subgroup within GCB-DLBCL using, respectively, a Burkitt lymphoma-like or *MYC*/*BCL2*-DH-founded gene expression signature.[25-27] This subgroup, termed molecular high-grade (MHG) in the study by Sha et al, is enriched in cases with *MYC*/*BCL2*-DH, and more importantly includes other poor prognosis cases without the double-hit, which are not readily identified by other methods.[25,28]

Intriguingly, although MHG-DLBCL is enriched in *MYC*/*BCL2*-DH, a proportion of cases with *MYC*/*BCL2*-DH do not have the MHG gene expression signature and these cases show no worse survival than that of conventional GCB-DLBCL (non-MHG).[25] To understand the genetic basis underlying MHG-DLBCL and its aggressive clinical behaviour, we used targeted sequencing to investigate 697 cases of DLBCL, particularly enriched for those with *MYC*/*BCL2*-DH (TH) (n=62).

METHODS

Case selection and materials

Of the 697 DLBCL cases included, 400 were from the REMoDL-B trial (28 *MYC* translocation positive) and 297 (97 *MYC* translocation positive) from a UK population based cohort.[25,28] The vast majority of cases in the population based cohort were from the Haematological Malignancy Research Network (HMRN) (www.hmrn.org), which tracks all haematological

105 malignancies across 14 hospitals diagnosed by a centralised fully-integrated
106 haematopathology laboratory.[29] Case selection in the present study was biased toward
107 those with a *MYC* translocation, which, together with *BCL2* and *BCL6* translocations, was
108 determined at the time of diagnosis (HMRN) or retrospectively collected from pathology
109 records or by performing interphase fluorescent in situ hybridisation (FISH) on TMA
110 (REMoDL-B).[18,25] Among the population based cohort, 5 cases had a previous history of
111 follicular lymphoma (FL), and a further 28 cases had a histological evidence of concurrent FL.
112 For the REMoDL-B trial, patients with a previous history of low grade lymphoma was
113 excluded.

114 Formalin-fixed paraffin-embedded (FFPE) diagnostic tissue biopsy was available in each case
115 and local ethical guidelines were followed for the use of these tissue materials for research
116 with the approval of the ethics committees of the involved institutions (05-Q1604-10, 04-
117 Q1205-125, 10-H0504-79).

118 **DNA extraction and quality assessment**

119 Haematoxylin and eosin stained FFPE tissue slides were reviewed and tumour rich areas
120 (>40%) in consecutive sections were isolated by crude macrodissection in each specimen.
121 DNA was extracted using the QIAamp DNA Micro Kit (QIAGEN, Crawley, UK) and quantified
122 using a Qubit® Fluorometer (Life Technologies, UK). The quality of DNA samples was
123 assessed by PCR of variably sized genomic fragments using a standardised protocol.[30]

124 **Targeted sequencing by HaloPlexHS enrichment and Illumina HiSeq sequencing**

125 This was essentially carried out as described previously.[31] Briefly, 100ng genomic DNA was
126 subjected to targeted enrichment of 70 genes (Table S1), which are recurrently mutated in

aggressive B-cell lymphomas using a customised HaloPlexHS probe library (Agilent Technologies). The HaloPlexHS probes incorporate molecular barcodes, hence allowing removal of PCR errors during data analysis. Library preparation was performed according to the manufacturer's instructions for FFPE tissue samples. The pooled libraries were sequenced on an Illumina HiSeq4000 (2 × 150 bp end sequencing protocol) or HiSeq2500 (Rapid Run Mode 2 × 150 bp end sequencing protocol). As stipulated by our previous study, DNA samples amenable for PCR of ≥400bp genomic fragments were investigated in a single replicate, while those amenable for PCR of 300bp were analysed in duplicates, with reproducible variants being considered as a true change.[31]

Variant calling and annotation

Sequence data analysis and variants calling were performed using a previously validated in-house protocol.[31] Briefly, SNV were called using UnifiedGenotyper with no downsampling.[32] As this was unable to call SNVs at <8% AAF reliably, MuTect2 was additionally employed for detection of hotspot mutations at low AAF values. Indel detection was separately carried out on the recalibrated bam files using Pindel v0.2.5,[33] which allowed detection of indels as low as 2% AAF. Variant calling files were concatenated to produce one library vcf each for the SNV and Indel pipelines, and then filtered using vcftools v0.1.15 and bedtools v2.25 for read depth, quality score, and known PCR/sequence artefacts. Further filtering was carried out to remove variants in intronic regions outside essential splicing sites, SNPs with a minor allele frequency ≥0.1% (dbSNP database, 1000 Genomes Project, the ExAC exome sequencing database) and synonymous changes. In addition, missense variants predicted to be benign by at least 7 out of 9 functional prediction tools (SIFT, Polyphen2 HDIV, Polyphen2 HVAR, LRT, MutationTaster, MutationAssessor, FATHMM, SVM score and LR score) were

excluded. The resulting variants were further scrutinised by reviewing the bam file to eliminate potential PCR and sequence artefacts. Only variants above the cut-off value (20 alternative allele depth for DNA samples amenable for PCR of ≥400bp, 15 alternative allele depth in both replicates for DNA samples amenable for PCR of 300bp) were considered to be a true change.[31] Finally, extensive search of COSMIC database and published literature was carried out to retain those known and confirmed to be somatic variants. The final mutation list can be found in Table S2.

Molecular subtyping by gene expression profiling

Whole genome gene expression profiling was performed on mRNA extracted from FFPE diagnostic tissue specimens using the Illumina whole genome DASL array.[34] Data analyses and COO classification were carried out using the "DLBCL automatic classifier" (DAC).[35] The MHG group was identified based on a Burkitt lymphoma-like signature as defined in previous studies.[25,36]

Interphase fluorescence in situ hybridisation (FISH)

Chromosome translocation status was available from routine haematopathological diagnosis or previous studies for *MYC*, *BCL2* and *BCL6* in 550, 233 and 218 cases respectively.[18,25] In the REMoDL-B and HMRN cohort, *MYC* translocation was screened with Dako *MYC* break-apart probe, and those showing no evidence of *MYC* translocation but with MHG phenotype were further investigated with Abbott *MYC* break-apart and *MYC*/*IGH* dual fusion probe. In the remaining cases from other UK hospitals, *MYC* translocation was investigated with Abbott *MYC* break-apart probe.

Interphase FISH was performed for *BCL2* and *BCL6* translocation in 433 and 366 cases in the present study. In cases with *MYC* translocation, additional FISH was performed with *MYC*/*IGH* (Abbott) (if not yet done), then *MYC*/*IGK* and *MYC*/*IGL* (Cytocell) dual fusion probes in those without any evidence of *MYC* and *IGH* fusion. All FISH was carried out on tissue microarray or whole tissue section as described previously.[18,25]

MYC immunohistochemistry

Data on MYC protein expression by immunohistochemistry on tissue microarrays (TMA) slides were available from our previous study.[25] The immunostained slides were scanned and MYC protein expression was quantified and presented as percentage of positive nuclear staining in lymphoma cells using IHC Profiler Image-J software according to the instructions for nuclear protein targets (https://imagej.net/).[37]

Statistical analysis

The probability of *MYC* hotspot mutations occurring by chance was assessed by Poisson regression. Associations among chromosomal rearrangements, mutations and clinical variables were analysed using Fisher's exact test. Survival analysis was carried out using Cox proportional hazards and likelihood ratio tests in R (https://cran.r-project.org). All quoted *P* values are two-sided.

RESULTS

Among the 697 cases investigated by targeted sequencing, 553 were investigated for chromosomal translocations by interphase FISH; and *MYC, BCL2,* and *BCL6* translocations

were found in 125, 136 and 97 cases respectively, with *MYC*/*BCL2*/*BCL6*-TH in 11, *MYC*/*BCL2*-DH in 51 (*BCL6* translocation data unknown in 8), and *MYC*/*BCL6*-DH in 22 (Figure 1&2).

The mutation profile of DLBCL with *MYC/BCL2*-DH *or BCL2*-SH suggests their derivation from follicular lymphoma

In general, DLBCL with *MYC*/*BCL2/BCL6*-TH and *MYC*/*BCL2*-DH had a similar mutation profile, and were characterised by a higher mutation load and more frequent mutations in *BCL2*, *KMT2D*, *CREBBP*, *EZH2* and *TNFRSF14* than those with isolated *MYC* translocation (Figure 2A, Figure S1&S2). Interestingly, these mutations are the cardinal features of follicular lymphoma,[38-42] and were similarly seen in DLBCL with *BCL2*-SH (Figure 2B). The *BCL2* mutation profile was almost identical between *BCL2* translocation positive DLBCL and follicular lymphoma (Figure 2C).[42] These findings suggest that DLBCL with a *BCL2* translocation may be derived from a follicular lymphoma or its precursor lesion. In support of this suggestion, DLBCL with *MYC*/*BCL2*-DH (TH) and those with *BCL2*-SH also harboured an additional mutation profile (*MYC*, *GNA13*, *TP53*, *P2RY8, PIM1*, *CCND3*, *B2M, EBF1* and *S1PR2*), which was associated with high-grade transformation of follicular lymphoma as shown by several previous studies (Figure 2B).[38-41] Furthermore, both groups (28% and 50% respectively) frequently presented with either a previous or concurrent follicular lymphoma (Figure 2D). Intriguingly, DLBCL with *MYC*-*BCL2*-DH(TH) were more often associated with a previous, but not concurrent follicular lymphoma than those with *BCL2*-SH. Nonetheless, 65% of BCL2 tr+ve cases lacked documented evidence of follicular lymphoma at diagnosis (Figure 2D). With the exception of *MYC* mutations, there was no significant difference in the mutation profile between *BCL2* translocation positive cases with and without follicular

lymphoma (Figure S3). *MYC* mutation was more frequent in BCL2 translocation positive cases without follicular lymphoma, and this was primarily due to a high frequency of *MYC* translocation in this group.

As expected, DLBCLs with *MYC/BCL2*-DH(TH) were either MHG (56%) or GCB (38%), the remaining cases being unclassifiable (6%) (Figure 3). Similarly, the majority of DLBCL with *BCL2*-SH were GCB (74%), with the remaining cases distributed among MHG (14%), ABC (4%) and unclassified (8%). It is worth noting that the three cases of ABC-DLBCL with *BCL2*-SH lacked both *EZH2* and *GNA13* mutations that were nearly exclusively seen in GCB (MHG)-DLBCL.

MHG-DLBCL with *MYC* translocation are enriched with *MYC* mutations that enhance its stability and transforming capacity

Our previous study showed that among patients with a *MYC* translocation, MHG-DLBCL had a significantly worse survival than GCB-DLBCL (non-MHG).[25] To understand the genetic basis underlying MHG-DLBCL and its aggressive clinical behaviour, we compared the mutation profile among MHG, GCB and ABC subtype, and also between MHG and GCB within *MYC*/*BCL2* double hit groups (Figure 4). This revealed a significantly higher frequency of *MYC* mutations in the MHG group (Figure 4B).

MYC is a known target of somatic hypermutation machinery, and as expected many of the *MYC* mutations were in the RCY-motif (R=A/G, Y=C/T), with their extent attenuating when further downstream from the promoter (Figure 5A). In comparison with synonymous mutations, there was a significant enrichment of nonsynonymous changes in codons 57, 58 and 59 (Figure 5A). Additionally, an in-frame deletion of codons 56-60 was seen in one case.

These mutations are likely to be functional, pathogenic and selected during lymphoma development as they affect the phosphorylation site (T58) and its neighbouring amino acids, which are important for MYC protein degradation.[43] Several lymphoma derived MYC mutants, including P57S and T58A, have been previously shown to dramatically increase the half-life of MYC protein, and also confer increased transforming capacity.[44,45]

These *MYC* hotspot mutations were seen in a subset of DLBCL with *MYC* translocation, more frequently in those with *MYC*/*BCL2*-DH, and the majority (74%) were MHG (Figure 5B). In contrast, cases with *MYC* non-synonymous mutations in other codons did not show any association with molecular subtype although occurred predominantly in those with *MYC*/*IG* translocation (Figure 5B&C). *MYC* hotspot mutations were significantly more frequent in cases with *MYC*/*IG* (41%) than those with *MYC*/non-*IG* translocation (8%), and together had a considerable overlap with MHG phenotype (Figure 5C). Cases with *MYC* hotspot mutations had a significantly higher MYC protein expression than those with other *MYC* mutations (Figure 5D) as shown by immunohistochemistry and quantitative analysis of the scanned immunostained slides.[25] Finally, *MYC* pathogenic mutations had a potential adverse effect on patient survival (Figure 6). Even within the MHG group, cases with *MYC* pathogenic mutations had significantly worse overall survival than those without these mutations in the REMoDL-B trial, and a similar trend was also seen in HMRN's population-based cohort (Figure 6 A&B). In a separate analysis within cases with *MYC*/*BCL2*-DH irrespective of their MHG status, cases with *MYC* pathogenic mutations also had significantly worse overall survival than those without these mutations in the REMoDL-B trial, albeit not in HMRN's population-based cohort (Figure S4). In multivariable analysis adjusting for MHG and *MYC*/*BCL2*-DH, *MYC* pathogenic mutations retained statistical significance in the REMoDL-B group, albeit not in the HMRN cohort.

A low allelic frequency (4-5% AAF) of the above *MYC* pathogenic mutations was seen in 3 cases, likely representing a subclonal change. Among these 3 cases, 2 had molecular subtyping data and 1 was MHG.

DLBCL with *MYC/BCL6*-DH or *BCL6*-SH, or *MYC*-SH are heterogeneous in their mutation profile and molecular subtype

There was no apparent mutation signature, nor biased molecular subtype associated with *BCL6* translocation with exception of *BCL10* and *NOTCH2* mutations which were significantly enriched in cases with *MYC/BCL6*-DH or *BCL6*-SH BCL6 (Figures 2A, 3 & S5). Similarly, there was no specific mutation signature in DLBCL with *MYC*-SH with the exception of high frequent *MYC* mutations. Cases with *MYC*-SH varied in their molecular subtype, nonetheless MHG (39%) and GCB (29%) accounted for the majority. Interestingly, 6 of the 11 MHG-DLBCL with *MYC*-SH had *TP53* mutations.

DISCUSSION

Using integrated analyses of chromosome translocation, somatic mutation profiling of a panel of 70 genes that are recurrently mutated in aggressive B-cell lymphoma, and molecular subtype in a large cohort of DLBCL, the present study made two novel observations.

Firstly, we have provided several strands of evidence indicating that DLBCL with *MYC*/*BCL2*-DH and those with *BCL2*-SH are most likely derived from a low-grade follicular lymphoma or its precursor lesion. These include finding: 1) a cardinal mutation signature (*BCL2*, *KMT2D*, *CREBBP*, *EZH2*, and *TNFRSF14*) associated with follicular lymphoma development; 2) a

mutation profile (*MYC*, *GNA13*, *TP53*, *P2RY8, PIM1*, *CCND3*, *B2M*, *EBF1* and *S1PR2*) associated with follicular lymphoma high-grade transformation; and 3) frequent presence of a previous or concurrent follicular lymphoma in DLBCL with *BCL2* translocation.

We acknowledge the limitation of the relatively small gene panel used in the present study, and the above speculation needs to be confirmed by more comprehensive genetic profiling. Nonetheless, the speculation is supported by the finding that transformed follicular lymphomas also show frequent *MYC* hotspot mutation.[41] In support of our study, the mutation signature associated with follicular lymphoma was also the characteristic change in high grade B-cell lymphoma with *MYC* and *BCL2* translocations,[46] and in the EZB or C3 genetic subgroup, which are enriched by *BCL2* translocation.[23,24]

Intriguingly, DLBCL with *MYC*/*BCL2*-DH had strong mutation signatures associated with follicular lymphoma, but less frequent evidence of a concurrent follicular lymphoma than those with *BCL2*-SH (28% vs 50%). Given the highly proliferative nature of DLBCL with *MYC*/*BCL2*-DH, these high-grade lymphoma cells may frequently efface the low-grade follicular lymphoma lesion, potentially leading to its underdetection. In addition, a single lymph node is commonly biopsied for histological diagnosis, increasingly needle core rather than excision biopsies. This would underestimate the true incidence of follicular lymphoma in patients with DLBCL. Alternatively, DLBCL with *MYC*/*BCL2*-DH may be derived from a precursor lesion, such as a common mutated precursor cell population. In fact, transformed follicular lymphomas are more commonly (66-83%) derived from a common mutated precursor cell (CPC) population, in a process of divergent evolution.[38-41] The tissue compartment containing the CPC population is likely to be in-situ follicular neoplasia (ISFN), the precursor lesion of FL, albeit this remains to be confirmed in future investigations. It is pertinent to speculate that *BCL2* translocation positive DLBCL could be similarly derived

from an ISFN lesion, regardless of any evidence for parallel follicular lymphoma development.

Secondly, we have identified frequent *MYC* hotspot mutations that affect the phosphorylation site (T58) and its adjacent amino acids, which are critical for FBXW7 mediated proteasome degradation (Figure 5A).[43] Such lymphoma derived MYC mutants (T58A, P57S) have been shown to increase the half-life of MYC protein from 30 to 110 minutes, and also confer increased transforming capacity, but are defective in promoting apoptosis due to failure to activate BIM.[44,45,47] Thus, these hotspot mutations are likely to be pathogenic and selected during lymphoma development.

Although these *MYC* hotspot mutations were seen in a subset of cases with *MYC* translocation, they were predominantly in MHG. This may explain, at least in part, the heterogeneous molecular subtype and clinical outcome of DLBCL with *MYC* translocation, including those with *MYC*/*BCL2*-DH. *MYC* hotspot mutations (pathogenic changes) were significantly more frequent in cases with *MYC/IG* than those with *MYC*/non-*IG* translocation (41% vs 8%, *P*=0.005), potentially explaining in part why these cases showed a worse prognosis than those with non-IG gene as the *MYC* partner.[48,49] Cases with *MYC* pathogenic mutations were significantly associated with higher MYC protein expression as assessed by immunohistochemistry and quantitative imaging analysis. This could potentially explain in part the variability (20-100%) of MYC protein expression in tumour cells of *MYC* translocation positive DLBCL.[50] The above findings are also consistent with the observation that the adverse prognosis of the *MYC*/*BCL2*-DH group is primarily due to those with IG as MYC translocation partner.[48,51]

It is worth noting that the above *MYC* hotspot mutations were also seen at a sub-clonal level, albeit in only a few cases. This finding raises an interesting question about whether tumour cells carrying these mutations are more resistant to therapies, and could thus be enriched in resistant or relapsed disease. If this is the case, the clinical impact of these *MYC* mutations may be under-estimated in the present study, as the investigation was exclusively based on diagnostic tissue biopsies.

More recently, a second hotspot of tumour associated *MYC* mutations was identified in codons 243-249 through meta-analysis of mutation data from Burkitt lymphoma.[52] These mutants (T244A, P245A) phenocopy the aforementioned mutations in their enhanced MYC protein stability, transforming capacity, and also defective BIM activation.[52] We did not observe any hotspot mutations in this region, but this could be due to the relatively small number of cases in the present study, or different mutation spectra between Burkitt and DLBCL. Mutation in codon 138 has also been suggested to enhance MYC protein stability and thus regarded as pathogenic change in DLBCL.[53,54] Nonetheless, mutation in codon 138 was frequently accompanied by change in codon 58, and its independent impact remains uncertain due to a very limited number of mutant cases identified.[54]

In the 2017 WHO lymphoma classification, *MYC*/*BCL6*-DH DLBCL are included in the double-hit category, without any distinction from those with *MYC*/*BCL2*/*BCL6*-TH or *MYC*/*BCL2*-DH.[55] We show here that DLBCL with *MYC*/*BCL6*-DH are significantly different in their mutation profile and molecular subtype from those with *MYC*/*BCL2*/*BCL6*-TH or *MYC*/*BCL2*-DH. This observation is further supported by a recent study albeit based on a smaller cohort.[46] In fact, *MYC*/*BCL6*-DH DLBCL are highly heterogeneous in their molecular subtypes, indicating their diverse COO, notwithstanding the high prevalence of *NOTCH2*

mutations.[23,24] *BCL6* translocation is recurrently seen in both follicular and marginal zone lymphoma.[56-59] It would be interesting to explore whether *MYC*/*BCL6*-DH DLBCL also result from high-grade transformation of a low-grade lesion such as follicular or marginal zone B-cell lymphoma or their precursor lesions, and their heterogeneous molecular subtypes reflect their inherent features from their derived low-grade lesion. These heterogeneities, in addition to the small numbers of cases available for each study, may explain the disparate clinical outcomes reported for *MYC*/*BCL6*-DH DLBCL.[14,17,18] In light of this, *MYC*/*BCL6*-DH DLBCL should not be regarded as a single group, and their biology and clinical management need to be explored in the context of their respective molecular subtype, rather than within the double-hit category.

In summary, DLBCL with *MYC*/*BCL2*-DH harbour the characteristic mutation signatures that are associated with follicular lymphoma development and its high-grade transformation, suggesting their derivation from follicular lymphoma or its precursor lesion, probably following acquisition of a *MYC* translocation. Our study also identifies the novel association of MHG-DLBCL with *MYC* hotspot mutations that enhance its stability and transforming capacity, and further highlight the pathogenic role of these mutations and their clinical significance, beyond transcriptional deregulation as a result of translocation.

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FIGURE LEGENDS

Figure 1: Summary of cases of DLBCL included in the study. A total of 697 cases were studied, including 400 from the REMoDL-B trial and 297 cases from population based cohort,

mainly from the Haematological Malignancy Research Network (HMRN). Laboratory data on chromosome translocations and molecular subtypes by gene expression profiling are indicated.

Figure 2: DLBCL with *BCL2* translocation harbor the cardinal mutation signature of follicular lymphoma. A) Heatmap illustration of mutation distribution according to chromosome translocation status; Where data available, evidence of previous or concurrent follicular lymphoma is indicated. B) DLBCL with *BCL2* translocation, particularly those with *MYC*/*BCL2*-DH, harbor the cardinal mutation signature of FL, and also the mutation profile associated with its high-grade transformation.[38-41] Representative mutation data in FL and transformed FL are from the study by Kridel et al,[41] with *EZH2* mutation considered as the core changes associated with FL.[38-41] C) Comparison of *BCL2* mutation profile between *BCL2* translocation positive DLBCL in the present study and FL in the study by Huet et al.[42] D) DLBCL with *BCL2* translocation often have a previous or concurrent follicular lymphoma. *MYC*/*BCL2*-DH: *MYC*/*BCL2*-double-hit; TH: *MYC*/*BCL2*/*BCL6*-triple hit; SH: single hit; tr-ve: translocation negative. FL: follicular lymphoma.

Figure 3: Molecular subtype of DLBCL according to translocation status. MHG: molecular high-grade; GCB: germinal center B-cell like; ABC: activated B-cell like; UNC: unclassified.

Figure 4: A) Comparison of mutation profile among MHG, GCB and ABC subtypes. The panel includes only the genes (n=57) that had a mutation frequency ≥5% in at least one molecular subtype to make the figure legible. B) Mutation comparison between MHG and GCB within the *MYC*/*BCL2*-DH(TH) group. Only genes showing a significant or apparent difference are included in the figure panel, with *BCL2* mutation included as a reference.

Fisher exact test was used to analyse the difference of mutation frequency between various groups with statistical significance indicated.

Figure 5. *MYC* pathogenic mutations and their relationship to molecular subtype and genetic changes in DLBCL. **A)** Distribution of *MYC* variants with hotspot mutations clustered at the phosphorylation site (T58) and its neighbouring amino acids that are important for *MYC* degradation. *One case shows an in-frame deletion of codons 56-60 and all other mutations are missense changes. The codons 57, 58 and 59 hotspot mutations and the in-frame deletion are likely pathogenic and selected during lymphoma development. *MYC* variants are annotated according to transcript ENST00000377970.6 in keeping with the literature. **B)** *MYC* hotspot mutations in codons 57, 58 and 59 are seen in a subset of cases with *MYC* translocation, more frequent in those with *MYC/BCL2-DH*, but are significantly enriched in MHG-DLBCL. **C)** *MYC* hotspot mutations are more commonly seen in *MYC/BCL2-DH* DLBCL with IG gene as the *MYC* translocation partner, with a considerable overlap with MHG phenotype. **D)** DLBCL with *MYC* mutation in codons 57-59 show a significantly higher MYC protein expression than those with *MYC* translocation, but lacking these pathogenic mutations. The MYC protein expression was investigated by immunohistochemistry, quantified using IHC Profiler Image-J software and presented as percentage of positive nuclear staining in lymphoma cells. Unpaired t-test was used to compare the two groups. MHG: molecular high-grade; GCB: germinal center B-cell like; ABC: activated B-cell like; UNC: unclassified.

Figure 6. Prognostic value of *MYC* codons 57-59 mutations in DLBCL. **A)** Differential impact on survival between *MYC* mutations in codons 57-59, and others. **B)** MHG-DLBCL with *MYC* mutations in codons 57-59 show the worst overall survival in comparison with GCB-DLBCL.

C) Single variable Cox proportional hazards regression analysis of progression-free survival in GCB-DLBCL. *In multivariable analysis adjusting for MHG and *MYC*/*BCL2*-DH, *MYC* pathogenic mutations in codons 57-59 retain statistical significance in the REMoDL-B cohort, albeit not in the HMRN cohort.

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Figure 1

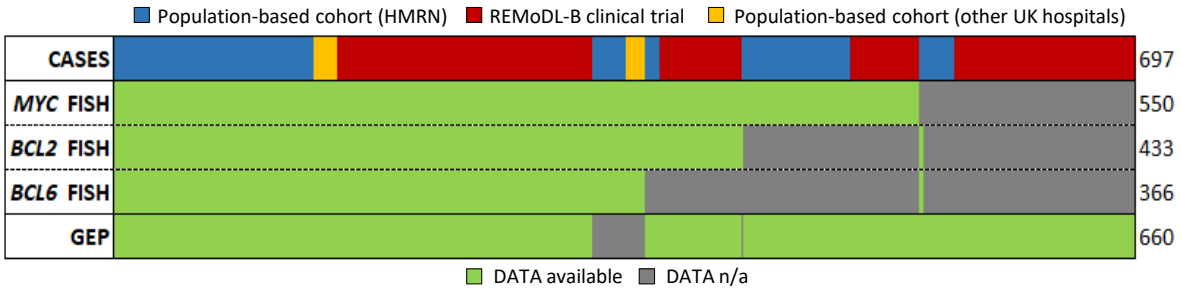
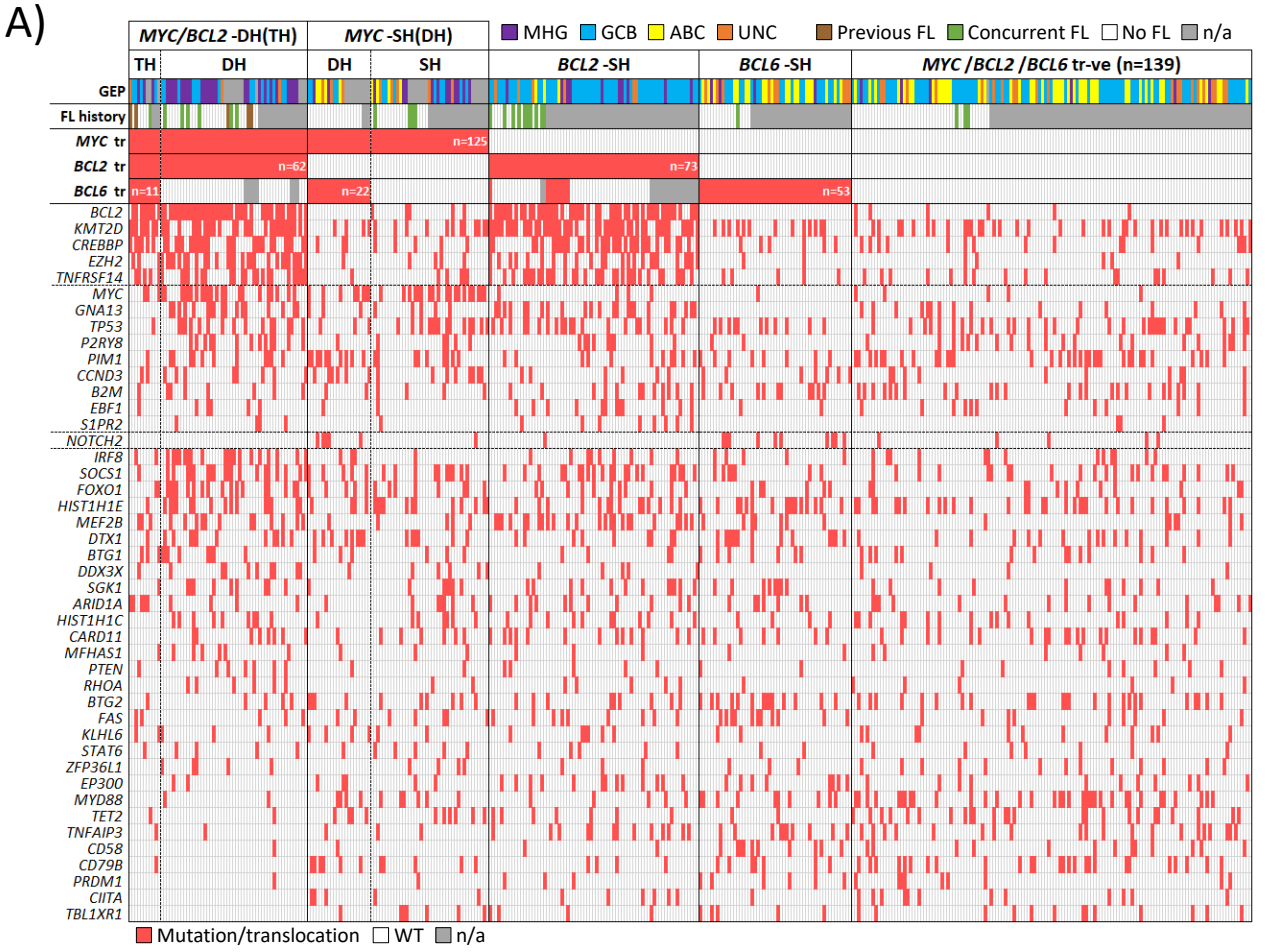

Population-based cohort (HMRN)
REMoDL-B clinical trial
Population-based cohort (other UK hospitals)
CASES
MYC FISH
BCL2 FISH
BCL6 FISH
GEP
697
550
433
366
660
DATA available
DATA n/a

Figure 2


A)
MYC/BCL2 -DH(TH)
MYC -SH(DH)
TH
DH
DH
SH
MHG
GCB
ABC
UNC
Previous FL
Concurrent FL
No FL
n/a
BCL2 -SH
BCL6 -SH
MYC /BCL2 /BCL6 tr-ve (n=139)
GEP
FL history
MYC tr
BCL2 tr
BCL6 tr
n=125
n=62
n=73
n=11
n=22
n=53
BCL2
KMT2D
CREBBP
EZH2
TNFRSF14
MYC
GNA13
TP53
P2RY8
PIM1
CCND3
B2M
EBF1
S1PR2
NOTCH2
IRF8
SOCS1
FOXO1
HIST1H1E
MEF2B
DTX1
BTG1
DDX3X
SGK1
ARID1A
HIST1H1C
CARD11
MFHAS1
PTEN
RHOA
BTG2
FAS
KLHL6
STAT6
ZFP36L1
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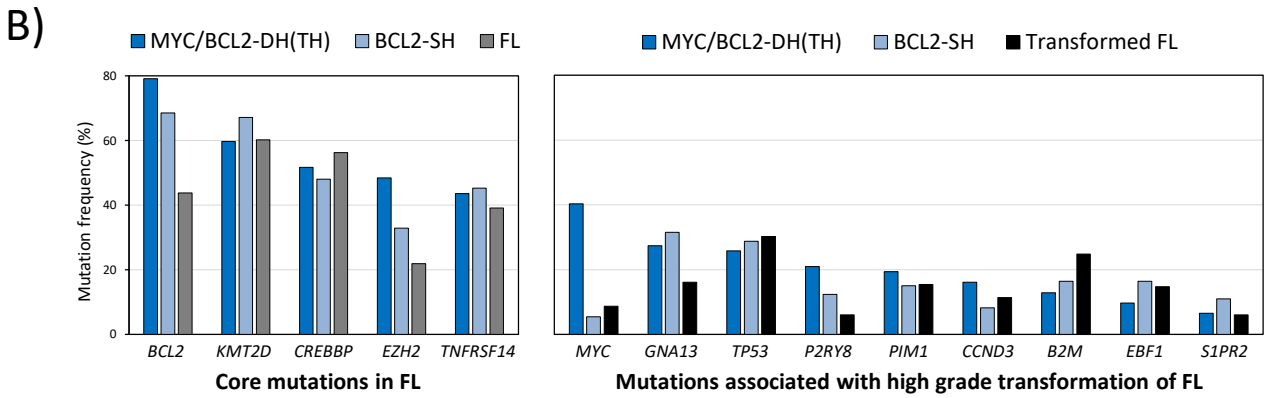
PRDM1
CIITA
TBL1XR1
Mutation/translocation
WT
n/a


B)
MYC/BCL2-DH(TH)
BCL2-SH
FL
Mutation frequency (%)
BCL2
KMT2D
CREBBP
EZH2
TNFRSF14
Core mutations in FL
MYC/BCL2-DH(TH)
BCL2-SH
Transformed FL
MYC
GNA13
TP53
P2RY8
PIM1
CCND3
B2M
EBF1
S1PR2
Mutations associated with high grade transformation of FL

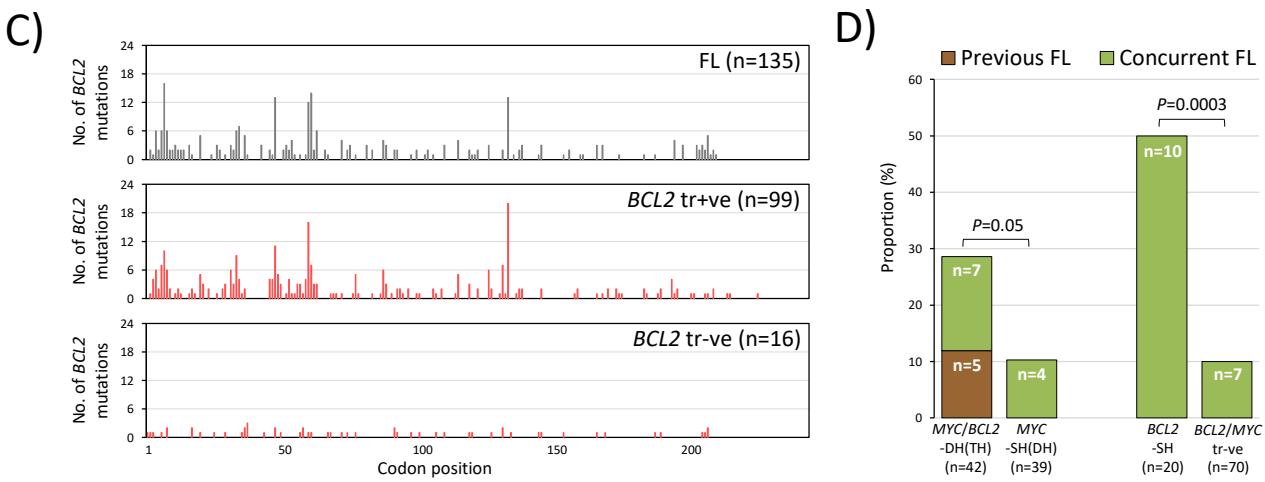

C)
No. of BCL2 mutations
FL (n=135)
BCL2 tr+ve (n=99)
BCL2 tr-ve (n=16)
Codon position
D)
Previous FL
Concurrent FL
Proportion (%)
P=0.05
P=0.0003
n=7
n=5
n=4
n=10
n=7
MYC/BCL2 -DH(TH) (n=42)
MYC -SH(DH) (n=39)
BCL2 -SH (n=20)
BCL2/MYC tr-ve (n=70)

Figure 3

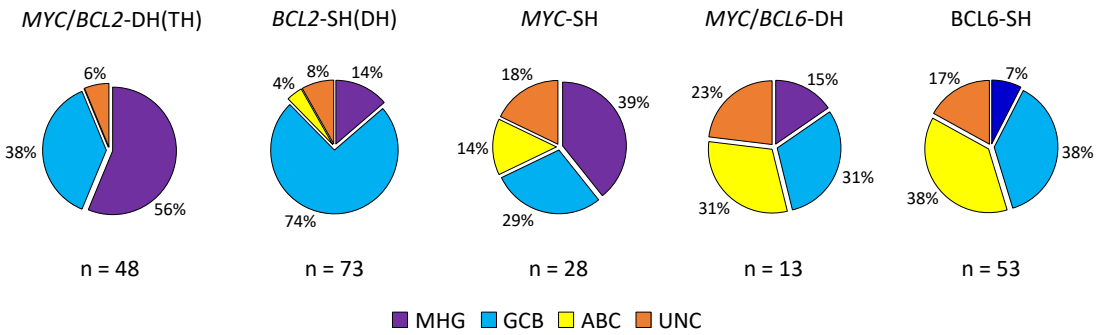
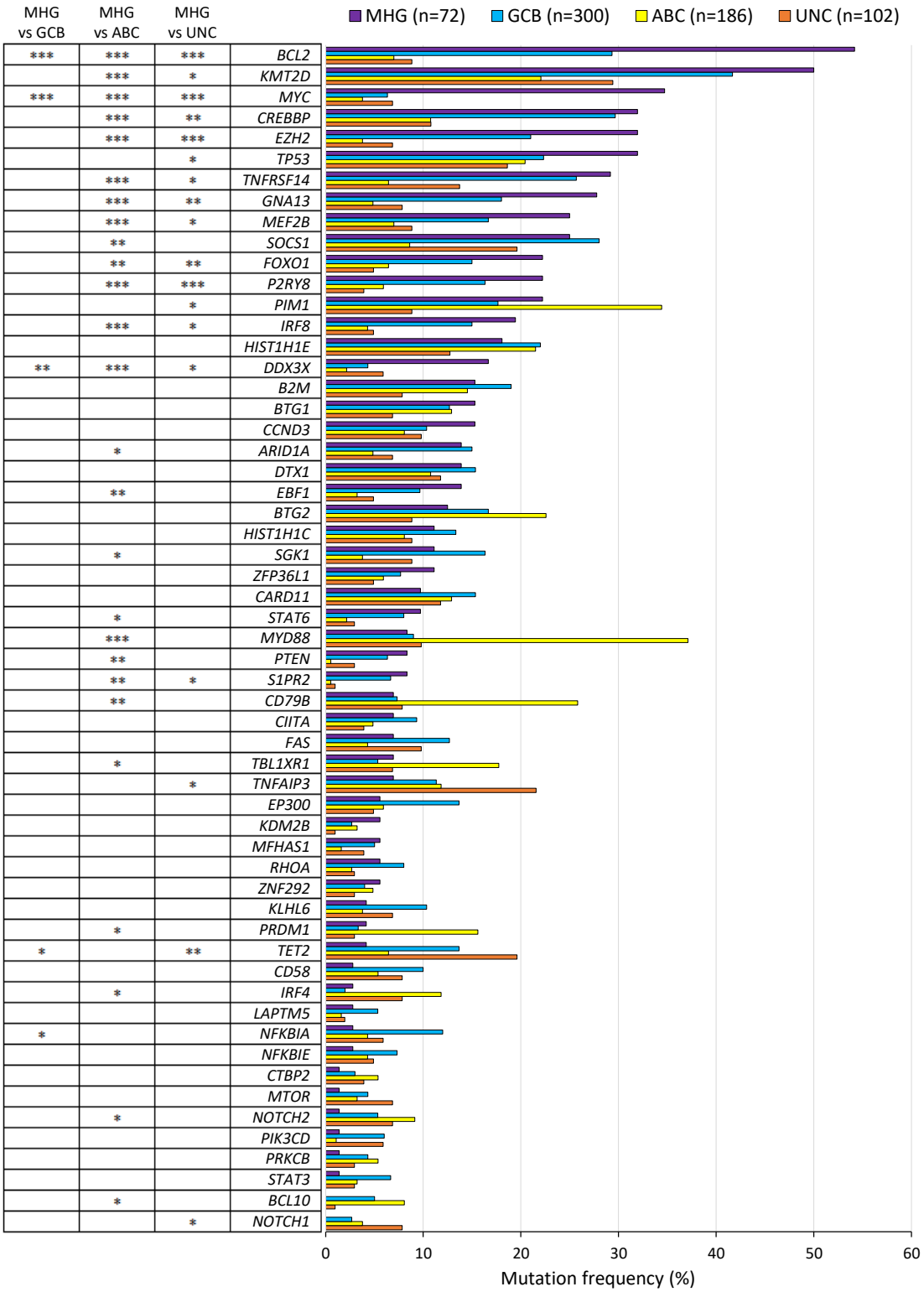

MYC/*BCL2*-DH(TH)
6%
38%
56%
n = 48
BCL2-SH(DH)
8%
14%
4%
74%
n = 73
MYC-SH
18%
39%
14%
29%
n = 28
MYC/*BCL6*-DH
23%
15%
31%
31%
n = 13
BCL6-SH
17%
7%
38%
38%
n = 53
MHG
GCB
ABC
UNC

Figure 4

A)


MHG vs GCB
MHG vs ABC
MHG vs UNC
MHG (n=72)
GCB (n=300)
ABC (n=186)
UNC (n=102)
BCL2
KMT2D
MYC
CREBBP
EZH2
TP53
TNFRSF14
GNA13
MEF2B
SOCS1
FOXO1
P2RY8
PIM1
IRF8
HIST1H1E
DDX3X
B2M
BTG1
CCND3
ARID1A
DTX1
EBF1
BTG2
HIST1H1C
SGK1
ZFP36L1
CARD11
STAT6
MYD88
PTEN
S1PR2
CD79B
CIITA
FAS
TBL1XR1
TNFAIP3
EP300
KDM2B
MFHAS1
RHOA
ZNF292
KLHL6
PRDM1
TET2
CD58
IRF4
LAPTM5
NFKBIA
NFKBIE
CTBP2
MTOR
NOTCH2
PIK3CD
PRKCB
STAT3
BCL10
NOTCH1
0
10
20
30
40
50
60
Mutation frequency (%)

B)

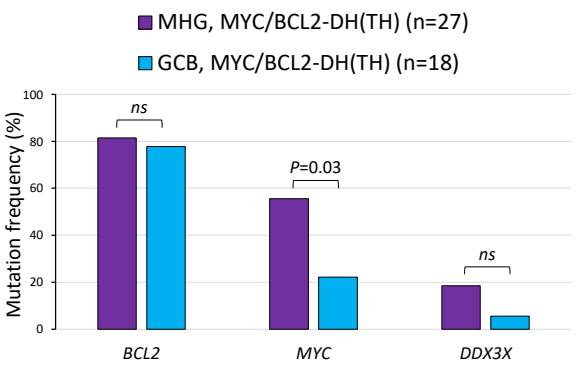

MHG, MYC/BCL2-DH(TH) (n=27)
GCB, MYC/BCL2-DH(TH) (n=18)
Mutation frequency (%)
100
80
60
40
20
0
ns
P=0.03
ns
BCL2
MYC
DDX3X

Figure 5

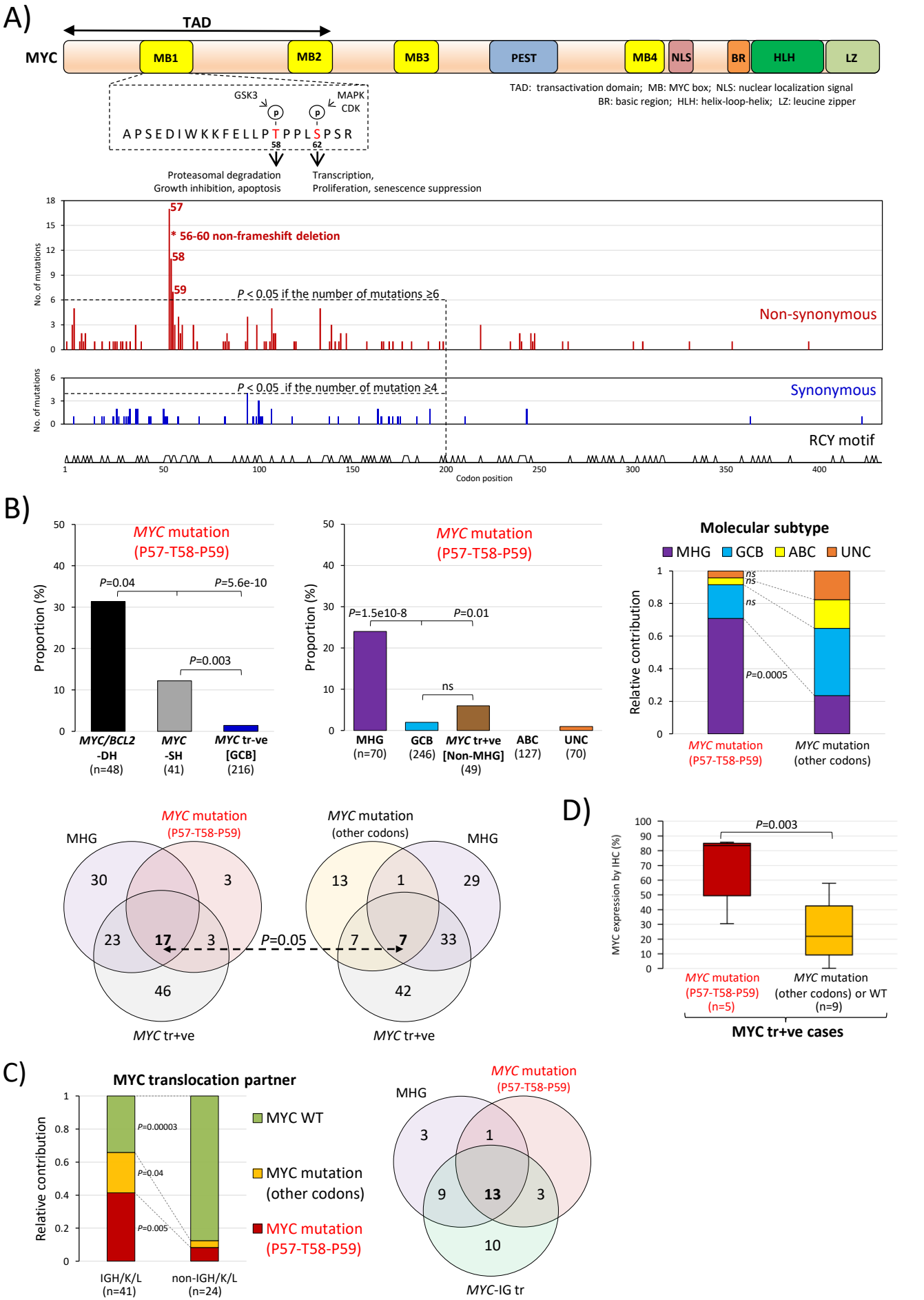
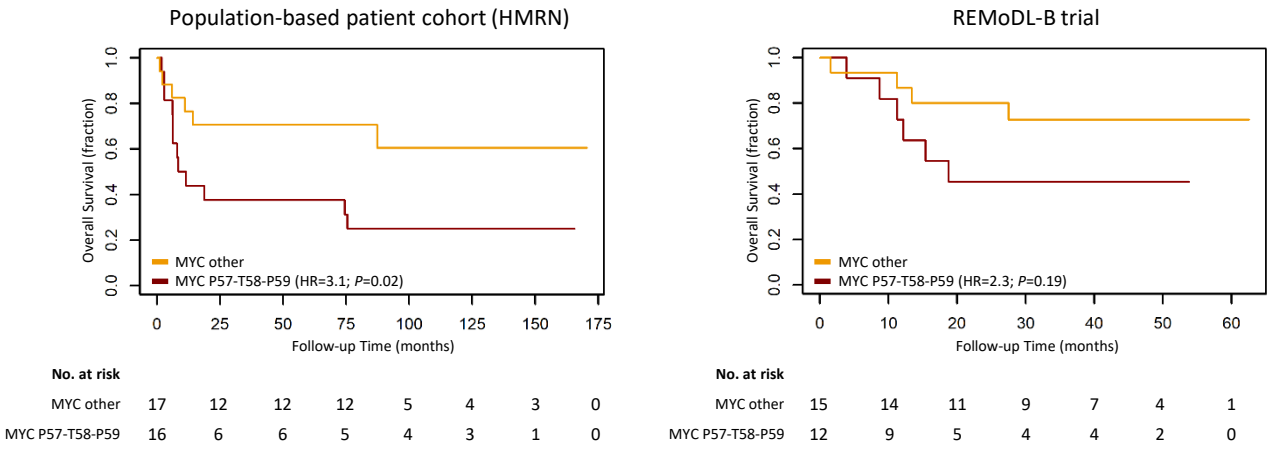
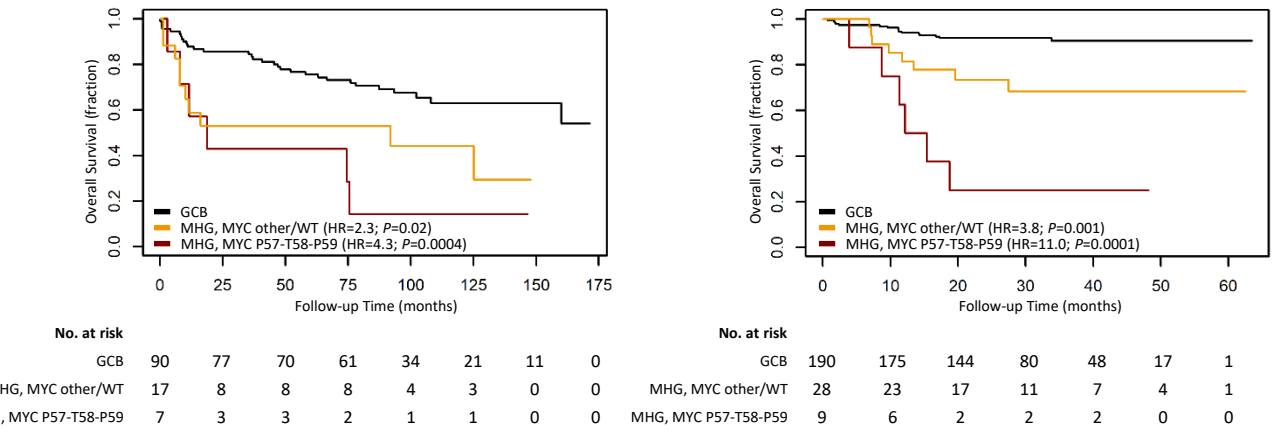

A)
TAD
MYC
MB1
MB2
MB3
PEST
MB4
NLS
BR
HLH
LZ
TAD: transactivation domain; MB: MYC box; NLS: nuclear localization signal
BR: basic region; HLH: helix-loop-helix; LZ: leucine zipper
GSK3
MAPK
CDK
APSEDIWKKFELLPTPPLSPSR
58
62
Proteasomal degradation
Growth inhibition, apoptosis
Transcription,
Proliferation, senescence suppression
57
* 56-60 non-frameshift deletion
58
59
P < 0.05 if the number of mutations ≥6
Non-synonymous
No. of mutations
P < 0.05 if the number of mutation ≥4
Synonymous
RCY motif
Codon position
B)
MYC mutation
(P57-T58-P59)
Proportion (%)
P=0.04
P=5.6e-10
P=0.003
MYC/BCL2
-DH
(n=48)
MYC
-SH
(41)
MYC tr-ve
[GCB]
(216)
P=1.5e10-8
P=0.01
ns
MHG
(n=70)
GCB
(246)
MYC tr+ve
[Non-MHG]
(49)
ABC
(127)
UNC
(70)
Molecular subtype
MHG
GCB
ABC
UNC
Relative contribution
P=0.0005
MYC mutation
(P57-T58-P59)
MYC mutation
(other codons)
MYC mutation
(other codons)
MHG
30
3
23
17
3
46
MYC tr+ve
P=0.05
13
1
29
7
7
33
42
D)
MYC expression by IHC (%)
P=0.003
MYC mutation
(P57-T58-P59)
(n=5)
MYC mutation
(other codons) or WT
(n=9)
MYC tr+ve cases
C)
MYC translocation partner
Relative contribution
P=0.00003
P=0.04
P=0.005
MYC WT
MYC mutation
(other codons)
MYC mutation
(P57-T58-P59)
IGH/K/L
(n=41)
non-IGH/K/L
(n=24)
MHG
3
1
9
13
3
10
MYC-IG tr

Figure 6

A)



B)



C)

Univariate Cox proportional hazards regression analysis of progression-free survival in GCB-DLBCL

		Population-based cohort (HMRN)		REMoDL-B trial	
		HR (95% CI)	***P* value**	**HR (95% CI)**	***P* value**
IPI	**Low (0 - 1)**	Reference		Reference	
	Intermediate (2 - 3)	2.1 (0.8, 5.7)	0.13	2.6 (1.1, 6.4)	0.032
	High (4 - 5)	5.2 (1.9, 14.0)	0.0013	3.4 (1.2, 9.6)	0.02
Age	**<60 year**	Reference		Reference	
	60 year	1.2 (0.63, 2.2)	0.61	0.89 (0.47, 1.7)	0.71
Stage	**I/II**	Reference		Reference	
	II/IV	2.8 (1.5, 5.5)	0.002	2.2 (1.0, 4.8)	0.042
MHG	**non-MHG**	Reference		Reference	
	MHG	1.9 (1.0, 3.7)	0.049	3.0 (1.6, 5.8)	0.001
TP53	**wild type**	Reference		Reference	
	mutation	1.6 (0.85, 2.9)	0.15	2.0 (1.1, 3.8)	0.03
***MYC* translocation**	**No**	Reference		Reference	
	Yes	1.8 (0.97, 3.2)	0.062	2.4 (1.1, 5.3)	0.034
***TP53* mutation & *MYC* translocation**	**No**	Reference		Reference	
	Yes	4.5 (1.8, 12)	0.0017	4.7 (1.8, 12)	0.0014
MYC* mutations in P57-T58-P59	**No**	Reference		Reference	
	Yes	1.4 (0.55, 3.5)	0.49	4.9 (2.1, 12)	0.0003
***MYC*/*BCL2*-DH**	**No**	Reference		Reference	
	Yes	2.1 (1.1, 3.9)	0.017	4.0 (1.8, 9.1)	0.0008