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Differences between BCL2-break positive and negative follicular lymphoma unraveled by whole-exome sequencing

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Depending on disease stage follicular lymphoma (FL) lack the t(14;18) in ~15–~50% of cases. Nevertheless, most of these cases express BCL2. To elucidate mechanisms triggering BCL2 expression and promoting pathogenesis in t(14;18)-negative FL, exonic single-nucleotide variant (SNV) profiles of 28 t(14;18)-positive and 13 t(14;18)-negative FL were analyzed, followed by the integration of copy-number changes, copy-neutral LOH and published gene-expression data as well as the assessment of immunoglobulin N-glycosylation sites. Typical FL mutations also affected t(14;18)-negative FL. Curated gene set/pathway annotation of genes mutated in either t(14;18)-positive or t(14;18)-negative FL revealed a strong enrichment of same or similar gene sets but also a more prominent or exclusive enrichment of immune response and N-glycosylation signatures in t(14;18)-negative FL. Mutated genes showed high BCL2 association in both subgroups. Among the genes mutated in t(14;18)-negative FL 555 were affected by copy-number alterations and/or copy-neutral LOH and 96 were differently expressed between t(14;18)-positive and t(14;18)-negative FL ($P < 0.01$). N-glycosylation sites were detected considerably less frequently in t(14;18)-negative FL. These results suggest a diverse portfolio of genetic alterations that may induce or regulate BCL2 expression or promote pathogenesis of t(14;18)-negative FL as well as a less specific but increased crosstalk with the microenvironment that may compensate for the lack of N-glycosylation.

INTRODUCTION

Follicular lymphoma (FL) is a germinal center (GC)-derived indolent lymphoma. Most patients eventually succumb to disease because of clinical progression or transformation to an aggressive variant.¹

Most FL harbor the t(14;18)(q32;q21) translocation, causing aberrant expression of the anti-apoptotic protein BCL2.¹ However, depending on the clinical stage, around ~15–~50% lack this translocation.^{2–4}

Nevertheless, BCL2 expression was observed in ~69–86% of t(14;18)-negative FL in a recent study.³ The underlying genetic mechanisms leading to BCL2 expression in t(14;18)-negative FL are, however, widely unknown.

Previous studies had reported high similarities in gene expression (GE), miRNA and copy-number (CN) profiles between t(14;18)-positive and -negative FL.^{4,5} Moreover, no significant survival differences were found in patients with advanced stage disease.³ Nevertheless, a significant enrichment of post-GC, NFκB as well as immune response (IR)-signatures and a downregulation of miR16 were found in t(14;18)-negative FL,

suggesting a late GC phenotype in this subgroup.^{4,5} Interestingly, these observations were more recently supported by the findings that t(14;18)-negative FL tend to transform into activated B-cell type diffuse large B-cell lymphoma (ABC-DLBCL).⁶

The genetic landscape of translocation-positive FL shows a number of genetic alterations, including frequently mutated genes such as *KMT2D* (*MLL2*), *CREBBP* and *EZH2*⁷ and some of these mutations might have prognostic impact.⁸ Moreover, immunogenetic features in FL have been widely studied, showing that the gain of novel N-glycosylation sites in immunoglobulin (IG) genes due to somatic hypermutation (SHM) may be an important mechanism driving FL pathogenesis.^{9–12}

In order to identify alternative mechanisms of BCL2 upregulation and apoptosis resistance and to search for mutations driving pathogenesis of t(14;18)-negative FL, we performed a comprehensive whole-exome (WE) single-nucleotide variant (SNV) profiling of BCL2-translocation-negative FL and complemented our analysis with CN and GE-profiling as well as the assessment of IG N-glycosylation sites.

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MATERIALS AND METHODS

Study cohorts

Fresh frozen (FF) and formalin-fixed paraffin-embedded (FFPE) material was available from 41 FL grades 1–3A. *BCL2*-breakpoints were assessed in either FFPE- or FF-tissue using fluorescence *in situ* hybridization with *BCL2*-BAP probes (Abbott Molecular, Ludwigshafen, Germany).^{13,14} Since *BCL2*-translocation partners other than *IGH* are rare in FL,¹⁵ we use the terms t(14;18)-positive and t(14;18)-negative FL throughout the manuscript. WE-sequencing (WES) and SNP6.0-arrays were performed in FF-tumor samples of 11 FL (10 t(14;18)-negative, one t(14;18)-positive) retrieved from the files of the Institute of Pathology, University of Würzburg (Supplementary Table S1). *BCL2* expression in these cases was assessed by immunohistochemistry in FFPE tissue using the clones 124 (DAKO, Glostrup, Denmark) and E17 (Cell Marque, 1:100, Rocklin, CA, USA). Whole-genome sequencing was performed within the International Cancer Genome Consortium (ICGC)-MMML-Seq project on FF-material of 30 FL specimens (three t(14;18)-negative, 27 t(14;18)-positive) and their corresponding normal controls as described previously.¹⁶ Readily filtered SNV data and information on the *BCL2*-breakpoint status of the ICGC cohort was provided by the ICGC MMML-Seq consortium for further downstream-analysis (see also Bioinformatics Evaluation). Sequencing of the IG variable (IgV)-regions was accomplished using FF-material of 10 t(14;18)-negative FL, also implemented in the WES.⁴ GE data and the t(14;18)-status of additional 184 FL was available from previous publications.^{4,17} The study was approved by the Ethics committees of the Universities Kiel and Würzburg and by the recruiting centers.

Whole-exome sequencing

The WE-libraries (37 Mb, excluding UTRs) were generated starting from 30 ng DNA using the Nextera Enrichment DNA Sample Preparation Kit (Illumina, San Diego, CA, USA) and sequenced with 72 bp paired-end reads on the GAllx (Illumina) according to the manufacturer's instructions.

SNP6.0 microarray analysis

DNA was isolated from the 10 FF WE-sequenced t(14;18)-negative FL using the Allprep Kit (Qiagen, Hilde, Germany) followed by hybridization to SNP6.0-arrays (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's protocol. CN- and loss of heterozygosity (LOH)-data were analyzed using the genotyping console and the CHAS-software from Affymetrix.

Sequencing of the immunoglobulin variable region

Ig-sequences were analyzed using the CE-IVD certified LymphoTrack Dx IGH FR1 Assay MiSeq kit and/or the Lymphotrack IGH Somatic Hypermutation Assay—PGM (Invivoscribe, San Diego, CA, USA) according to the manufacturer's protocols.

Bioinformatics evaluation

Raw sequences, revealed by WES, were analyzed using CASAVA (configureBclToFastq.pl, Illumina), BWA (Burrows-Wheeler Alignment Tool) and the HaploTypeCaller of GATK (Genome Analysis Toolkit) (Supplementary Table S2). Annotations of SNVs (e.g. to amino acid (AA)-exchanges, gene-symbols) and mutated genes (e.g. curated gene sets (including pathways and signatures), chromosomal sites, gene-families) were performed using SeattleSeq, GSEA,¹⁸ STRING¹⁹ as well as bioDBnet,²⁰ respectively. In addition, WES data was analyzed using the published bioinformatics pipeline of the ICGC which was also used for the whole-genome sequencing data.¹⁶ SNPs listed in 1000 genomes and dsbSNPv138 were excluded from the data sets if not specified otherwise. SNVs corresponding to the regions of the Nextera design file were extracted from the whole-genome sequencing data and integrated in our analysis. The overlap of SNV data with CN/LOH and published GE-microarray data of 147 t(14;18)-positive and 17 t(14;18)-negative FL^{4,17} was accomplished using short python-scripts. The highest scoring Ig-sequence was analyzed using IG-Blast and gains or losses of N-glycosylation sites were identified by visual inspection as previously described.¹² Clustering analysis was performed with the Cluster software from Michael Eisen.²¹ WES and SNP6.0 array data in this paper are deposited in the EGA with accession number EGAS00001002164 (see also Supplementary Methods).

RESULTS

Low-stage FL are enriched in the t(14;18)-negative FL sub-cohort. Out of seven t(14;18)-negative WE-sequenced FL with clinical information available, four patients had early-stage disease (Supplementary Table S1). Immunohistochemistry analysis of 10 t(14;18)-negative FL collected for WES revealed *BCL2* expression in 7/10 (70%) FL.

Sequencing data output and quality control

WES revealed a ~80–~120× mean-coverage on target and uniformity at 1× of at least ~94% (Supplementary Table S2). Excluding SNPs/SNVs listed in 1000 genomes and dsbSNPv138, 1250 unique SNVs were called in the 11 WE-sequenced FL cases (10 t(14;18)-negative, 1 t(14;18)-positive) affecting 1057 genes using our in-house bioinformatics pipeline (Supplementary Table S3a) and 1642 SNVs, affecting 1477 genes using the ICGC pipeline (Supplementary Table S3b). Whole-genome sequencing in the ICGC cohort (3 t(14;18)-negative, 27 t(14;18)-positive FL plus corresponding normal DNA) revealed 1583 SNVs affecting 1305 genes after tumor-normal-subtraction (Supplementary Table S3c). The validation rate of the WES data was 85%. A validation approach focusing on genes, called by both bioinformatics pipelines in t(14;18)-negative FL, revealed a validation rate of 98%. Curated gene set/pathway annotation was restricted to those genes that carried SNVs called by both bioinformatics pipelines and genes mutated in the cases of the ICGC cohort (t(14;18)-negative: 456 genes (~35 genes/case), t(14;18)-positive: 1102 genes (39 genes/case) (Supplementary Table S4). In total, 148 SNVs of t(14;18)-negative WE-sequenced FL were validated by Sanger sequencing (Supplementary Table S5).

The SNV profiles of t(14;18)-negative tumors support a diagnosis of FL.

Our FL cohort (13 t(14;18)-negative, 28 t(14;18)-positive) was affected by mutations in genes known to be recurrently mutated in FL such as *KMT2D* (*MML2*), *BCL2*, *TNFRSF14*, *CREBBP*, *HIST1H1 B-E*, *EZH2*, *MEF2B* and *STAT6* (Table 1).^{6,8,22–27} Specifically, t(14;18)-negative FL of the current cohort were affected by *CREBBP* in ~38%, *STAT6* in ~23%, and *EZH2*, *EP300* and *HIST1H1C-E* in ~15% of the cases, respectively.

t(14;18)-negative FL show recurrent mutations in genes with oncogenic potential

Around 10% ($n=255$) mutated genes were present in both t(14;18)-negative and -positive FL (Supplementary Table S6a), while 1435 and 1234 genes were exclusively mutated in t(14;18)-negative or -positive FL, respectively (Supplementary Table S6b and c). Of note, *MEF2B* and *BCL2* carried SNVs in 3 out of 28 (~11%) and 13 out of 28 (~46%) t(14;18)-positive FL, respectively, but in none of the t(14;18)-negative FL. Recurrent mutations were detected in 193 (Supplementary Table S7a and b) and 118 (Supplementary Table S7c and d) genes in t(14;18)-negative and t(14;18)-positive FL, respectively. Out of these, 135 genes were exclusively mutated in t(14;18)-negative FL (Supplementary Table S7a) and 106 genes exclusively mutated in t(14;18)-positive FL (Supplementary Table S7c). Fifty-eight and 12 genes were mutated in both cohorts, but more frequently in t(14;18)-negative and t(14;18)-positive FL, respectively (Supplementary Table S7b and d). Exclusion of point-mutations only called in the 10 t(14;18)-negative WE-sequenced FL by only one of the two bioinformatics pipelines or not called in any of the three t(14;18)-negative FL for which germline SNV filtering was available, resulted in a list of 88 genes recurrently mutated in t(14;18)-negative FL. Among these, were cancer-associated genes such as *ASH1L*, *SLITRK4*, *ANK2* and *LRP1B* which were exclusively mutated at frequencies ranging from ~23% (3/13) to ~31% (4/13) (Supplementary Table S6b) and

Table 1. SNV profile of FL with and without t(14;18) with respect to genes recurrently mutated in FL according to the literature^{6,8,22–28}

Gene/%	t(14;18)-neg FL	t(14;18)-pos FL	FL (lit)	SMZL (lit)	NMZL (lit)	EMZL-MALT (lit)	ABC-DLBCL (lit)	DLBCL, NOS (lit)	GCB-DLBCL (lit)
KMT2D (MLL2)	~ 8	~ 46	67–89	11	34		41	32–38	46
BCL2	0	~ 46	25–76	0	0		1		24–45
TNFRSF14	~ 15	~ 18	25–36	0	11		2	22	13–17
CREBBP	~ 38	~ 61	33–75	5	6		6–13		31–32
HIST1H1C-E	~ 15	~ 15	8–28	0	9		25		9
EZH2	~ 15	~ 14	12–31	0	0		0		18–22
TNFAIP3/A20	~ 8	0	3–11	7–8	9–33	15–30	15–30		2–11
MEF2B	0	~ 9	10–31	0	0		12	11	23
EP300	~ 15	~ 7	9–23	5	6		4–15		2–14
STAT6	~ 23	~ 14	9–12	0	0		0		14
IRF8	~ 15	~ 4	6–9	0	0		9		15
TP53	~ 8	~ 4	5–12	15	0	19	18–19		16–26

Framed in bold: genes recurrently mutated in FL but not in marginal zone lymphoma (MZL).

Table 2. Reduced list of recurrently mutated genes that were exclusively or more frequently mutated in t(14;18)-negative FL

Gene	Gain	Loss	cnLOH	Chr	PhastCons (sample 1, 2, ...)	Polyphen (sample 1, 2, ...)
SUMF2 ^a	Yes	No	No	7	0.99, 1.00	Damaging, damaging
NEO1 ^a	No	No	Yes	15	0.99, 1.00	Damaging, damaging
MYO10 ^a	No	No	No	5	1.00, 1.00	Damaging, damaging
ATF7IP ^a	No	No	Yes	12	0.99, 0.96	Damaging, damaging
SOGA2 ^a	No	No	No	18	0.79, 0.98	Damaging, damaging
RNF169 ^{a,b}	No	No	Yes	11	1.00, 1.00	Damaging, damaging
CUBN ^a	No	No	No	10	0.49, 0.35	Damaging, damaging
DOCK10 ^a	No	No	No	2	0.01, 1.00	Damaging, damaging
ASH1L ^a	Yes	No	Yes	1	1.00, 1.00	Damaging, damaging
CENPI ^{a,c}	No	No	Yes	X	1.00	Damaging
FAM35A ^{a,d}	Yes	No	No	10	0.66, 0.24	Damaging, damaging
MCTP2 ^a	No	No	No	15	0.65, 0.00	Damaging, benign
LRP6 ^a	No	No	No	12	0.99, 1.00	Damaging, benign
CEP78 ^a	No	No	No	9	0.91, 1.00	Benign, damaging
ZBED5 ^a	No	No	No	11	1.00, 1.00	Damaging, benign
MLH3 ^{a,c}	No	No	No	14	0.45	Benign
ANK2 ^a	Yes	No	No	4	1.00, 0.24, 1.00, 0.97	Damaging, benign, damaging, benign
MS4A1 ^a	No	No	No	11	0.00, 0.00	Benign, benign
PCLO ^a	No	No	No	7	1.00, 0.00	Damaging, benign
PLEKHH2 ^a	Yes	No	No	2	0.99, 1.00	Damaging, damaging
SCIN ^a	No	No	No	7	0.99, 0.00	Damaging, benign
STARD9 ^a	No	No	Yes	15	0.01, 0.00	Unknown, unknown
USH2A ^e	No	No	No	1	1.00	Damaging

In a first step, point-mutations only called in the 10 t(14;18)-negative in-house FL by only one of the two bioinformatics pipelines were excluded. Subsequently, only SNVs were kept that were called by IntOGen v 2.2.4, located in highly conserved regions according to PhastCons, leading to a structural change according to PolyPhen or affected genes of biological relevance according to the literature. Finally, only genes are depicted in this list that carry SNVs validated by Sanger sequencing (see Supplementary Table S5). Of note, validation was limited to samples retrieved from the files of the Institute of Pathology in Würzburg.

^aExclusively mutated in t(14;18)-negative FL. ^bSNV listed in dbSNP138 (not listed in Supplementary Table S3). ^c1 SNV in a whole-exome sequenced sample that was collected from the files of the institute of pathology in Würzburg and 1 SNV in a sample of the ICGC cohort. ^d1 sample with indel (indels not reported in Supplementary Table S3). ^eMutated in both subgroups, but more frequently mutated in t(14;18)-negative FL.

further cancer-associated genes which were not exclusively but more frequently mutated in t(14;18)-negative FL (Supplementary Tables S6b and S8).^{29–40} Finally, focusing on genes that carried SNVs called by IntOGen v 2.2.4⁴¹ revealed high conservation scores according to PhastCons (>0.9), were damaging according to PolyPhen or were of biological relevance according to literature-data resulted in a list of 23 core genes more frequently mutated in t(14;18)-negative FL (Table 2). Approximately one-third of these genes accumulated in chromosomes 1q, 7 and 15q (Table 2). These results suggest that several cancer-associated genes are recurrently mutated in t(14;18)-negative FL and less frequently or not at all mutated in t(14;18)-positive FL.

Enrichment of mutations in apoptosis-related and BCL2-associated genes in t(14;18)-positive and -negative FL

Curated gene set annotation of genes affected by SNVs found in either t(14;18)-negative ($n=456$) or -positive FL ($n=1102$) demonstrated the majority of mutations to be significantly enriched in apoptosis, cell cycle/proliferation, epigenetic processes and immune/inflammatory response signatures (Figure 1 and Supplementary Table S9a and b) by P -values and FDR- q values of <0.001. Specifically, 40/100 top gene sets (40%) were identical and 73–81% identical or similar among t(14;18)-positive and -negative FL. A comparison between t(14;18)-negative FL with and without BCL2 expression also revealed an enrichment of apoptosis-signatures in both subgroups; however, the enrichment

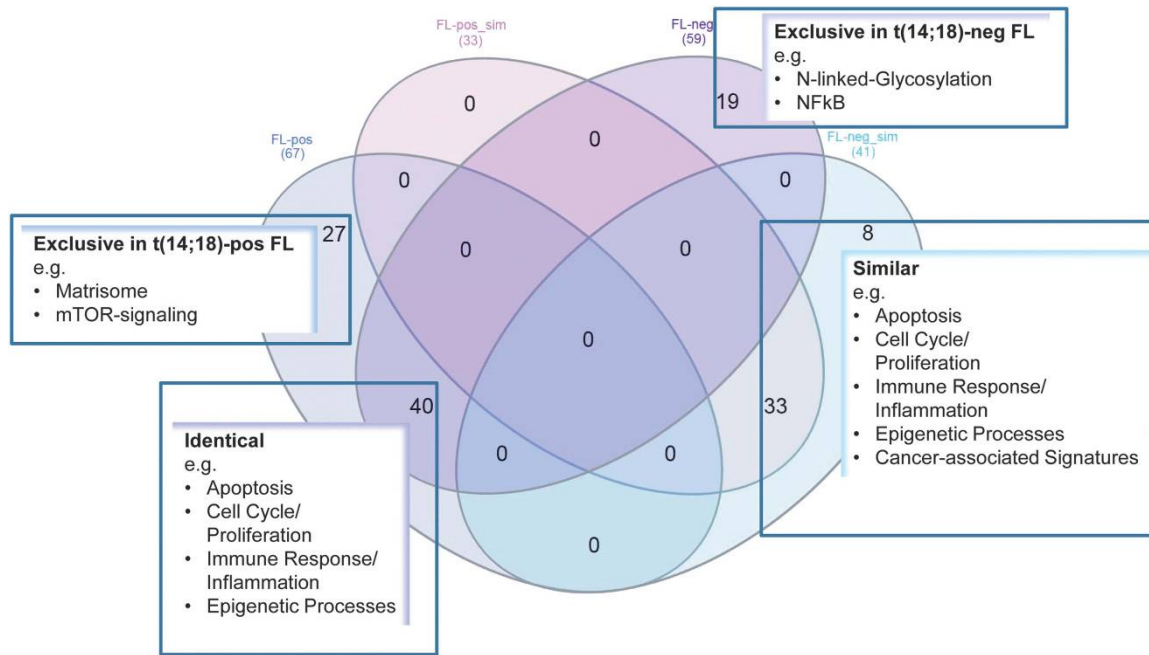


Figure 1. Signatures/pathways enriched in t(14;18)-positive and -negative FL according to GSEA annotation (C2) of genes affected by SNVs, called in either t(14;18)-positive or -negative FL. Only genes were annotated called by both bioinformatic pipelines or in samples of the ICGC data set (t(14;18)-negative: 456 genes, t(14;18)-positive: 1102 genes). First 100 pathways with a *P*-value of < 0.001 and an FDR of < 0.05 are depicted (see also Supplementary Table S9a and b). Venn diagram: *InteractiVenn* (<http://www.interactivenn.net>).

was more prominent in the cases with BCL2 expression (Supplementary Table S10a and b). These observations and the fact that BCL2 is also expressed in t(14;18)-negative FL suggests that BCL2 is an important player in FL pathogenesis. Thus, we investigated BCL2 association of mutated genes in all FL subcohorts by STRING analysis.

Comparing t(14;18)-positive and -negative FL almost all mutated genes clustered together with BCL2 within the same STRING-network using low confidence settings (Table 3a and Supplementary Figure S1). At high confidence, the network still comprised 40–50% of mutated genes in both cohorts (Table 3a and Supplementary Table S11a and b). Moreover, only minor differences were found in the BCL2 association level comparing the WES t(14;18)-negative FL with and without BCL2 expression (Table 3b and Supplementary Table 11c and d). Among the genes mutated in t(14;18)-negative but BCL2-positive FL having direct BCL2 association at highest confidence were for instance *MAPK8* and *PTPA/PPP2R4* (Table 3c). Overall, these results indicate that both t(14;18)-positive and -negative FL carry mutations in BCL2-associated and apoptosis-related genes to a significant extent.

Many SNVs detected in t(14;18)-negative FL correlate with differential mRNA expression and other genetic lesions

Assuming that genes with a high relevance in FL-pathogenesis may accumulate different genetic alterations, we performed an integrative analysis of SNVs, GE, CNAs and cnLOH. An overlap of genes with significantly ($P < 0.01$) different mRNA expression according to previously published data⁴ with genes harboring non-synonymous SNVs in t(14;18)-negative FL exclusively revealed 96 candidates (Supplementary Table S12a). Subsequently, these genes were scored by different criteria (e.g. SNV frequency, biological relevance, BCL2 association, occurrence of CN alteration or cnLOH) and supervised clustering was performed of top-scoring genes (≥ 4) such as the TS CDC73, and the MAPK8 and MAP3K9 kinases to visualize differences between FL with and without t(14;18) (Figure 2 and Supplementary Table S12b). Furthermore,

Table 3a. Amount of mutated genes with BCL2 association (directly or indirectly) according to STRING analysis in t(14;18)-negative and t(14;18)-positive FL

Confidence	BCL2 association of genes mutated in FL with or without t(14;18)	
	t(14;18)-negative FL (n = 1435) (%)	t(14;18)-positive FL (n = 1234) (%)
Low	~ 99	~ 96
Medium	~ 83	~ 76
High	~ 46	~ 40
Highest	~ 29	~ 20

Table 3b. Amount of mutated genes with BCL2 association (directly or indirectly) according to STRING analysis in t(14;18)-negative FL with and without BCL2 expression

Confidence	<i>BCL2</i> association of genes mutated in t(14;18)-neg FL with or without <i>BCL2</i> -expression	
	t(14;18)-negative FL – <i>BCL2</i> (%)	t(14;18)-negative FL + <i>BCL2</i> (%)
Low	~ 57	~ 64
Medium	~ 24	~ 36
High	~ 12	~ 12
Highest	0	~ 1

we focused on CNAs and cnLOH affecting the same genomic sites targeted by non-synonymous SNVs in the t(14;18)-negative WES cohort and revealed 555 matches (Figure 3 and Supplementary Table S13a and b). Genes targeted by SNVs and gains were significantly enriched ($P < 0.001$ and FDR < 0.05) at chromosomes

Table 3c. Mutated genes with direct BCL2 association at highest confidence in t(14;18)-negative FL

#node1	node2	score > 0.9	CN_gain	CN_loss	cnLOH	differential_GE_p < 0.01
MTOR	BCL2	0.976	No	No	No	No
DIABLO	BCL2	0.971	No	No	No	No
CTNNB1	BCL2	0.925	No	No	No	Yes
MAPK8	BCL2	0.999	No	No	Yes	Yes
PRKACB	BCL2	0.913	No	No	Yes	No
PTGS2	BCL2	0.942	Yes	No	Yes	No
PPP2R4/PTPA	BCL2	0.943	No	No	Yes	No
BCL2	ITPR1	0.979	No	No	No	Yes
BCL2	MMP2	0.929	No	No	No	No

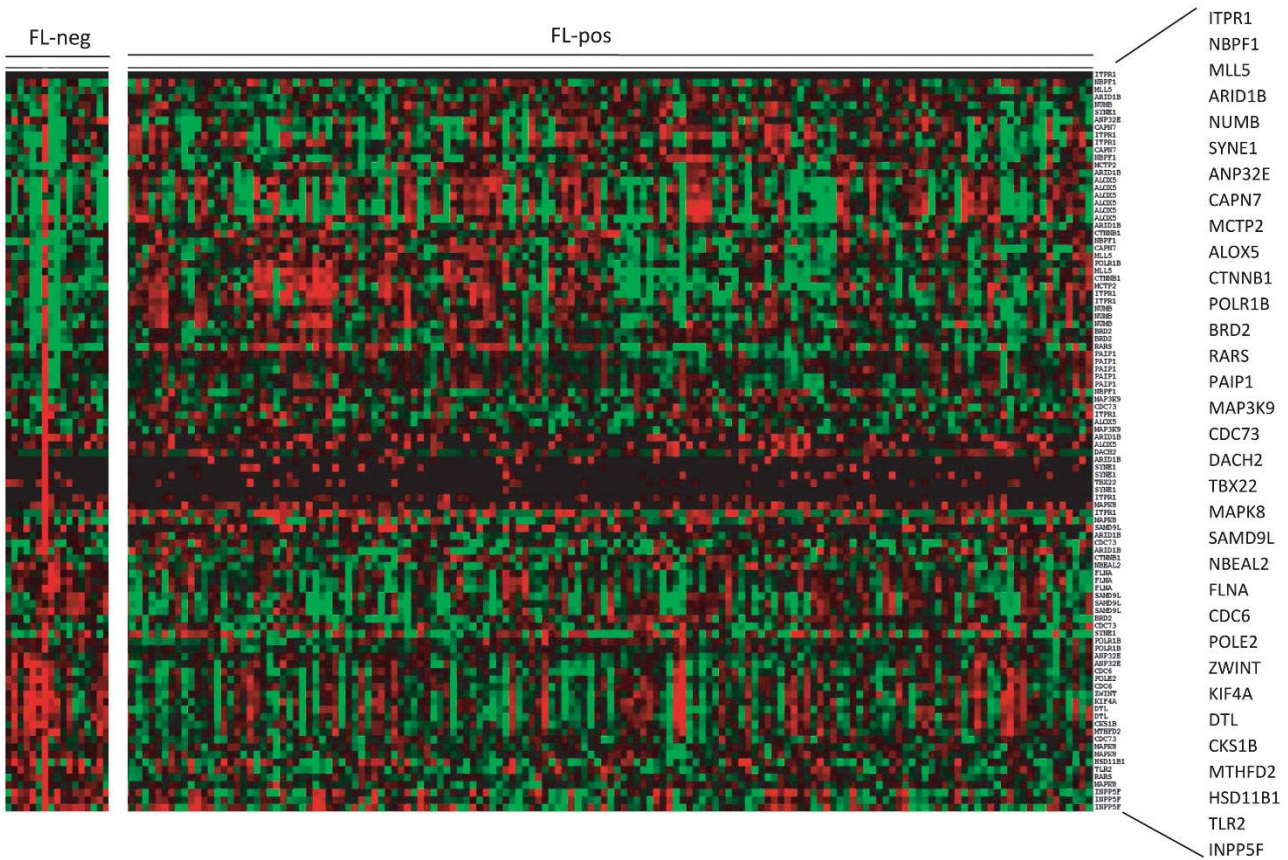


Figure 2. Supervised clustering of mutated genes differentially expressed between t(14;18)-positive and -negative FL ($P < 0.01$). Depicted are genes with scores ≥ 4 (see Supplementary Table S12 (columns Y, Z)). Notably, the cluster comprises several probesets for some genes. The zoomed-in gene list, however, depicts each gene only once.

1q21 and 1q32, 2p22 and 17q25. For example, the recurrently mutated methyltransferase and cancer-driver gene *ASH1L* (1q22) showing SNVs by WES in four t(14;18)-negative FL was in addition affected by both a gain in two patients with SNV and a cnLOH in one additional patient (Table 2). Genes affected by SNVs and losses were significantly enriched at 6q22 and 11q23 and genes that were targets of SNVs and cnLOH were for example enriched at 1p21 and 1p34, 5q31 and 9q34 ($P \leq 0.001$ and $FDR < 0.05$) (Figure 3 and Supplementary Table S14a–c). Finally, 49 genes were targets of SNVs, CNA and/or cnLOH and showed a significantly different expression between FL with and without t(14;18) such as *ZWINT*, associated with kinetochore function,⁴² the kinase *MAPK8* and the tumor suppressor (TS) *CDC73*. In addition 27 out of these 49 candidates (e.g. *ZWINT*, *MAPK8* and *CDC73*) were associated

with BCL2 by STRING-network analysis at high confidence (Figure 3 and Supplementary Tables S11a–c, S12 and S13). To summarize, several genes, including the kinase *MAPK8*, the TS *CDC73* or the methyltransferase *ASH1L*, which are targeted by SNVs in t(14;18)-negative FL exclusively in the current study cohort, are in addition differentially expressed between FL with and without t(14;18) and/or affected by CNAs/cnLOH at the same genomic site.

t(14;18)-negative FL show a reduced level of N-glycosylation. Despite the highly similar signature/pathway profile of t(14;18)-positive and t(14;18)-negative FL revealed by curated gene set annotation of genes mutated in either t(14;18)-positive or

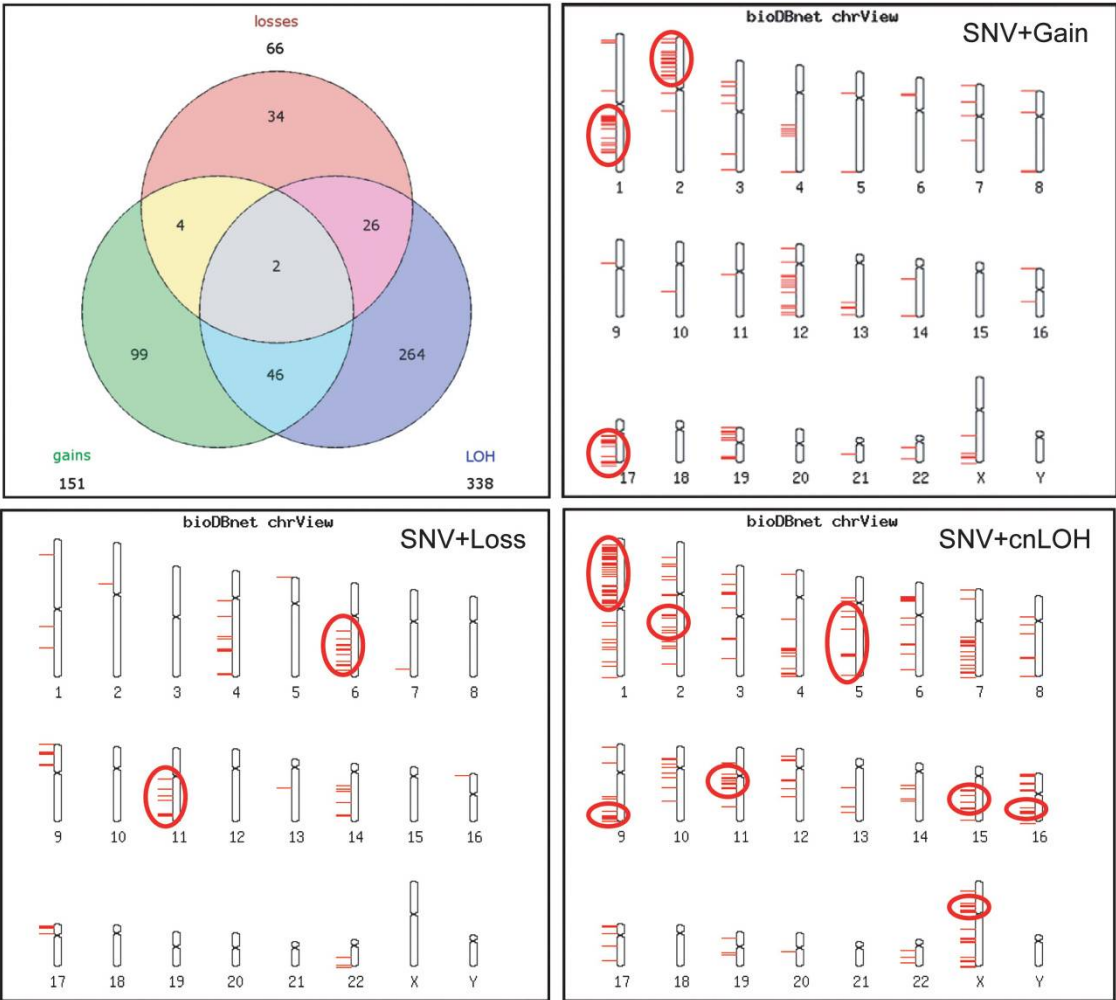


Figure 3. Overlap of CNAs and cnLOH with SNVs detected in t(14;18)-negative FL at the same genomic site. This overlap revealed 555 candidates. Significant hotspots (circled in red) were assessed by GSEA C1 annotation.

	SHM	AA-exchange > N	N-glycosylation sites
t(14;18)-neg	5.8–10.6%	0/13 (0%)	1/6 (~17%)
t(14;18)-pos/Historical FL cohort	2.7–13.5% ^a	6/28 (~21%)	55/70 (79%) ^b 24/24 (100%) ^c

Abbreviation: SHM, somatic hypermutation. ^aAarts *et al.*⁵⁷ ^bZhu *et al.*¹² ^cZabalegui *et al.*⁴⁴

-negative FL, we observed a more prominent enrichment of IR/ inflammatory-signatures and an exclusive and significant enrichment of an N-glycosylation pathway in t(14;18)-negative FL, including genes such as UGGT2, MAN1C1 and PDIA3 (Figure 1 and Supplementary Table S9a and b). Moreover, a manual screening approach, including all *IGV* genes affected by mutations in the WES data set revealed AA exchanges to asparagine exclusively in t(14;18)-positive FL (Table 4 and Supplementary Figure S2). Using

the highly sensitive CE-IVD certified LymphoTrack assay⁴³ confirmed these preliminary findings in the same cases. While IG N-glycosylation sites were present in 79–100% of t(14;18)-positive FL in other study cohorts,^{12,44} we observed new N-glycosylation sites in only one out of six (~17%) successfully sequenced t(14;18)-negative FL (Table 4), despite a comparable IG gene somatic hypermutation-level between t(14;18)-positive and -negative FL. Recapitulating these findings, we found that SNVs are enriched in genes associated with the microenvironment and N-glycosylation in t(14;18)-negative FL. However, at the same time we learned that the gain of novel Ig N-glycosylation sites is a less common event in t(14;18)-negative FL as compared with t(14;18)-positive FL.

DISCUSSION

FL may be even more heterogeneous than previously assumed. Around 15% of stage III/IV and ~50% of stage I/II lack the t(14;18); however, the majority of t(14;18)-negative FL express BCL2 by so far unknown mechanisms.³ In addition, previous investigations showed that the delineation of t(14;18)-positive and -negative FL might be clinically relevant,⁶ and non IG markers for the detection of minimal residual disease (MRD) in t(14;18)-negative FL do not exist, so far.

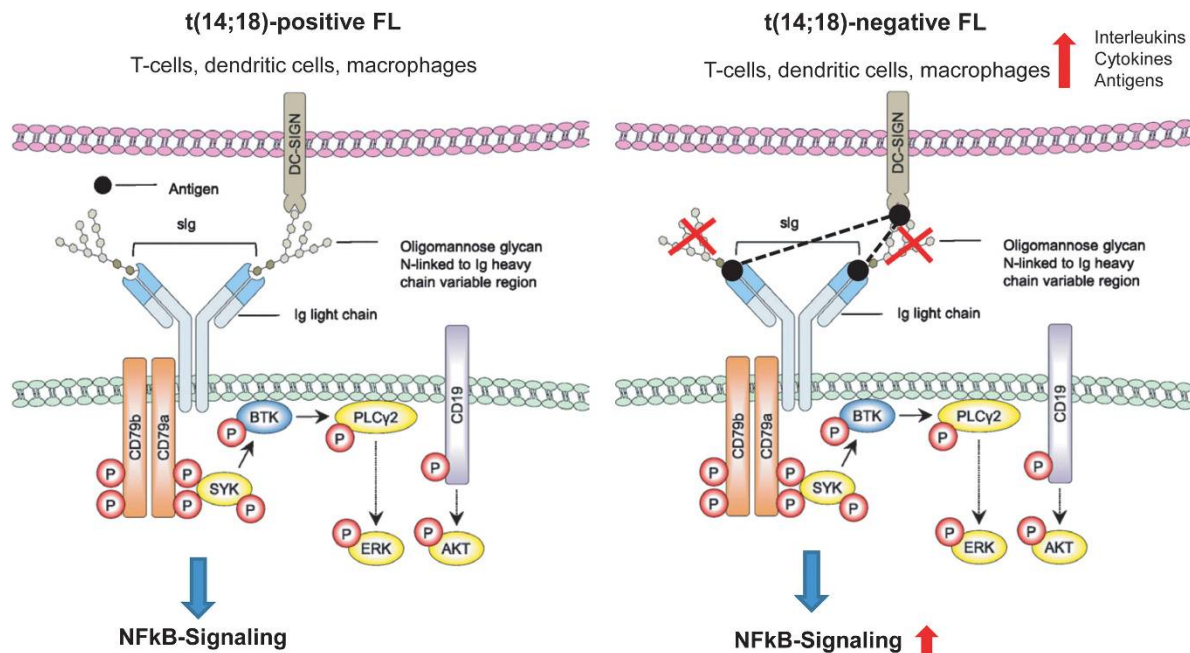


Figure 4. Hypothesis-driven model of N-glycosylation signaling in FL with and without t(14;18) based on current knowledge.^{3–6,58} Figure adapted and modified from Matthew P Strout, BLOOD, 2015^(ref. 58) by permission from BLOOD, the American Society of Hematology.

Therefore, we performed a comprehensive integrative analysis, including SNV-, CNA/cnLOH- and GE-profiling, to obtain a clearer view on the pathogenesis of t(14;18)-negative FL.

In support of our view that t(14;18)-negative cases are part of the spectrum of FL, we observed a high number of mutations typically associated with FL, such as in *EZH2* and *STAT6* which are only rarely mutated in, for example, marginal zone lymphoma.^{45–49} In contrast, mutations in *PTPRD* reported in nodal marginal zone lymphoma⁴⁹ were not found in any FL of the current study. Moreover, although the called SNVs affected different genes, they accumulated in the same or similar signatures in t(14;18)-positive and -negative FL.

The significant enrichment of apoptosis-associated genes in t(14;18)-positive and t(14;18)-negative FL, which were targeted by SNVs in an almost mutually exclusive fashion in the two subgroups, led us to speculate that some of these genes might be regulated by BCL2 in t(14;18)-positive FL or trigger BCL2 expression in t(14;18)-negative FL. In order to clarify to which level the mutated genes show BCL2 association, we predicted bioinformatic signaling networks encompassing genes that were mutated in either t(14;18)-positive or -negative FL and BCL2. Even at high confidence, almost half of the genes clustered together with BCL2 within the same network in both cohorts and only minor differences were observed in the BCL2 association level between the two groups. Moreover, the BCL2 association level between t(14;18)-negative FL with and without BCL2 expression was similar. One resilient hypothesis is that BCL2-negative FL bypass the need for BCL2 by using other anti-apoptotic mechanisms, as shown by several lesions in genes belonging to apoptosis-regulating pathways. In contrast to this, mutations in genes directly associated with BCL2 at high confidence in t(14;18)-negative FL with BCL2 expression such as *MAPK8* and *PPP2R4/PTPA* might indeed trigger BCL2 expression and compensate for the t(14;18). A MAPK8-signature was for instance enriched upon the annotation of mutated genes in t(14;18)-negative FL expressing BCL2 and knockdown of PTPA induces apoptosis, suggesting that PTPA has— similar to BCL2— anti-apoptotic properties.^{50,51} Moreover, both genes were in addition affected

by cnLOH. However, SNVs and cnLOH did not co-occur in the same patient but affected different patients.

Overall, the similar pathway/signature usage in t(14;18)-positive and -negative FL suggests parallels in the molecular pathogenesis and a central role for anti-apoptotic mechanisms in the pathogenesis of both t(14;18)-positive and t(14;18)-negative FL.

Another novel finding of this study was the detection of recurrent mutations in well-defined cancer-associated genes only present in t(14;18)-negative FL that therefore may specifically trigger tumor pathogenesis in this subgroup. Among these are the cancer-driver genes *ASH1L*, *ATFIP*, *PCLO* and *LRP6* which were affected by SNVs in conserved regions leading to structural changes according to bioinformatics predictors.^{30,34,39,52} In addition to those, many genes carrying both an SNV and a CNA in the current data set were significantly enriched in chromosomal regions previously described to be frequently altered in FL, for example, del6q, +1q, +17q and +2p.⁵³

Moreover, 49 core genes were identified in t(14;18)-negative FL that carried not only an SNV but also a CNA or cnLOH and were shown to be differentially expressed between FL with and without t(14;18) such as the kinase *MAPK8* and the TS *CDC73*, possibly underlining special importance of these genes in the pathogenesis of t(14;18)-negative FL. Most interestingly, the cancer-driver gene *ASH1L* (1q22), a methyltransferase, carried a SNV in four patients by WES. Two of these patients also showed a CN gain and an additional patient showed a cnLOH affecting *ASH1L*. Sanger sequencing allowed the detection of two of the SNVs, while the other two mutations were probably affecting a minor subclone not detectable by Sanger sequencing. Thus, in total 5 out of 10 t(14;18)-negative FL patients with both sequencing and CNA/cnLOH data available were affected by genetic aberrations in *ASH1L*. Since FL has been described to be a tumor of the epigenome,⁷ due to its frequent occurrence of mutations in methyltransferases such as *EZH2* and other proteins of the epigenome, this finding might be of special interest. *ASH1L* mutations were reported in context with other cancer entities,^{30,54,55} but to our knowledge not reported in FL, so far. In consideration of the small study cohorts one, however, has to

be aware that the statistical power to detect differentially mutated genes is limited, especially in the context of multiple testing. Therefore, these results warrant further validation in more extensive FL series.

In support of previous findings and the theory of a late GC phenotype in t(14;18)-negative FL,^{3–6} we also observed a significant enrichment of SNVs in genes associated with NFkB-activation in t(14;18)-negative FL as well as mutations in genes associated with GCB-DLBCL in t(14;18)-positive FL⁵⁶ (Supplementary Figure S3 and Supplementary Table S15).

The more prominent occurrence of mutations in genes associated with N-glycosylation in t(14;18)-negative FL that led to a significant enrichment of a N-glycosylation pathway in this sub-cohort by annotating genes exclusively mutated in t(14;18)-negative FL, gave rise to the hypothesis of differences in the usage of N-glycosylation between t(14;18)-positive and -negative FL. Indeed, we found gains of new N-glycosylation sites less frequently in t(14;18)-negative FL,^{12,44} suggesting that the SNVs that accumulated in genes associated with N-glycosylation in t(14;18)-negative FL might be responsible for this lack. However the level of on-target somatic hypermutation, involving IG genes, was high and comparable to that seen for a historical FL cohort.⁵⁷ The on-target somatic hypermutation in translocation-negative FL therefore seems to be less prone to cause new N-glycosylation sites, probably indicating a different type of selective pressure which might rely more on classical antigen presentation than on lectin binding. These observations, together with the more prominent enrichment of mutations in genes associated with IR/inflammation or the enrichment of SNVs in genes associated with NFkB-signaling in t(14;18)-negative FL, may suggest that at least some cases of this sub-cohort might bypass lectin-mediated BCR-signaling via N-glycosylation⁵⁸ by a more diffuse, increased or aberrant signaling stemming from the bystander cells/microenvironment or NFkB-associated genes, supporting previous observations of more impact of the enriched microenvironment and a late GC phenotype in t(14;18)-negative FL⁴ (Figure 4).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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