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### Follicular Lymphoma grade 3B. A separate entity?

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# CHAPTER 6

## Summary & Discussion

The aim of this thesis was to further characterize a specific subgroup of NHL, namely follicular lymphomas grade 3B (FL3B). As with all cancers, lymphomas were originally categorized based on morphology and clinical behaviour. Subsequently, the use of antibodies against cell surface markers together with morphological criteria made it possible to assign a lymphoma to a diagnostic entity. However, within each lymphoma category, there is a heterogeneity of clinical behaviour. For example the majority of published studies show a significantly more aggressive clinical course for FL grade 3<sup>(1,2)</sup> than for other follicular lymphomas (FL). Therefore, FL grade 3 cases are typically treated with combination chemotherapy that is also used for diffuse large B-cell lymphomas (DLBCL) patients, but also appear to have an increased likelihood of relapses as seen in FL grade 1 and 2<sup>(3)</sup>. The International Prognostic Index (IPI) is based on several pre-treatment criteria and can be used to subdivide in categories and to provide useful prognostic information. Despite these detailed classification and prognostic index procedures, different clinical behaviours persists within the same IPI groups<sup>(4)</sup>.

Recurrent genetic abnormalities can provide clues for an improved classification and a more reliable prognostic index and therefore lead to tailored treatment strategies for lymphoma subtypes. A large number of techniques can be used to study genetic abnormalities in lymphomas such as cytogenetics, molecular genetics, fluorescence in situ hybridization (FISH), CGH micro-array and gene expression profiling.

### Summary discussion/results

Our study was initially based on a cytogenetic investigation of lymphomas with the diagnosis FL3B. Based on these results, the cases were divided into 3 subgroups: I, with 3q27 aberrations and without a t(14;18); II, without a 3q27 aberration and without a t(14;18); III, with a t(14;18) but without 3q27 aberrations. A remarkable finding was the mutual exclusiveness of a t(14;18) and a 3q27 aberration<sup>(5)</sup>. This is in contrast with findings in FL grade 1,2,3A and DLBCL where cases have been reported with both a t(14;18) and 3q27 aberrations. Our own, unpublished, data revealed a combined t(14;18) and 3q27 aberration in 5 out of 125 FL cases (4%) and in 4 out of 135 DLBCL cases (3%). In view of the low frequencies of 3q27 aberrations in the FL group in general, the combination of these two aberrations in these 5 cases (50% of all cases with a 3q27 abnormality share the t(14;18)) is remarkable. In DLBCL cases, t(14;18) was observed in 25 out of 135 cases (18%) and 3q27 aberrations in 40 out of 135 cases (29%) and a combination of the two in 4 cases. Thus, the mutual exclusiveness of a 3q27 aberration and the t(14;18) seems to be a characteristic finding in FL3B.

FL3Bs are thought to be pathogenetically related to either FL or DLBCL based on the presence of a t(14;18)/*BCL2* or a 3q27/*BCL6* rearrangement. Besides these, other genetic aberrations might characterize the third subgroup without t(14;18) or 3q27 rearrangements. These genetic aberrations may be small deletions or mutations which have not been identified, since the basis of this study was conventional cytogenetics. The finding of the mutual exclusiveness may point to different pathways of tumorigenesis for FL3B. Cases with t(14;18)/*BCL2* rearrangement are probably more related with the FL1,2 and 3A, which is also in agreement with a significant higher percentage of gain of chromosome 7 and an almost significant higher percentage of CD10 positive cases in this subgroup. In parallel, FL3B cases with a 3q27/*BCL6* rearrangement might be related to 30-40% of the DLBCL cases with *BCL6* breakpoints.

However, our current and previously published data indicate that there are essential differences in the exact breakpoint positions at 3q27/*BCL6* between FL3B and DLBCL. In general, the great majority of rearrangements of the *BCL6* gene in DLBCL occur in the 4kb major breakpoint cluster region (MBR)<sup>(6)</sup>. An alternative breakpoint cluster region (ABR) within a genomic region between 245 and 285 kb 5' of the *BCL6* gene was identified in a small number of FL<sup>(7)</sup>. Our findings on 3q27/*bcl6* rearrangements indeed showed a very high frequency (6/9) of *BCL6* breaks in the ABR and a low frequency (2/9) in the MBR in FL3B, and a reverse pattern in DLBCL. The cases with a breakpoint in the ABR were either

primary FL3B or had a history of FL1,2. These data strongly indicate that a FL3B with a 3q27/*BCL6* rearrangement do not simply represent a follicular counterpart of DLBCL with a 3q27/*BCL6* rearrangement, but instead suggest a separate pathway leading to this particular type of FL.

In cases with a breakpoint at 3q27, *BCL6* can be juxtaposed to a large number of different translocation partners. Most translocations at the MBR will lead to promoter-substitution of the *BCL6* gene where the promoter is removed and replaced by foreign promoters, for instance the germline transcript (I $\mu$ ) promoter in cases of t(3;14) with a switch- $\mu$  breakpoint<sup>(8)</sup>. These breakpoints in the MBR of *BCL6* likely are generated during *Ig* rearrangements or class switch recombination processes or by somatic hypermutations (SHM)<sup>(9)</sup> and the generation of DNA double strand breaks and DNA repair mechanisms. In some cases mutations alone are already sufficient to deregulate *BCL6* since they may disrupt negative autoregulatory enhancer sequences of *BCL6*, thereby leading to constitutive activation of *BCL6*<sup>(10)(11)</sup>. Translocations and mutations at the MBR of *BCL6* therefore can deregulate the *BCL6* gene in two essentially different ways<sup>(12)</sup>. In contrast, breakpoints at the ABR likely are mediated by other, yet unknown, mechanisms and probably leaves the *BCL6* promoter intact. In consequence it is not known how the breakpoint is generated and also not how *BCL6* is deregulated in these cases<sup>(13,14)</sup>. All our cases reveal *BCL6* protein expression indicating that *BCL6* protein expression is not only caused by *BCL6* rearrangements but also by other mechanisms.

Similarly, *BCL2* protein expression is also detected in almost all FL3B cases including cases without *BCL2* rearrangements. Thus, *BCL2* and *BCL6* protein expression in FL3B are independent of the presence of *BCL2* and *BCL6* gene rearrangements and are also present in translocation negative cases. This is in accordance with findings in FL1,2,3A and DLBCL<sup>(15,16)</sup>.

#### *Additional aberrations*

Additional chromosomal aberrations like del(6q) and +7 are often seen as secondary aberrations and as a reflection of progression. Tumor progression during the course of the disease is often characterized by histological transformation to large cell lymphoma. The additional chromosomal aberrations in FL3B cases are comparable with those in DLBCL although a higher percentage of secondary aberrations was found in the t(14;18) subgroup (Table 1). In cases with evidence of histologic transformation from a low to a high grade lymphoma *MYC* rearrangement and *TP53* mutation are often observed. Moreover, *TP53* is rare in FL1,2 and mostly seen in cases with progression. These two aberrations are observed in a very low frequency in the FL3B group, again suggesting that

FL3B is a separate entity with different pathogenetic mechanisms (Table 1). P53 expression is seen in 55% of our cases with no difference within one of the three subgroups and is comparable with findings in FL and DLBCL; no correlation is seen with CD10 expression.

The three FL3B subgroups show similarities with both DLBCL and FL. The only statistically significant and almost significant differences that provide further evidence of the existence of different subgroups are the frequent gain of chromosome 7 as a secondary aberration and the high percentage of CD10 overexpression respectively in the t(14;18) subgroup.

In summary, a conservative conclusion may be that the FL3B group can be divided in at least 2 subgroups based on the presence of a t(14;18), with the remark that the t(14;18) negative group can be further subdivided in 3q27 positive and 3q27 negative cases.

**Table 1: Summary of FL3B results and comparison with FL1,2,3A and DLBCL**

(see also Table 1 General Introduction)

	<u><i>BCL2</i></u>	<u><i>BCL6</i></u>	<u><i>myc</i></u>	<u><i>TP53</i></u>	<u><i>CD10</i></u>	<u>+7</u>	<u>del (6q)</u>
FL1,2,3A	80-90%	5-15% (ABR)	<5%	<5%	~ 100%	50%	10-20%
DLBCL	20-30%	30-40% (MBR)	5-15%	20-30%	<40%	15%	30-40% <sup>1)</sup>
<b>FL3B</b>	<b>37%</b>	<b>27%</b>	<b>16%</b>	<b>9%</b>	<b>41%</b>	<b>45%</b>	<b>40%</b>
<b>I: 3q27+</b> (n=10)	<b>0%</b>	<b>80% #</b>	<b>10%</b>	<b>10%</b>	<b>33%</b>	<b>30%</b>	<b>30%</b>
<b>II</b> (n=9)	<b>0%</b>	<b>0%</b>	<b>11%</b>	<b>0%</b>	<b>22%</b>	<b>33%</b>	<b>44%</b>
<b>III: t(14;18)</b> (n=11)	<b>100%</b>	<b>0%</b>	<b>28%</b>	<b>18%</b>	<b><u>67%</u></b>	<b><u>73%</u></b>	<b>45%</b>

#: 6 cases with ABR break, 2 cases with MBR break.

<sup>1)</sup>: higher percentage in testicular lymphomas (88%), DLBCL-CNS and AIDS related DLBC lymphomas; up to 70%

### *Clinical classification*

We analyzed the clinical data and treatment outcome and of 28 patients with the diagnosis FL3B from which representative cytogenetic data were available. See for supplementary clinical data, treatment results and additional immunohistochemistry data Tables A1, A2a, A2b, A3 (Appendix)

In 18 patients the primary diagnosis was FL3B whereas in 10 patients NHL was diagnosed and treated before the biopsy was obtained on which the diagnosis FL3B was made.

No significant differences were seen in the treatment efficacy of the primary and secondary FL3B cases with respect to response to therapy, number of relapses and overall survival. It is impossible to compare these results because almost all patients underwent a different therapy and the time interval of collection of material between the first and last patient exceeded 20 years. Although some investigators<sup>(21)</sup> reported that cases with *BCL6* rearrangements have a better survival, others<sup>(16,22)</sup> associate *BCL6* rearrangement not with a favorable prognosis.

### *Biological Classification*

Expression patterns of *BCL6*, *CD10* and *MUM1* have been suggested as important tools to classify DLBCL in GCB and non-GCB groups<sup>(23,24,25)</sup>. *CD10* and *BCL6* are common markers of GCB DLBCL and cases were classified as GCB if both *BCL6* and *CD10* were positive or if *CD10* alone was positive. *MUM1* expression is associated with ABC DLBCL. We performed this immunostaining on our panel of FL3B cases and results could be drawn from 25 cases. These results showed that almost all primary FL3B cases can indeed biologically be classified as GC lymphoma whereas the majority of the cases with an ABC signature were secondary FL3B cases with an antecedent diagnosis of DLBCL. All cases with a t(14;18) rearrangement showed a GC signature (Figure 1, Table 2 and 3).

There was no clear difference between the ABC and GC cases with respect to number of relapses or overall survival. Because of the relatively small number of patients and the differences in therapy no firm conclusion can be drawn. More data are needed e.g. by combining our results with those of other investigators with respect to (cyto) genetic data on FL3B. This can result in a sufficient number of patients for meaningful comparison of clinical and biological data.

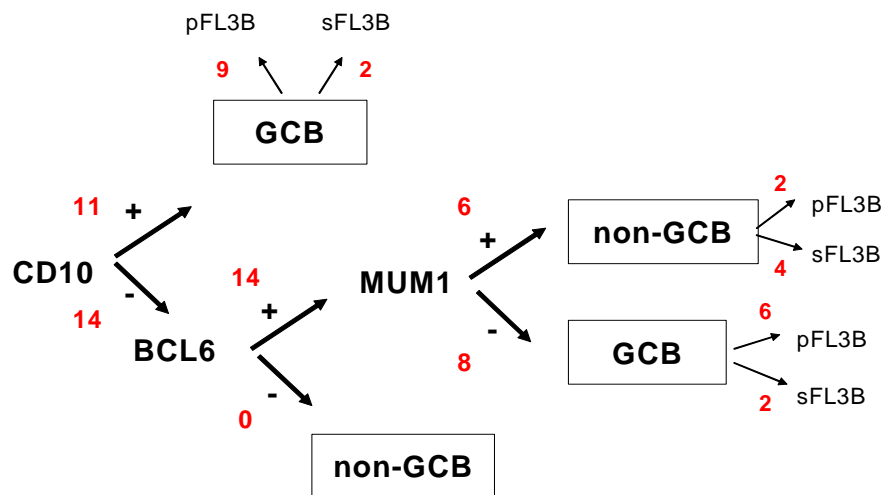


Figure 1: Decision tree for histochemical staining and the classification of 25 cases with the diagnosis FL3B.

Table 2: Biological classification results of 25 FL3B cases with and without a t(14;18).

	t(14;18) +	t(14;18) -
<b>GCB</b>	<b>8</b>	<b>11</b>
<b>non-GCB</b>	<b>0</b>	<b>6</b>

Table 3: Biological classification results of 24 primary and secondary FL3B cases.

	primary FL3B	secondary FL3B
<b>GCB</b>	<b>14</b>	<b>4</b>
<b>non-GCB</b>	<b>2</b>	<b>4</b>



*Postulated relation of FL3B with FL1,2 and DLBCL*

Transformation of FL to DLBCL might occur via two possible pathways: (1) with acquired *MYC* and *TP53* alterations which leads to FL3B as a separate entity, or (2) via FL3B to a histological DLBCL (Fig. 2). A third possibility is that the FL acquires a 3q27 aberration in combination with an existing t(14;18) during the transformation route, but our results show a mutual exclusiveness for *BCL2* and *BCL6* rearrangements in FL3B and again suggests the status of a separate entity.

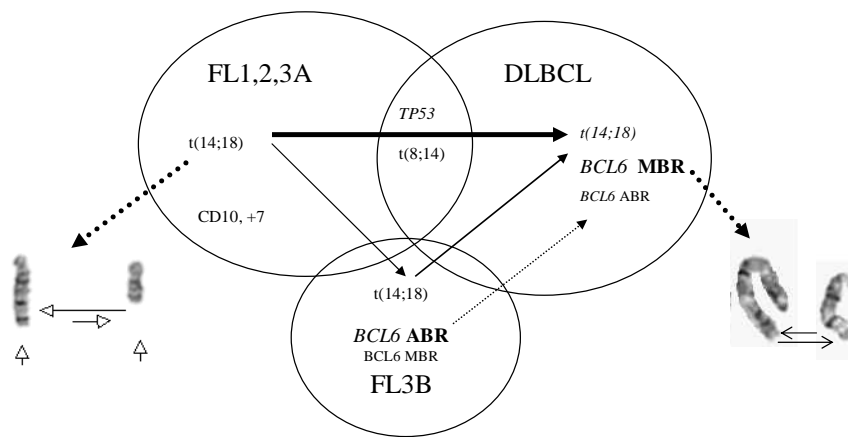


Figure 2

A postulated model of the relationship of FL3B, ship between FL1,2,3A, and DLBCL based on the data of this study. MBR: major breakpoint region, ABR: alternative breakpoint region.

## **Overall Conclusion**

With respect to our (cyto) genetic findings we demonstrated that FL3B is a separate entity with genetic and histologic properties belonging to both the FL and DLBCL and suggest treatment strategy should be based on genetic findings. This is in concordance with the basic principle of the presently used WHO classification in which the different subgroups are divided based on genetic, immunophenotypic, biologic, clinical and morphologic features. Based on our data, it might be reasonable to create two subgroups: one with and one without a t(14;18) breakpoint. The FL3B cases with a t(14;18) and higher frequencies of CD10 expression and gain of chromosome 7 should be incorporated in the FL1,2,3A group whereas the other cases without a t(14;18) and with or without a 3q27 rearrangement appear to be more closely related to DLBCL.

*Testicular lymphomas*

Deletion or partial deletion of the long arm of chromosome 6 has been reported in high frequencies (up to 70%) in testicular DLBCL, central nervous system (CNS) DLBCL and AIDS related DLBCLs. In our study of testicular lymphomas 88% (15 out of 17) showed a partial deletion of the long arm of chromosome 6, with the smallest region of overlap (RMD) located at 6q16.3-6q21 (104Mb-113Mb) and 6q23.3 (137.5Mb-138.8Mb). In one case we observed a 2.7-Mb homozygous deletion ranging from 135.3Mb to 138.0Mb, that partly overlapped with the smallest region of overlap at 6q23.3. The overlap is approximately 0.5 Mb, and contains three RefSeq genes: IL22RA2 and IFNGR1, and OLIG3. Whether a decreased or absent IL22RA2 and/or OLIG3 expression plays a role in the development of testicular lymphoma is presently unclear. IFNGR1 encodes the ligand-binding chain (alpha) of the heterodimeric interferon gamma receptor. The interferon gamma receptor is involved in the regulation of expression of HLA class II genes. HLA class II downregulation is frequently found in testicular DLBCL and CNS DLBCL<sup>(17,18)</sup> and was associated with a very poor prognosis of DLBCL<sup>(19)</sup>. Another gene, IL20RA is deleted in 10 out of 16 cases, and also lies within the homozygous deletion of case #5. In contrast with the other genes mentioned above, IL20RA is highly expressed in normal testis and skin (<http://genome.ucsc.edu>).

In a very recent study by Pasqualucci et al, the authors identified BLIMP1/PRDM1 as a possible tumor suppressor gene at 6q<sup>(20)</sup>. These authors showed that in 24 percent of activated B cell like (ABC) DLBCL the BLIMP1 gene is inactivated by structural alterations. The genomic alterations included deletions on one allele and mutations on the other allele, leading to loss of function at the protein level. A majority of these cases (77%) lack BLIMP1 expression despite the presence of mRNA. BLIMP1 is localized at 6q21-q22.1, which partly overlaps our RMD located at 6q16.3-q21. BLIMP1 mRNA expression levels as detected by the Affymetrix U133 plus2 oligonucleotide arrays were not consistently low in the cases of testicular DLBCL with 6q deletion (M.Booman, personal communication).

In conclusion, aberrations affecting chromosome 6 are often seen in systemic DLBCL but the frequency is clearly higher in testicular lymphomas and CNS lymphomas. Our study revealed in 88% of the investigated cases a deletion of the long arm of chromosome 6 with two deletion hot spots, mapping at 6q16.3-q21 and 6q23.3 respectively. This suggest that functional loss of one or more genes within these RMDs may be a crucial event in the pathogenesis of testicular lymphomas.

## Future perspectives

In future studies a large number of FL3B cases, preferably in a large prospective international study, should be analyzed in which all patients are treated with the same regimens during the course of the disease. Presence or absence of *BCL6* and *BCL2* rearrangements should be determined and related to overall survival of the patients.

Besides the markers and aberrations studied in this thesis, future studies should also include MIB1/Ki-67, array-CGH and expression profiling. An alternative for grading the follicular lymphomas is application of the proliferation index as measured with the MIB1/Ki-67 antibody with prognostic significance. However, as explained above we would advise to use this method only in FL1,2,3 cases with proven t(14;18) and to consider the other FL3 without this translocation as a separate entity.

Array-CGH provides another potent tool to analyze the pathogenesis of FL3B lymphomas. These arrays cover the whole genome, with a very high resolution down to the level of 1Mb, or even a complete tiling array. In Chapter 5 the strength of this technique was demonstrated by identification of small deletions in cases which revealed no aberrations with conventional cytogenetics or FISH. As our starting point was cytogenetics and subsequent molecular genetic investigation that also reveal translocations, applying array-CGH and enlarging the amount of cases in the future might reveal additional genetic aberrations in the different subgroups and give a more reliable basis for grouping the FL3Bs with either FL1,2,3A, DLBCL or as a separate entity.

Another approach that can be followed is gene expression profiling. The enormous number of data points resulting in a pattern of expressed genes, a signature, can be used for several goals in the study of the pathogenesis of lymphomas or even for the development of novel treatment approaches for these diseases. A disadvantage of these techniques is that the percentage of tumor cells is always less than 100% with variable admixture of normal cells that may influence the results. Combining laser dissection microscopy (LDM) with expression profiles could prove to be a potent tool in clarifying the real nature of FL3B.

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