Follicular Lymphoma grade 3B. A separate entity?
Bosga-Bouwer, Annigje Geesje

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2006

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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CHAPTER 4

Molecular, cytogenetic and immunophenotypic characterization of Follicular Lymphoma grade 3B; A separate entity or part of the spectrum of Diffuse Large B-cell Lymphoma or Follicular Lymphoma?

Anneke G Bosga-Bouwer; Anke van den Berg; Eugenia Haralambieva; Debora de Jong; Ronald Boonstra; Philip Kluin; Eva van den Berg; Sibrand Poppema

Department of Medical Genetics (A.G.B.B., E.v.d.B.), Department of Pathology & Laboratory Medicine (A.v.d.B., E.H., D.d.J., R.B., P.K., S.P.), University Medical Center Groningen, and University of Groningen, the Netherlands.

Human Pathology 2006; 37:528-533
Abstract

We studied a histological homogeneous group of 29 cases with the diagnosis follicular lymphoma grade 3B (FL3B). In a previous study we subdivided this group in 3 subgroups based on I: aberrations of the 3q27 region, II: lack of 3q27 and t(14;18) and III: the presence of a t(14;18). In this study we further characterized the FL3B lymphomas, that is currently part of the spectrum of follicular lymphoma in the WHO classification, taking into account other cytogenetical aberrations, immunohistochemistry for P53, BCL2, BCL6 and CD10, rearrangement of the proto-oncogene MYC and mutation of the tumor suppressor gene TP53. With respect to P53, BCL2, BCL6 expression, MYC rearrangement and TP53 mutation FL3B represents a homogeneous group. CD10 expression and gain of chromosome 7, considered to be typical follicular lymphoma markers were more common in the FL3B t(14;18) positive subgroup. The lack of CD10 expression and gain of chromosome 7 in the majority of cases in the other two subgroups suggest that those cases have a closer relation to DLBCL.
Introduction

Many Non-Hodgkin lymphomas (NHL) are characterized by clonal chromosomal abnormalities. Molecular characterization of the affected breakpoint regions has led to the identification of target genes mapping at or close to the breakpoint regions. Several of these clonal chromosomal abnormalities have now been shown to be specific for different NHL subgroups and can be used to discriminate between different NHL subtypes. Follicular lymphomas (FL) are characterized by a translocation t(14;18) affecting the \( BCL2 \) gene and Burkitt lymphomas (BL) by a t(8;14) involving the \( MYC \) gene. Translocations involving the \( BCL6 \) gene, located on chromosome 3q27, are frequently seen in diffuse large B-cell lymphomas (DLBCL). Several other chromosomal abnormalities have been associated with other entities. Frequently, however, these abnormalities are not as specific as initially described.

Cytogenetic studies indicate that follicular lymphomas which undergo histologic transformation retain the t(14;18) translocation and generally acquire multiple, often complex secondary chromosomal abnormalities. Until now no predominant genetic locus is suggested to be responsible for this histologic transformation. FL grade 3B (FL3B) can be distinguished from diffuse large B-cell lymphoma (DLBCL) by the presence of at least a focal follicular pattern. In contrast to FL grade 1 or 2, the predominant cell type of FL3B is the centroblast, which is also the typical cytological feature of DLBCL.

We previously demonstrated that three distinct subsets can be identified among cases with the histological diagnosis FL3B: (I) with the presence of a breakpoint 3q27/\( BCL6 \) rearrangement without t(14;18), (II) with the presence of cytogenetic aberrations without 3q27/\( BCL6 \) rearrangements and without a translocation t(14;18) and (III) with the presence of a translocation t(14;18) without 3q27/\( BCL6 \) rearrangements. Based on these findings, a conservative conclusion may be that FL3B with a translocation t(14;18)/\( BCL2 \) rearrangement are part of the same entity as the other FL grade (1, 2 or 3A). The cases with 3q27/\( BCL6 \) rearrangements or other unrelated translocations are potentially more closely related to the majority of DLBCL. The cases with rearrangements affecting 3q27, an aberration also frequently observed in DLBCL, was studied in further detail to discriminate between the Alternative Breakpoint Cluster Region (ABR) and the Major Breakpoint Region (MBR) mapped into the first non-coding exon of the \( BCL6 \) gene. In the majority of these FL3B cases we detected a breakpoint in the ABR, whereas a breakpoint in MBR was commonly observed in a control group of DLBCL cases. Although the presence of 3q27 aberrations suggests a similar pathogenesis for this group of FL3B and DLBCL, the
consistent difference observed in BCL6 breakpoint regions might suggest an alternative pathogenesis for the FL3B cases.

These observations led us to further examine FL3B cases to assess the presence of other genetic and immunophenotypic characteristics that might allow discrimination of cases closer related to either FL1,2,3A or DLBCL. We selected characteristics that are thought to play an important role in the histological transformation from low-grade to high grade NHL or markers considered to be typical for follicular lymphomas or diffuse large B-cell lymphomas. We studied TP53 mutation analysis, presence of MYC rearrangements, and BCL2, BCL6, CD10 and P53 expression. Mutation of the tumor suppressor gene TP53 frequently associates with cases resulting from the histologic transformation of FL11,13 and appears in low percentages in de novo DLBCL12. The human TP53 gene encodes a 393 amino acid residues long nuclear phosphoprotein, P53, which is involved in a number of processes like programmed cell death or apoptosis, inhibition of tumor growth, maintenance of genome integrity or stability and cell cycle arrest (14). The nuclear phosphoprotein MYC located on chromosome 8q24, has been implicated as a potent regulator of cellular proliferation and may also play an important role in the transformation process of FL15,16. The majority of precursor B cells as well as a subset of follicular center cells express CD10 antigen, while other mature B-cells, plasma cells and B-cell lymphomas only occasionally do17,18. The immunophenotype of the malignant lymphocytes in FL suggests that they are derived from germinal center cells and mostly they do express CD10, although some grade III follicular lymphomas are negative19.

We report the further molecular cytogenetic and immunophenotypic characterization of a group of 29 patients with the diagnosis FL3B with single or combined expression or abnormalities of P53, CD10, BCL2, BCL6, MYC rearrangement and TP53 mutation.

Materials and Methods
Patients
In the present study, 30 successive cases from 29 patients with the histological diagnosis follicular lymphoma grade 3B (FL3B), were studied for other aberrations associated with malignant transformation. FL3B was defined according to the WHO classification as a follicular lymphoma with at least a partially follicular pattern and with neoplastic follicles composed of solid sheets of centroblasts without residual centrocytes. All of the cases had at least 25% follicularity. Most of the cases also had diffuse areas with centroblasts with similar morphology as those in the neoplastic follicles. The primary diagnosis at
first presentation was FL3B in 19 of these biopsies. In 11 biopsies the primary diagnosis was other than FL3B (Table). From one patient two sequential biopsies were investigated (case #15 and #22). Due to lack of paraffin and frozen tissue, two cases of our previous study were not included in the present study (case #3 and #23).

**Fluorescence in situ hybridization**

The detection of translocation breakpoints at 8q24 involving the *MYC* gene were performed, using standard in situ hybridization protocols on nuclear suspensions isolated from frozen tissue samples or paraffin slides. The following breakpoint flanking probes were used in a FISH segregation assay: cosmids 4663 and 4665 (green), pooled together with PAC103G5 (ES4745) (red) for 5’ breakpoints and the cosmids 4664 and 4671 (green) pooled with PAC117K22 (ES8071) (red), for 3’ breakpoints. All probes were labeled by nick translation with biotin-16-dUTP or digoxigenin-11-dUTP (La Roche, Basel, Switzerland) and the reaction was visualized by indirect immunofluorescence as a dual color break leading to segregation of the green and red signal. The cosmid clones were kindly provided by Dr. C. Croce (Jefferson Cancer Institute, Philadelphia) and the PAC’s were obtained from the RPCI-1 Human PAC Library of the Roswell Park Cancer Institute (available at the Leiden Genome Technology Center, Leiden, the Netherlands).

**Immunohistochemistry**

Immunostaining for P53 and CD10 was performed on paraffin sections. We used a mouse anti-human P53 antibody (Bp53-12-1, BiogeneX, San Ramon, CA, USA) and a mouse anti-human CD10 (Novocastra Laboratories, Newcastle upon Tyne, United Kingdom). Peroxidase labeled rabbit anti mouse followed by peroxidase labeled goat anti-rabbit antibodies (DAKO, Copenhagen, Denmark) and peroxidase enzyme staining with diaminobenzidine and H$_2$O$_2$ were used to visualize the P53 and CD10 protein positive cells.

The P53 protein staining was designated as +/- (weak positive), with a couple of scattered P53 protein positive tumor cells, + (moderate positive), with a substantial proportion of P53 protein positive tumor cells, varying in intensity or ++ (strong positive), with almost all the tumor cells strongly P53 protein positive. CD10 protein staining was designated as ++ (strong positive), + (positive), +/- (weak positive) or – (negative).

Immunostaining for bcl-2 and bcl-6 was performed on frozen tissue samples according to standard methods, using the following antibodies: mouse anti-human monoclonal antibody to BCL2 protein (clone 124; Dakopatts, Glostrup, Denmark) and PG-B6p for BCL6 protein (a gift of Dr. B. Falini,
Molecular, cytogenetic and immunophenotypic characterization of FL3B

Institute of Hematology, University of Perugia, Italy). The intensity of the staining was graded as ++ (strong positive), + (positive), +/- (weak positive) or – (negative).

**Mutation Analysis**
Detection of TP53 mutations was performed with polymerase chain reaction (PCR) followed by Denaturing Gradient Gel Electrophoresis (DGGE) and sequencing of aberrant bands: DNA amplification was carried out as previously described with GC-clamped primers for

exon5: 5’-[40GC]CTCTGCTCTCCCTCCTCCCAACCAGGCCCTGTCGTCTCTC-3’
exon6: 5’-[40GC][12AT]GGCCTCTGAATTCCTCACTGACAGAGACCCCAGTTGCAAC-3’
exon7: 5’-[40GC]ACAGGTCTCCCCAAAGGCACCAGTGGAACGGTGGAAGCTG-3’
exon8: 5’-[40GC]GCCTCTTGCTCTTTTCTCCCCATAACTGCACCCTTGTCTC-3’

Each amplification round was carried out in a standard PCR buffer and 1µl DNA was added to each reaction. Samples were amplified for 33 cycles; denaturating at 94 ºC for 30s, annealing at 61 ºC and elongation at 72 ºC for 45s. For each PCR run, control samples included: a blank (no DNA) and a known positive. Each amplification was followed by heteroduplexing; denaturing at 94 C for 10 min. and hybridization at 61 ºC for 45 min. DGGE is used for comprehensive analysis of the exons 5, 6, 7 and 8 of the TP53 gene: DNA sequencing: Amplified and purified PCR products from cases with an aberrant banding pattern after DGGE gel electrophoresis were subjected to single stranded automated sequencing. From some cases the heteroduplex bands from the aberrant DGGE banding patterns of DNA from paraffin-embedded material were excised from the gel and sequenced after re-amplification and purification.

**Results**

**FISH**
With probes flanking the breakpoints at 8q24 involving the MYC gene, a rearrangement was detected in four cases (Table), generally confirming the cytogenetic results. Case #17 showed an 8q24 aberration by classical cytogenetics but did not show a MYC rearrangement by FISH.

**Cytogenetics**
Previously we identified three distinctive subsets based on: (I) the presence of a breakpoint 3q27 / BCL6 rearrangement; (II) the presence of cytogenetic aberrations without 3q27 / BCL6 rearrangements and without a t(14;18) / BCL2
rearrangement; and (III) the presence of a t(14;18) / BCL2 rearrangement without 3q27 / BCL6 rearrangements (Table). Frequencies of various other structural and numerical cytogenetic aberrations, like a deletion of the long arm of chromosome 6, deletions of chromosome 17p/q, 8q24 rearrangements and gain of chromosome 7 were compared within the 3 groups and revealed a significant difference only for chromosome 7 gain. Gain of chromosome 7 was observed more frequently in the t(14;18) positive subgroup (8/11=73%) in comparison to the two t(14;18) negative subgroups (25% and 36%) (Table 1). The differences between the three separate subgroups did not reach significance, due to the low number of cases in each subgroup. But comparison of the t(14;18) positive subgroup with the two t(14;18) negative subgroups did yield a significant difference (p=0.029, Pearson Chi-Square). Although gain of chromosome 7 was observed less frequently in the 3q27 positive group (2/8=25%) in comparison with the 3q27 negative group (12/22=54%) this did not reach significance (0.196, Pearson Chi-Square).

Immunohistochemistry
Bcl-2 staining was present in the majority of cases, with the lowest proportion of strong-positive (++) or positive (+) cases in the group with a BCL6 rearrangement. Bcl-6 staining was found in all cases, consistent with a follicular center cell origin. CD10 protein expression was seen in about 30% of the FL3B cases. It was observed at a higher frequency in t(14;18) positive subgroup (6/9=67%) than in the two t(14;18) negative subgroups (28% and 27%). Again the differences between the three subgroups did not reach significance due to the small number of cases. Comparison of CD10 protein expression in the t(14;18) positive subgroup with the two t(14;18) negative subgroups revealed a much higher frequency in the t(14;18) positive subgroup (p=0.053, Pearson Chi-Square analysis). Almost half of the cases showed a positive or weak positive P53 protein expression with no clear relation between the cases with or without a t(14;18) or 3q27 aberrations. There was no obvious relation between the CD10 and P53 expression. (Table).

Mutation analysis
TP53 mutation was detected in only 3 cases (9%); #2, #13 and #14. The following nucleotide change and amino acid change were detected: #2: TAC->TGC, TYR>CYS (codon 234), #13: GAC->GAA, ASP->GLU (codon 259), #14: GTG->GGG, VAL->GLY (codon 218). The primary diagnosis of #2 and #13 was FL3B, the primary diagnosis of #14 was FL1.
Table: Molecular cytogenetic and immunophenotypic characterzation of 30 FL3B cases.

<table>
<thead>
<tr>
<th>Case nr</th>
<th>Primary diagnosis</th>
<th>genetic abnormality MYC breakpoint</th>
<th>Immunohistochemistry***</th>
<th>7p53 mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>gain of chr. #7</td>
<td>Bcl-2</td>
<td>Bcl-6</td>
</tr>
<tr>
<td>Group I: 3q27 / BCL6 break and no t(14;18)#</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>FL3B</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>FL3B</td>
<td>.</td>
<td>.</td>
<td>+</td>
</tr>
<tr>
<td>17</td>
<td>FL1/2→FL3B</td>
<td>+/−</td>
<td>+</td>
<td>.</td>
</tr>
<tr>
<td>22*</td>
<td>FL3B</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>25</td>
<td>FL3B</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>29</td>
<td>FL3B</td>
<td>.</td>
<td>+/−</td>
<td>+</td>
</tr>
<tr>
<td>30</td>
<td>FL1/2→FL3B</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>DLBCL→FL3B</td>
<td>+/−</td>
<td>+</td>
<td>.</td>
</tr>
<tr>
<td>Total group I</td>
<td></td>
<td>12%</td>
<td>25%</td>
<td>28%</td>
</tr>
<tr>
<td>Group II: no 3q27 / BCL6 break and no t(14;18)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Imm.bl→FL3B</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>DLBCL→FL3B</td>
<td>+/−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>FL3B</td>
<td>-</td>
<td>.</td>
<td>+</td>
</tr>
<tr>
<td>16*</td>
<td>FL3B</td>
<td>.</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>18</td>
<td>FL3B</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>19</td>
<td>FL3B</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>24</td>
<td>?FL3B*</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>32</td>
<td>FL3B</td>
<td>.</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>FL3B</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>FL3B</td>
<td>.</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Total group II</td>
<td></td>
<td>9%</td>
<td>36%</td>
<td>27%</td>
</tr>
</tbody>
</table>
### Table: Group III: t(14;18) and no 3q27/BCL6 break

<table>
<thead>
<tr>
<th></th>
<th>FL12→FL38</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>ND</th>
<th>ND</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>FL12</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>FL38</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>.</td>
</tr>
<tr>
<td>11</td>
<td>FL38</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
<td>.</td>
<td>+</td>
<td>.</td>
</tr>
<tr>
<td>13</td>
<td>FL38</td>
<td>.</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>.</td>
<td>.</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>FL12→FL38</td>
<td>.</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>20</td>
<td>FL38</td>
<td>.</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>.</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td>21</td>
<td>FL38</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>26</td>
<td>FL38</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>&lt;5%</td>
<td>.</td>
</tr>
<tr>
<td>27</td>
<td>FL38</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td>28</td>
<td>FL12→FL38</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>.</td>
<td>&lt;1%</td>
<td>.</td>
</tr>
<tr>
<td>31</td>
<td>FL12→FL38</td>
<td>.</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td>.</td>
<td>.</td>
</tr>
</tbody>
</table>

**Total**

|   |   |   |   |   |   |   |   |   |
|---|---|---|---|---|---|---|---|
| FL12→FL38 | 25% | 73% | 97% | 40-60% | 18% |

---

# two cases with a breakpoint at the MBR (= major breakpoint region) and 5 cases with a breakpoint at the AER (= alternative breakpoint region) detected with FISH with specific probes 10.

* data unknown

** cases with a sequential biopsy

***++ strong positive, + positive, + weak positive, - negative.

ND: not done
Discussion

In a previous study we identified three different subgroups within a group of follicular lymphoma grade 3B (FL3B); I: the presence of a breakpoint 3q27 / BCL6 rearrangement, II: the presence of cytogenetic aberrations without 3q27 / BCL6 rearrangements and without a t(14;18) / BCL2 rearrangement and III: the presence of a t(14;18) / BCL2 rearrangement without 3q27 / BCL6 rearrangements. These findings may point to at least three different pathways of oncogenesis. Presence of BCL2 rearrangements, as observed in FL3B subgroup III, suggests a relationship to the other follicular lymphomas (grade 1, 2, 3A). The 3q27 aberrations observed in FL3B subgroup I, suggest a relationship to DLBCL of germinal center cell origin. In this study we attempted to establish further proof for a possible relationship with either FL or DLBCL. Comparison of the three distinct subgroups indicates that expression of CD10 is more frequent in the t(14;18) positive subset as compared to the other two t(14;18) negative subsets; 67% vs 27% and 28% respectively and almost reached significance. Gain of chromosome 7 is also more frequent in this t(14;18) positive subset, 73% vs 36% and 25%, and is statistically significant (P=0.029). The two t(14;18) negative subgroups show no differences besides the presence or lack of 3q27 aberrations and appear to present as a homogeneous entity. However, negative or weak positive staining for BCL2 was observed in a higher frequency in the cases with a 3q27 rearrangement.

We found a higher percentage over-expression of CD10 in the t(14;18) positive subset which might be in accordance with previous findings that both CD10 and t(14;18) are typical FL markers and appear in low percentages in DLBCLs in particular in DLBCL with a germinal center cell expression pattern. The initial diagnosis of most of the cases with over-expression of CD10 was primary FL3B.

Cytogenetic studies indicate that FL which undergo histological transformation retain their translocation t(14;18) and often have gain or loss of chromosomes and multiple, complex secondary chromosomal abnormalities. Although some of these secondary chromosomal aberrations tend to recur, none accounts for a predominant fraction of the total abnormalities.

In the t(14;18) positive subgroup, gain of chromosome 7 was observed more frequently than in the t(14;18) negative subgroups. This is in accordance with the finding that +7 is more frequently seen in cases with primary NHL-associated
abnormalities like e.g. t(8;14) in Burkitt lymphoma and t(14;18) in follicular lymphoma. A higher number of secondary aberrations in lymphomas with a t(14;18) compared to t(14;18) negative lymphomas is previously described.

Several studies indicate that transformation of follicular lymphoma to a higher grade NHL is associated with over-expression of P53 and mutations of the TP53 gene. A transformation pathway implicates the transition from a preexisting follicular NHL to a lymphoma with higher grade histology. Cases following this pathway often harbor rearrangements of bcl-2 and TP53 mutations. Whereas the bcl-2 rearrangement is already present in the follicular NHL phase, TP53 mutations are gained during histological transformation. In our study an over-expression of P53 was seen in about half of the cases, both primary and secondary FL3B. A TP53 mutation was only detected in three cases, also both primary and secondary FL3B. No differences were seen in the distribution over the different subgroups. Our data suggest that there is no critical role for the TP53 mutations in FL3B pathogenesis.

The MYC proto-oncogene, located on chromosome 8q24, also has been reported to frequently play a role in the transformation process. Several high grade lymphomas with evidence of histological transformation from a follicular lymphoma have been reported to carry both a t(14;18) and a translocation affecting chromosome 8q24 involving the MYC locus.

MYC gene rearrangements were found in 4 cases with cytogenetic aberrations on 8q24. The fifth case with an 8q24 aberration did not have a break in the MYC region possibly due to a break outside the MYC region in 8q24 as covered by the FISH probes.

In conclusion, the results of single and associated lesions of MYC, BCL2 and BCL6 expression, P53 expression and TP53 mutation did not reveal consistent or significant differences within the three cytogenetically different FL3B subgroups. Overall, positive CD10 expression and gain of chromosome 7 in the t(14;18) positive subgroup were the only distinctions between the t(14;18) positive and the two t(14;18) negative subgroups. This confirms the suggestion that the t(14;18) positive subgroup may have more in common or even be derived from the indolent FLs (1, 2 and 3A). Based on the difference in BCL6 breakpoint region observed in the 3q27 positive subgroup in comparison with the breakpoint regions generally observed in DLBCL the 3q27 positive subgroup may constitute a separate entity.
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