Contiguous follicular lymphoma and follicular lymphoma in situ harboring N-Glycosylated sites

by Emilie Mamessier, Charlotte Drevet, Florence Broussais-Guillaumot, Marie-Laure Mollichella, Sylvain Garciaiz, Sandrine Roulland, Maxime Benchetrit, Bertrand Nadel, and Luc Xerri

Haematologica 2014 [Epub ahead of print]

doi:10.3324/haematol.2014.115782

Publisher's Disclaimer.
E-publishing ahead of print is increasingly important for the rapid dissemination of science. Haematologica is, therefore, E-publishing PDF files of an early version of manuscripts that have completed a regular peer review and have been accepted for publication. E-publishing of this PDF file has been approved by the authors. After having E-published Ahead of Print, manuscripts will then undergo technical and English editing, typesetting, proof correction and be presented for the authors' final approval; the final version of the manuscript will then appear in print on a regular issue of the journal. All legal disclaimers that apply to the journal also pertain to this production process.
Contiguous follicular lymphoma and follicular lymphoma in situ harboring N-Glycosylated sites

Emilie Mamessier 1,2,3, Charlotte Drevet 1,2,3, Florence Broussais-Guillaumot 3, Marie-Laure Mollichella 1,2,3, Sylvain Garcia 1,2,3, Sandrine Roulland 1,2,3, Maxime Benchetrit 4, Bertrand Nadel 1,2,3,°, Luc Xerri 5,°

1INSERM U1104, Marseille, France
2Centre d’Immunologie de Marseille Luminy, Université de la Méditerranée, Marseille, France
3CNRS UMR7280, Marseille, France
4Service d’Onco-hématologie adulte, Institut Paoli Calmettes, Marseille, France
5Laboratoire DIAG, Nice, France
6Service de Biopathologie, Institut Paoli Calmettes, Marseille, France

° Contributed equally to this work.

Correspondence

Emilie Mamessier, INSERM U1104, Marseille, France
Centre d’Immunologie de Marseille Luminy, Université de la Méditerranée, Marseille, France. E-mail: mamessier@ciml.univ-mrs.fr
Follicular lymphoma in situ (FLIS) are composed of a clonal B cells population harboring the typical t(14;18) hallmark of follicular lymphoma (FL), forming unconventional BCL2BrightCD10+ cells foci in an otherwise normal reactive lymph node (LN). The diagnosis of FLIS is made on the fortuitous discovery of unconventional BCL2BrightCD10+ cells foci (1). Several studies recently demonstrated that FLIS are already advanced precursors in follicular lymphomagenesis, but not necessarily committed to malignant transformation (2, 3). However, the relationship between FLIS and FL still remains unclear, as only a minority (<5%) of FLIS patients might eventually develop FL. This is in line with the usually indolent progression course of the disease, and the genomic instability observed in FLIS cells, which can engage FL precursor cells either in an evolutionary malignant process, or to an evolutionary dead end (4).

We report herein the case of a 35 years old male patient who presented with a cervical adenopathy. Histological examination of the excised LN displayed an altered architecture suggestive of FL, consisting of high number of monomorphic large follicles, uniformly spread in the cortical and medullary areas. Most follicles contained a predominant population of small cleaved cells with scant macrophages and mitoses. The mantle zone was reduced or absent. However, in a minor cortical area, a few follicles showed features mimicking residual classical GCs, including a smaller size, higher cell polymorphism, and a preserved mantle zone (Figure 1A).

The BCL2 immunostaining (clone 100) was negative in follicles displaying a typical FL pattern. In contrast, follicles located in the pseudo-residual area were BCL2Bright, i.e.
more strongly stained than the surrounding mantle zone and reactive T cells (Figure 1B). Most follicles were only slightly positive for Ki67 (Supplemental Figure 1A). Both BCL2− and BCL2+ follicles were CD10 positive (Supplemental Figure 1B) and contained a BCL2/JH breakpoint evidenced by FISH analysis (Figure 1C). Altogether these results suggested the diagnosis of simultaneous occurrence of BCL2− FL (grade I/II) and of BCL2+ FLIS in the same LN. We decided to further analyze those two lesions independently, and performed macrodissection in order to proceed with individual molecular analyses when required. Sanger sequencing revealed that both FLIS and FL shared the same BCL2/JH sequence at the t(14;18)+ breakpoint, and thus originated from the same clone (Figure 1D).

We tested two other anti-BCL2 antibodies (E17, SP66) directed against other epitopes, but the staining remained BCL2− in the FL area of the LN, similar to the anti-BCL2 antibody (clone 100) staining (Figure 1E-F). We thus sequenced exons 1 to 3 of the BCL2 gene (B-cell CLL/lymphoma 2, NG_009361.1). Punctual mutations, resulting in amino acid substitutions, were found in the FL component (Supplemental Table I), and were indeed located in the targeted aa41 to aa54 epitope of clone 100 (mutations found in aa45-47), in the aa61 to aa76 epitope of clone E17 (mutation found at position aa64) and in the N-terminal region epitope of clone SP66 antibodies (mutation in aa32). None of the registered mutations involved a stop codon. The FLIS and FL sequences were submitted to 3D-molecule Viewer (Vector NTI advanced 5.11.1®), which revealed that the acquired mutations in the FL dissected area resulted in an altered energy profile of the BCL2 protein, probably preventing a proper fixation of most anti-BCL2 antibodies (Figure 1G and Supplemental Figure 2).
We thus determined whether the FL cells expressed at least the BCL2 transcript. mRNA was extracted from microdissected FL follicles and qRT-PCR was performed. A ≈7-fold relative increase in BCL2 transcripts was found compared to microdissected benign reactive lymphadenitis (the Log2(BCL2/GAPDH) was of 3.7 in FL follicles as compared to 0.5 in benign reactive lymphadenitis), indicating that the absence of BCL2 staining was not due to transcriptional down-regulation.

Taking into account that the FL follicles were composed of more than 80% of FL B-cells, it is unlikely that contaminating T cells could be entirely responsible for this high level of BCL2 transcription, which is thus likely related to FL B-cells. Although we cannot exclude that a post-transcriptional mechanism could have induced down-regulation of the BCL2 protein, these data are in accordance with the view of the FL component presenting with multiple mutations inducing a conformational change of the BCL2 protein, which may or may not have altered BCL2 function. Sustained activity of activation-induced cytidine deaminase (AID) has been shown to be partly responsible for somatic mutations in FL. AID expression was indeed present in both FLIS and FL areas of our sample (Supplemental Figure 3A).

Despite the concomitant FL/FLIS localization, these alterations were lacking in the FLIS dissection, suggesting that they were acquired in the FL component after divergence from a common founder clone. To our knowledge, only rare cases of FLIS with concomitant FL or DLBCL were reported previously, and usually observed in distinct LNs (5). These reports suggested that FLIS clones were probably remnants of an earlier colonization by t(14;18)+ B cells that have preceded FL (5, 6). Our case is reminiscent of previous reported cases of FLIS associated with FL, in which the associated FL was often negative for BCL2 protein (5-7). This either suggests that FLIS is more easily
detected in those cases because of the lack of BCL2 in the FL area, or that BCL2 mutations are frequently associated with progression from FLIS to FL, when compared to *de novo* or sporadic FL.

To further establish the clonal hierarchy between the FLIS and FL lesions, we investigated the immunoglobulin variable heavy chain (V\textsubscript{H}) gene region of FL cells, a region frequently mutated in FL (8). The V\textsubscript{H} region of the FL clone was identified as IGHV3-48*03/IGHD3-22*01/IGHJ4*02, with \(~85.4\%\) homology \((+/- 0.27)\) among the various FL sub-clones \((n=16\) analyzed sequences, corresponding to 7 different sub-clones) (Figure 2A, Supplemental Table II). We backtracked this specific IGHV3-48*03/IGHD3-22*01/IGHJ4*02 sequence in the FLIS and found the same rearrangement within 3 sub-clones (Supplemental Table II). Surprisingly, 2 of the FLIS subclones were more mutated than the corresponding FL sub-clones \((82.3\% +/- 2.3\) of homology), attesting of a strong and/or repeated somatic hyper mutation (SHM) activity. In contrast, when looking at non-identical IGHV3 sequences, *i.e.* in distinct VDJ clones isolated from the FLIS area that did not match the sequence of the FL clone (and possibly represent infiltrating normal mantle-zone B-cells), the homology was of 98.2% +/-2.4 \((n=14\) sequences) (Figure 2A). In addition, the intra-clonal variability was higher in the FLIS than in the FL component, which could be due to a dynamic trafficking, such as multiple GC re-entries of the FLIS clones. This is in line with two recent reports showing a subclonal heterogeneity among genomic alterations observed in FLIS (2, 3). Overall, our analysis reveals that the FLIS and the FL clones have evolved through a divergent evolution model, which postulates the existence of unique co-existing lesions.
and sub-clones selection, in a way similar to that reported in FL and relapsed FL (9) (Figure 2B).

Finally, among the mutations at the $V_H$ IGHV3-48*03 sites, we observed that some of them were responsible for the introduction of a recurrent N-glycosylation motif (N-X-S/T) in both FL and FLIS lesions (Supplemental Table III). These specific glycosylation sites introduce oligomannose glycans that are characteristic of FL, with an incidence nearly reaching 100%. They can occasionally be observed in some GC-derived tumors other than FL, but are very infrequent in normal B cells (10). Notably, a similar “N-I-S” motif was also found in the CDR2 region of a $V_H$ IGHV3-48*03 site sequenced from a FL sample (11). Functionally, added glycans terminate at high mannose, which might influence the behavior of FLIS or FL cells through opportunistic interactions between the B cell receptor (BCR) with mannose-binding lectins bearing cells (12). The engagement of those lectins with the N-glycosylated slg would substitute conventional antigen binding, and could represent a surrogate for antigen stimulation, providing necessary signaling for lymphoma cells survival (13). This chronic signaling through the BCR might not constitute an “oncogenic hit” per se, but might nonetheless favor the generation of long lived FLIS clones, increasing the chance to accumulate the hits that will further drive the clone’s fate (14). In addition to this role in B cells survival, chronic engagement of N-glycosylated slg may also contribute to a mucin-dependent evasion from immune surveillance and the induction of an immunosuppressive environment around the tumor (15). Since DC-SIGN and DC-SIGNR are two mannose-binding lectins able to bind mannosylated Igs in vitro and FL cells (13) and which were found, in public data sets, to
be up-regulated in lymphoid organs (http://biogps.org/#goto=genereport&id=10332, Supplemental Figure 4A-B), we have investigated their expression using IHC in our case. Positive immunostaining was only present in the lymphatic endothelium and sinus histiocytes, which were not directly in contact with FL cells (Supplemental Figure 3B). Although this result does not support a stimulation of FL cells by DC-SIGN and DC-SIGNR, one cannot rule out the possible role of other lectin-like receptors. In fact, lectins are rarely completely specific for particular sugars, and it is still unclear which oligosaccharides are responsible for lectin binding to lymphoma cells (12).

To conclude, this case provided the opportunity to simultaneously analyze synchronous and contiguous FLIS and FL lesions sharing a common ancestor. This is the first report showing that N-Glycosylation sites can already be present in a FLIS lesion, adding a new stone bridging the genomic instability with the micro-environment dependency of FL cells.

Authorship Contributions

ME, DC, MML and XL performed the experiments and corresponding analyses. FB, GS, RS and BM managed the clinical data and/or wrote the manuscript. ME, NB, XL designed the project and wrote the manuscript.

Disclosure of Conflicts of Interest

The authors have no conflict of interest to report.

Supplemental informations are available at Haematologica's website.
References


Figures legends

**Figure 1.** Description of BCL2 status in both the FLIS and the FL areas of a cervical lymph node.

A. Haematoxylin/eosin coloration showing the FLIS and FL zones.

B. Histochemical staining of BCL2 with the E100 clone. The staining was negative in the germinal center of the FL areas, whereas it was extremely intense within the GC of the FLIS containing area (stronger than BCL2+ cells of the extra follicular zones

C. FISH staining with the BCL2 break apart probe (LSI BCL2 break-apart probe, Vysis®) in the FLIS area. Similar results were obtained in the FL area.

D. Sanger sequencing of the BCL2/JH breakpoint, and the *denovo* inserted sequence, in the FLIS and FL areas.

E. Histochemical staining of BCL2 with the E17 clone in the FL area

F. Histochemical staining of BCL2 with the SP66 clone in the FL area

G. Energy profile obtained from the BCL2 sequence obtained from the FLIS and the FL. Fixation sites of the 3 tested antibodies are mentioned.

**Figure 2.**

A. The IGHV3-48*03/ IGHD3-22*01/ IGHJ4*02 sequences of the FL and FLIS were used to perform a hierarchical tree between clones.

B. Percentage of mutations found in all the IGHV3-48*03/ IGHD3-22*01/ IGHJ4*02 sequences of the FL and FLIS samples. Other germinal center B cells were used as control to have an idea of the percentage of mutations that can be observed on a similar population.
Figure 1.
<table>
<thead>
<tr>
<th>Table I.</th>
<th>BCL2 amino acid sequence, exon 1 to 3 (mutations compared to the WT sequence are highlighted in red)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Germline</strong></td>
<td>1- MAHAGRTGYDNREVMKYIHYYKLSQKGYEWDAGDVGAAPPGAAPAPGIFSSQPQHTHPAAASRDPAVARTSPLQTPAAGAAAAGPALSVPVPPVVHLLRQAAGDFSRRYRDFAEEMSSLHLTPFTARGRATVVEELFRDGVNGWGRIVAFFEFGGVMCVESVNLREMSPLVDNIALWMTEYLNLHHTWQDGNGGWDAFVELYGPMSRPLFDWSLKLTTLSALVGACITLGAYLGHK-239</td>
</tr>
<tr>
<td><strong>FLIS</strong></td>
<td>1- MAHAGRTGYDNREVMKYIHYYKLSQKGYEWDAGDVGAAPPGAAPAPGIFSSQPQHTHPAAASRDPAVARTSPLQTPAAGAAAAGPALSVPVPPVVHLLRQAAGDFSRRYRDFAEEMSSLHLTPFTARGRATVVEELFRDGVNGWGRIVAFFEFGGVMCVESVNLREMSPLVDNIALWMTEYLNLHHTWQDGNGGWDAFVELYGPMSRPLFDWSLKLTTLSALVGACITLGAYLGHK-239</td>
</tr>
<tr>
<td><strong>FL</strong></td>
<td>1- MAHAGRTGYDNREVMKYIHYYKLSQKGYEWDAPGDVGAAPPGAAPPSDIFSSQPQHTHPAAASRAPVARTSPLQTPAAGAAAAGPALNPVPPVVHLLRQAAGDFSRRYRDFAEEMSSLHLTPFTARGRATVVEELFRDGVNGWGRIVAFFEFGGVMCVESVNLREMSPLVDNIALWMTEYLNLHHTWQDGNGGWDAFVELYGPMSRPLFDWSLKLTTLSALVGACITLGAYLGHK-239</td>
</tr>
<tr>
<td>Sample</td>
<td>Top VH gene match</td>
</tr>
<tr>
<td>----------</td>
<td>-------------------</td>
</tr>
<tr>
<td>Other B cells #9</td>
<td>IGHV3-48*03</td>
</tr>
<tr>
<td>FLIS #1</td>
<td>IGHV3-48*03</td>
</tr>
<tr>
<td>FLIS #2</td>
<td>IGHV3-48*03</td>
</tr>
<tr>
<td>FLIS #3</td>
<td>IGHV3-48*03</td>
</tr>
<tr>
<td>FL #1</td>
<td>IGHV3-48*03</td>
</tr>
<tr>
<td>FL #2</td>
<td>IGHV3-48*03</td>
</tr>
<tr>
<td>FL #3</td>
<td>IGHV3-48*03</td>
</tr>
<tr>
<td>FL #4</td>
<td>IGHV3-48*03</td>
</tr>
<tr>
<td>FL #5</td>
<td>IGHV3-48*03</td>
</tr>
<tr>
<td>FL #6</td>
<td>IGHV3-48*03</td>
</tr>
<tr>
<td>FL #7</td>
<td>IGHV3-48*03</td>
</tr>
<tr>
<td>FL #8</td>
<td>IGHV3-48*03</td>
</tr>
<tr>
<td>FL #9</td>
<td>IGHV3-48*03</td>
</tr>
<tr>
<td>FL #10</td>
<td>IGHV3-48*03</td>
</tr>
<tr>
<td>FL #11</td>
<td>IGHV3-48*03</td>
</tr>
<tr>
<td>FL #12</td>
<td>IGHV3-48*03</td>
</tr>
<tr>
<td>FL #13</td>
<td>IGHV3-48*03</td>
</tr>
<tr>
<td>FL #14</td>
<td>IGHV3-48*03</td>
</tr>
<tr>
<td>FL #15</td>
<td>IGHV3-48*03</td>
</tr>
<tr>
<td>FL #16</td>
<td>IGHV3-48*03</td>
</tr>
</tbody>
</table>
**Supplemental Figure 1:**
Immunohistochemical pattern of Ki67 and CD10 expression

**Legend:** Most follicles belonging to the FL component displayed a relatively low Ki67 expression (panel A, arrow) when compared to the minor FLIS component (panel A, arrowhead). CD10 expression was observed in both FL and FLIS components (panel B, arrow and arrowhead, respectively).
Supplemental Figure 3

A. AID expression

Benign HP  FLIS Zone  FL Zone

B. C-type lectin expression in the patient’s lymph node
Supplemental Figure 4. A. DC-SIGNR (probset 2079295_s_at) expression among various human tissues and cell types
Supplemental Figure 4. B. CD209 (probeset 207277_at) expression among various human tissues and cell types.