Immunohistochemical differentiation between follicular lymphoma and nodal marginal zone lymphoma – combined performance of multiple markers

by Michiel van den Brand, Janneke J.M. Mathijssen, Mar Garcia-Garcia, Konnie M. Hebeda, Patricia J.T.A. Groenen, Brunangelo Falini, Sergio Serrano, and J. Han J.M. van Krieken

Haematologica 2015 [Epub ahead of print]

Citation: van den Brand M, Mathijssen JM, Garcia-Garcia M, Hebeda KM, Groenen PJ, Falini B, Serrano S, and van Krieken JH. Immunohistochemical differentiation between follicular lymphoma and nodal marginal zone lymphoma – combined performance of multiple markers. Haematologica. 2015; 100:xxx
doi:10.3324/haematol.2014.120956

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.
Immunohistochemical differentiation between follicular lymphoma and nodal marginal zone lymphoma – combined performance of multiple markers

Michiel van den Brand¹, Janneke J.M. Mathijssen¹, Mar Garcia-Garcia², Konnie M. Hebeda¹, Patricia J.T.A. Groenen¹, Brunangelo Falini³, Sergio Serrano², J. Han J.M. van Krieken¹

¹Department of Pathology, Radboud university medical center, Nijmegen, the Netherlands
²Department of Pathology, Hospital del Mar-IMIM, Universitat Autònoma de Barcelona, Barcelona, Spain
³Institute of Hematology, University of Perugia, Ospedale S. Maria della Misericordia, Perugia, Italy

Running title: Immunohistochemistry for NMZL versus FL

Correspondence:
Michiel van den Brand
Department of Pathology, Radboud university medical center
P.O. box 9100, 6500 HB Nijmegen
E-mail: Michiel.vandenBrand@radboudumc.nl
Acknowledgements: The authors would like to thank dr. Roncador for providing the MNDA antibody. This work was supported by grants from Instituto de Salud Carlos III FEDER (PT13/0010/0005), the “Xarxa de Bancs de Tumors sponsored by Pla Director d’Oncologia de Catalunya (XBTC)”. 
Although many lymphomas can be classified reliably according to the World Health Organization Classification of 2008\textsuperscript{1}, the differentiation between nodal marginal zone lymphoma (NMZL) and follicular lymphoma (FL) is problematic in some cases. In fact, NMZL is often diagnosed by exclusion, resulting in heterogeneity in the diagnostic category of NMZL. New markers for NMZL have been described, but they have not yet been tested in combination.\textsuperscript{2, 3} In this study, we compared multiple immunohistochemical markers for their use in distinguishing NMZL from FL. From the results, we constructed an algorithm that combines these markers to help distinguish between FL and NMZL. Importantly, this algorithm also contains a category of “B-cell lymphoma, unclassifiable”, stressing the difficulty that remains in distinguishing NMZL from FL.

For the initial test series, we selected 47 patients with FL with a chromosomal rearrangement of \textit{BCL2} and 44 patients with a diagnosis of NMZL or probable NMZL from the archive of the Department of Pathology at the Radboud university medical center (Nijmegen, the Netherlands). For all NMZLs, \textit{BCL2} translocations were excluded using fluorescent in-situ hybridization with split-signal probes. Patient characteristics are described in Supplementary Table 1. For a diagnosis of NMZL, the following diagnostic criteria were used: 1) effaced architecture of the lymph node, due to a small B-cell proliferation with a follicular, marginal zone, or diffuse growth pattern, 2) either centrocytoid or more round cells with intermingled centroblasts, 3) a mature B-cell immunophenotype with expression of BCL2, 4) in cases with a follicular/ nodular growth pattern, signs of follicular colonization (presence of BCL2 negative cells and high Ki67 staining), 5) not fitting a diagnosis of chronic lymphocytic leukemia/small lymphocytic lymphoma, mantle cell lymphoma, or lymphoplasmacytic lymphoma. Expression of germinal center markers was not considered an exclusion criterion for a diagnosis of NMZL in this study.
As expected, immunohistochemistry showed significant differences between NMZL and FL (Table 1). Overall, FLs were positive for germinal center markers (CD10, BCL6, LMO2, HGAL) and negative for MNDA and IRTA1 (Supplementary Figure 1). NMZLs mostly showed an opposite pattern with positivity for MNDA in approximately two thirds of cases, IRTA1 staining in approximately one fifth of cases and usually no staining with germinal center markers. However, all germinal center markers were positive in a subset of NMZLs, and similarly, FLs with expression of MNDA were also identified (Supplementary Figure 2).

Based on the immunohistochemistry results, a combination of markers was used to design an algorithm that helps to distinguish NMZL from FL (Figure 1). This algorithm was built empirically, allowing inclusion of a category of “B-cell lymphoma, unclassifiable” to prevent contamination of the NMZL category. As expected, this algorithm classified most lymphomas according to their original diagnosis (Table 2). However, in the initial test series, one case of FL was classified as NMZL, and 6 cases of NMZL were classified as FL by the algorithm; a significant proportion of cases (13%) were considered “B-cell lymphoma, unclassifiable” by the algorithm. Most (75%) of these unclassifiable cases had an original diagnosis of NMZL.

To validate the algorithm, a second validation group of 21 FLs and 13 NMZLs, collected from the archive of the Department of Pathology at the Hospital del Mar (Barcelona, Spain) was stained for the same markers as the initial group. Overall, staining results were comparable to those in the test group with a high sensitivity of BCL6 for FL and a high specificity of IRTA1 for NMZL (Table 1); CD10 expression had a higher sensitivity in comparison to the test group, but a lower specificity. LMO2 and HGAL were less sensitive but more specific.
In this validation group, the algorithm gave a concordant classification as either NMZL or FL in 85% of cases (Table 2). No follicular lymphoma was misclassified as NMZL, and only one NMZL was misclassified as FL. Four cases (12%) were considered unclassifiable, three of which had an original diagnosis of FL and one with an original diagnosis of NMZL.

The algorithm was designed based on a comparison of NMZLs with FLs with a translocation involving BCL2. However, because a BCL2 translocation can be demonstrated relatively easily, the actual problem we are faced with in daily practice is the separation of NMZL from FL without a BCL2 translocation. FLs with and without a BCL2 translocation might be different from each other, as has been suggested by a gene expression study, and also by a recent comparative genomic hybridization study. In the latter study, genetic aberrations in FLs without a BCL2 translocation showed more resemblance to those in NMZL than in FLs with a BCL2 translocation. To address this problem, we tested a small series of 6 FLs without a BCL2 translocation, which were all classified as FL by the algorithm. This supports the idea that this algorithm also applies to FLs without a BCL2 translocation, but confirmation will require the study of larger series.

Thus far, the majority of the markers in the algorithm has only been described in single studies. Kanellis and colleagues reported expression of MNDA in 75% of NMZLs versus 5% of FLs. In our series, a less pronounced, but similar difference was observed, with MNDA expression in NMZL and FL in 70% and 15%, respectively. In accordance with Falini and colleagues, IRTA1 also discriminated between NMZL and FL in our series. However, in their study 73% of NMZLs expressed IRTA1, compared to only 21% in our study. This difference could be caused by a difference in interpretation. In our hands, faint IRTA1
expression was quite frequently observed in NMZLs, but also in some FLs. Because the reproducibility of the scores assigned to these cases proved to be very poor amongst different observers, a case was only considered positive if 30% or more of the cells showed moderate or strong expression of IRTA1. This approach, which caused a strong improvement in the diagnostic value of IRTA1 in our series, explains the small proportion of NMZLs positive for IRTA1.

Expression of the germinal center markers HGAL and LMO2 in lymphomas has been described by the group of Natkunam, who showed expression in the majority of FLs and only very rare expression in NMZL.7-10 For both markers, they detected only a single case of NMZL that was positive.7, 8 In our series, we observed more frequent expression of both LMO2 and HGAL in NMZL. We believe this could be caused by differences in inclusion criteria between our study and the previous studies. In the study by Salama et al., which contains the large majority of NMZLs previously studied for LMO2 and HGAL, cases were excluded from the NMZL group if they expressed germinal center markers9, which explains why expression of germinal center markers was not detected in NMZLs in their study. A recent study by Dyhdalo and colleagues reported LMO2 staining in 2 out of 25 NMZLs, of which one case also expressed BCL6.11 In our study, expression of germinal center markers was not considered an exclusion criterion. We made this choice because, in our experience, typical cases of NMZL do occasionally express germinal center markers and expression of CD10 and BCL6 in NMZL has been reported previously.12-14

The FLs that were used to build the algorithm were all required to have a \textit{BCL2} break, which, together with morphology, ensured that the diagnosis of FL is correct. Therefore, misclassification of FL with a \textit{BCL2} break as NMZL has been excluded. Unfortunately, no markers for NMZL can compare with the \textit{BCL2} translocation for FL. Therefore, the NMZL
group can still be expected to be more heterogeneous than the FL group, with some cases representing FL or other lymphomas rather than NMZL. Indeed, ‘NMZLs’ were rather frequently considered FL (in 12%) or unclassifiable (in 18%). This illustrates the difficulty that remains in the definition and diagnosis of NMZL; the lack of a sharp definition and the lack of positive diagnostic markers for NMZL result in a heterogeneous diagnostic category.

The ultimate question is: what is the gold standard? For this study we have used the combination of morphology and phenotyping for follicular colonization as defining criteria for NMZL. The addition of extensive immunohistochemistry, including new markers, might help to create a better gold standard for NMZL. At present however, it is very difficult to compare different strategies to diagnose NMZL as no perfect positive marker for NMZL is available. Hopefully, elucidation of the pathogenesis of NMZL will provide us with better positive markers for NMZL. The results from this study could assist in achieving this goal; the addition of extensive immunohistochemistry to conventional criteria for NMZL will help to establish smaller, but potentially more homogeneous study groups, facilitating studies into the pathogenesis of NMZL.
Authorship and disclosures

MB and JJM performed the laboratory work for this study, MB, JHK, JJM, and MGG performed scoring of immunohistochemistry and analyzed the data. JHK, SS, KH, PG and MB designed the study and interpreted the results. BF contributed essential reagents. MB and JHK wrote the paper. The authors report no potential conflicts of interest.
References


### Table 1: Immunohistochemistry results

<table>
<thead>
<tr>
<th></th>
<th>Number of positive cases n (%)</th>
<th>Sensitivity and Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial series</td>
<td>Validation series</td>
</tr>
<tr>
<td></td>
<td>NMZL (n=44)</td>
<td>FL (n=47)</td>
</tr>
<tr>
<td>BCL6</td>
<td>5 (11)</td>
<td>44 (94)</td>
</tr>
<tr>
<td>CD10</td>
<td>8 (18)</td>
<td>42 (89)</td>
</tr>
<tr>
<td>HGAL</td>
<td>11 (25)</td>
<td>44 (94)</td>
</tr>
<tr>
<td>LMO2</td>
<td>12 (27)</td>
<td>41 (87)</td>
</tr>
<tr>
<td>4/4 GCM(^1)</td>
<td>2 (5)</td>
<td>36 (77)</td>
</tr>
<tr>
<td>MNDA</td>
<td>31 (70)</td>
<td>6 (13)</td>
</tr>
<tr>
<td>IRTA1</td>
<td>6 (14)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

\(^1\)4/4 GCM: 4 out of 4 germinal center markers positive. FL: follicular lymphoma; NMZL: nodal marginal zone lymphoma; Se: sensitivity; Sp: specificity.
### Table 2: Algorithm results

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Initial series n (%)</th>
<th>Validation series n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FL</td>
<td>NMZL</td>
</tr>
<tr>
<td>FL</td>
<td>43 (91)</td>
<td>6 (14)</td>
</tr>
<tr>
<td>B-cell lymphoma, unclassifiable</td>
<td>3 (6)</td>
<td>9 (20)</td>
</tr>
<tr>
<td>NMZL</td>
<td>1 (2)</td>
<td>29 (66)</td>
</tr>
</tbody>
</table>

FL: follicular lymphoma; NMZL: nodal marginal zone lymphoma
Figure legends

Figure 1. Immunohistochemical algorithm for separation of nodal marginal zone lymphoma (NMZL) from follicular lymphoma (FL). The algorithm starts at the top with a lymphoma that is considered to be either FL or NMZL. If all four germinal center markers (BCL6, CD10, LMO2, HGAL) are positive, a diagnosis of FL is made. If not, IRTA1 expression is determined. If IRTA1 is positive, a diagnosis of NMZL is made. If IRTA1 is negative, MNDA and germinal center markers are used to divide the remaining cases in three categories: NMZL for MNDA positive cases with positivity for none or only one germinal center marker, FL for MNDA negative cases with expression of 2 or 3 germinal center markers and low-grade B-cell lymphoma, unclassifiable for cases that do not fit into these other two categories.
FL vs. NMZL

4 germinal center markers positive?
- yes → FL
- no → IRTA1 positive?
  - yes → NMZL
  - no →
    - MNDA positive and 0 or 1 germinal center marker(s) positive → NMZL
    - Marker profile not fitting into the other two categories → LGBCL, unclassifiable
    - MNDA negative and 2 or 3 germinal center markers positive → FL