Nodal reactive and neoplastic proliferation of monocytoid and marginal zone B cells: an immunoarchitectural and molecular study highlighting the relevance of IRTA1 and T-bet as positive markers

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Aims: Marginal zone B cells (MZCs) and monocytoid B cells (MBCs) appear to be related lymphoid cells that take part in reactive and neoplastic marginal zone proliferations. These lesions are not yet well characterized, and the aim of this study was to find better diagnostic criteria for them.

Methods and results: We analysed 60 nodal lesions with MBC and/or MZC proliferation for their morphological, immunophenotypic, molecular genetic and IG gene rearrangement features. On the basis of the results of the rearrangement assay and immunoglobulin light chain restriction, the lesions were divided into reactive and neoplastic groups. Among the neoplastic lesions, polymorphic and monomorphic subgroups emerged. All reactive lesions had morphological features of the polymorphic subgroup. By immunohistochemistry, IRTA1 and/or T-bet expression was found in all reactive lesions and in 90% of neoplastic lesions. Conclusions: IRTA1 and T-bet are positive markers for the identification of MZC/MBC proliferations, and thus for the diagnosis of nodal marginal zone lymphoma (NMZL). Polymorphic and monomorphic subgroups of NMZL could be distinguished. Most morphological and immunophenotypic patterns in reactive and neoplastic nodal expansions of MZCs and MBCs overlapped. Therefore, PCR clonality assay of the immunoglobulin heavy and light chain gene loci is the most reliable method for their differentiation.

Keywords: IRTA1/MUM2, marginal zone expansion, monocytoid B cells, nodal marginal zone lymphoma, T-bet

Introduction

The marginal zone (MZ) is a lymphoid tissue compartment surrounding the mantle zone, and is predominantly composed of memory and naïve B cells with centrocytoid and monocytoid morphology, as well as transformed large B cells. These cells are observed in reactive and neoplastic lesions. The
MZ is usually more prominent in the spleen and mesenteric lymph nodes (LNs) than in LNs at other locations.1,2

In addition to the MZ, monocytoid B cells (MBCs) are usually found in and adjacent to subcapsular and intermediary sinuses of LNs. These cells were first encountered in toxoplasmic lymphadenopathy,3,4 and were subsequently observed in various reactive conditions, including reactions to Epstein–Barr virus (EBV), cytomegalovirus (CMV), and human immunodeficiency virus (HIV).5–8

Neoplastic expansions of cells with features of MBCs were first described by Sheibani et al.9 under the name MBC lymphoma. Later, Cousar et al.9 described a parafollicular B-cell lymphoma. The findings of ultrastructural, cytological and topographic similarities between MBCs and marginal zone B cells (MZCs) led to the categorization of MBC lymphomas and parafollicular lymphomas as ‘marginal zone lymphoma’ (MZL).10–12 MZLs occurring at extranodal sites and those in LNs were classified as different lymphoma types in the WHO 2008 classification.13 In particular, the nodal form of MZL is considered to develop primarily in LNs without prior or concurrent involvement of extranodal sites other than bone marrow, liver, or spleen.5 Specific morphological and immunophenotypic criteria for nodal reactive and neoplastic expansion of MZCs and MBCs are lacking. Several immunohistochemical markers, such as bcl-2, CD43, and p27, have been regarded as helpful diagnostic tools for differentiating reactive proliferation of MBCs and MZCs from MZL. However, their sensitivity and specificity do not appear good enough for use as diagnostic markers.14–18

In 2003, Falini et al.19,20 introduced a monoclonal antibody directed against IRTA1 (immunoglobulin superfamily receptor translocation-associated 1) as a specific marker for MBCs and a subset of MZCs, both in reactive and in neoplastic lesions. In 2004, Dorfman et al.21 reported that T-bet, a T box transcription factor that is consistently present in T-helper 1 (Th1) cells, was expressed in a subset of B-cell lymphomas [memory B-cell related lymphomas including chronic lymphocytic leukaemia (CLL), MZL, and hairy cell leukaemia (HCL), as well as B-cell acute lymphoblastic lymphoma/leukaemia (B-ALL), but not in mantle cell lymphoma (MCL), follicular lymphoma (FL) or large B-cell lymphoma]. Later, Jöhrens et al.22 described the expression of T-bet in reactive MBCs located in or adjacent to LN sinuses, and also in mucosal-associated lymphoid tissue, but not in MZCs of mesenteric LNs or spleen. CD27 showed an opposite expression pattern: MZCs of the mesenteric LNs and spleen were positive for CD27, but MBCs in toxoplasmic lymphadenopathy were negative.

Falini et al.23 studied >2000 B-cell lymphomas for IRTA1 expression, and showed that it is significantly associated with MZLs as compared with other B-cell non-Hodgkin lymphomas (NHLs). In parallel with this, we have investigated the morphological, immunophenotypic, molecular and genetic features of nodal MZC and/or MBC expansions, to refine the diagnostic criteria for, and to study the suitability of, T-bet and IRTA1 as positive markers for the neoplastic and reactive lesions of these cells.

Materials and methods

Case selection

Specimens of LN lesions with increased number of MBCs and/or expansion of the MZ during the years 2005–2010 were obtained from the archive of the Institute of Pathology, Campus Benjamin Franklin, Charité University Medicine Berlin, Germany. Cases with previous and/or concurrent extranodal lymphomas were excluded. Sixty cases with sufficient material for morphological, immunophenotypic, molecular and molecular genetic assays were included in the study. An overview of the patient characteristics is shown in Table 1.

Ten lymph nodes, five with reactive follicular hyperplasia and five with FL, were collected from the archive of Pathodagnostik Berlin and used as controls for some immunostains. The samples were reviewed by R.B. and H.S. The vast majority of the cases were consultation cases, and follow-up data of the patients were not available.

Polymerase chain reaction (PCR)

To analyse lesions for clonal immunoglobulin (IG) gene rearrangement by PCR, DNA was extracted from paraffin sections of all 60 lesions using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Fifty-seven of the 60 lesions had appropriate DNA quality for PCR. IGH PCR was performed with three different BioMed-2 primer combinations specific for IGH framework regions 1, 2, and 3, respectively, each in conjunction with the same JH primer. In lesions showing polyclonal IGH rearrangement, immunoglobulin light chain gene PCR was carried out with the respective BioMed-2 primer combinations for IGK (kappa genes; tubes A and B) and IGL (lambda genes; tube C). Fluorescence-labelled PCR
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N, neoplastic; R, reactive.
products were analysed by capillary electrophoresis (GeneScan; 3130 Genetic Analyser: Applied Biosystems, Darmstadt, Germany), and only lesions with identical dominant PCR products in two independent PCR runs were considered to harbour clonal B-cell populations.24

**MORPHOLOGY**

Haematoxylin and eosin-stained and Giemsa-stained sections from formalin-fixed paraffin-embedded (FFPE) tissue specimens were reviewed for architectural patterns and cytomorphological features. Topographic LN compartments were defined as follows: interfollicular area (IF) – spaces outside of and in between follicles; MZ – a corona surrounding the mantle zone with a sharp border with both the mantle zone and the IF; perifollicular area (PF) – annular spaces surrounding secondary follicles without a sharp margin with either the mantle zone or the IF; and sinus and parasinus (S/PS) – the regions within and adjacent to subcapsular and intermediary sinuses.

The following architectural patterns were assessed: expansion of the IF, MZ/PF, and S/PS; the presence and architecture of reactive secondary follicles; the presence of large and small MBCs according to the description of Plank and Hansmann25; and the presence of lymphoid blasts located in the IF.

**IMMUNOHISTOCHEMISTRY**

Immunohistochemical staining was performed on 4-μm-thick deparaffinized sections. The sections were pretreated by heating in a pressure cooker containing a citrate buffer (pH 6.0), then washing with distilled water and cooling to room temperature with Tris buffer. Sections were incubated with primary antibodies for 30 min, except those against IRTA1, annexin-A1, and T-bet, for which the incubation time was 60 min. After washing following primary antibody incubation, the bound antibody was visualized by the alkaline phosphatase anti-alkaline phosphatase (APAAP) method using the alkaline phosphatase detection system from Dako (Copenhagen, Denmark). Details of antigen retrieval conditions, antibody suppliers and dilutions are listed in Table 2. The antibodies applied to all lesions included those against IRTA1, T-bet, CD30, Ki67, CD3, CD20, PD1, and kappa and lambda proteins. Immunostaining for T-bet was performed with both the monoclonal and polyclonal antibodies.

The expression pattern of T-bet was compared with those of CD3 and CD20. To differentiate T-bet-positive B cells from Th1 cells, we performed T-bet/CD3 and T-bet/CD20 double staining in nine reactive and 22 neoplastic cases. The protocol used for double staining has been described elsewhere.26 Furthermore, five lymph nodes with reactive follicular hyperplasia and five nodal FLs serving as controls were also investigated with these double staining combinations. Immunostaining for kappa and lambda light chains was performed to detect plasma cellular differentiation and light chain restriction.

All neoplastic lesions were subjected to immunostaining with antibodies against CD10, bcl-6, bcl-2, cyclin D1, CD5, and CD23, to exclude FL, MCL and CLL. To exclude HCL, whose tumour cells are T-bet-positive, annexin-A1 was also applied to all neoplastic lesions. Also, IgD staining of the neoplastic lesions was performed to allow comparison with IgD staining patterns described in the nodal MZL (NMZL) subtypes by Campo et al.27 B-lymphoblastic lymphoma was excluded by morphology, supported by the demonstration of absence of TdT in a few cases.

Expression levels of IRTA1, T-bet, PD1 and CD30 were scored in a semiquantitative way, as follows: +, single scattered positive cells; ++, frequent small groups (2–4) of positive cells; ++++, frequent large aggregates (≥5) of positive cells; and ++++, sheets of positive cells.

A schematic illustration of our semiquantitative scoring system is given in Figure 1. Positive results for each of these markers were defined as follows.

**IRTA1**: we regarded ≥+ IRTA1 expression in lymphocytes as positive, as + IRTA1 expression can also be seen outside of sinuses in non-specific reactive lymphadenitis.20 Sinus endothelial cells that are IRTA1-positive were used as an internal positive control.

**T-bet**: we regarded lesions with ≥++ expression in the central follicular area and ≥+++ expression in the IF, PF, MZ and S/PS as positive, the reasoning being that, in specimens of reactive lymphoid tissue with follicular hyperplasia and also in FL cases, + to ++ T-bet expression by a mixture of B and T cells was seen in the IF, in agreement with a report by Dorfman et al.28

For IRTA1 and T-bet staining, not only the positivity but also the distribution pattern of the staining in the IF, MZ, PF and S/PS was assessed.

**CD30**: CD30 is an activation antigen, and is commonly expressed on a few large lymphoid cells at the periphery of follicle centres and in IF areas. We evaluated expression of CD30 by considering the density and location of the positive cells in the lymph node components.

**PD1**: this antigen is strongly expressed in follicular T-helper 2 (Th2) cells and weakly expressed in scat-
tered IF cytotoxic T cells.\textsuperscript{29,30} Therefore, we considered positivity for PD1 in relation to the strength and location of positive cells in lymph node components. The strongly PD1-positive follicular T cells in the remaining germinal centres served as an internal control for the intensity of PD1 expression.

CD21, CD27, IgM, CD38, CD138 and MUM1/IRF4 were used for further characterization of the lesions.

**EBV-ENCODED RNA IN-SITU HYBRIDIZATION**

**In-situ** hybridization (ISH) for EBV-encoded nuclear RNA (EBER) molecules was performed for all cases with probes specific for EBER1 and EBER2 prepared as digoxigenin-labelled run-off transcripts from the plasmids pJJJ1 and pJJJ2. For ISH, deparaffinized and rehydrated paraffin sections were exposed to 0.2 M HCl and 0.6 mg/ml pronase (Boehringer-Mannheim, Hannover, Germany), and this was followed by postfixation with 4% paraformaldehyde and dehydration through graded ethanols. After overnight hybridiza-
tion of the labelled probes (0.1 μg) at 50°C, bound probes were detected using a monoclonal digoxigenin-specific alkaline phosphatase-conjugated antibody (Boehringer-Mannheim) and an alkaline phosphatase detection system (Dako).31

FLUORESCENCE IN-SITU HYBRIDIZATION (FISH)

Four-micrometre-thick sections of FFPE tissue from 36 of the neoplastic lesions and 10 of the reactive lesions were subjected to interphase FISH following the procedure described in the Dako Histology FISH Accessory kit; a few optimizations were also applied. The FISH probes included: Vysis LSI BCL6 Dual Colour, Break Apart Rearrangement Probe (Abbott, Wiesbaden, Germany); CEP3 (D3Z1) Spectrum Orange (Abbott); CEP7 (D7Z1) Spectrum Aqua (Abbott); and CEP18 (D18Z1) Spectrum Green (Abbott). To evaluate FISH assays, the signal constellation in 100 non-overlapping interphase nuclei was scored using a Zeiss motorized Axio Imager Z1 microscope (Göttingen, Germany) with a cut-off level of 1.2% for BCL6 break and cut-off levels of 0.5%, 0.3% and 0.8% for polysomy of chromosomes 3, 7, and 18, respectively, as determined by interphase FISH experiments on sections of reactive tonsillar tissues.

Results

POLYMERASE CHAIN REACTION

Fifty-seven of 60 lesions contained amplifiable DNA and were subjected to PCR. Thirty-three lesions showed clonal rearrangement of IGH. In the remaining 24 lesions that showed polyclonal IGH rearrangement, a PCR for the rearrangement of the light chain loci was performed. Clonal rearrangement of IGK was seen in five of the 24 lesions; IGL rearrangement was not detected in any of the lesions. Altogether, 38 lesions were clonal by PCR. The remaining 19 were regarded as reactive lesions. For the three lesions not taken forward for PCR, due to degraded DNA, immunostaining for kappa and lambda light chains (as demonstrated below) showed restriction for one light chain; these results were considered to indicate clonality. All together, the results obtained by PCR and immunostaining led to classification of 41 of the 60 lesions as neoplastic.

MORPHOLOGY

Among the 41 neoplastic lesions, two distinct subgroups emerged. The first subgroup, comprising 36 (88%) of the 41 lesions, was characterized by partially preserved nodal architecture and expansion in one or more of the IF, PF and MZ nodal compartments, with a polymorphic cell population containing a mixture of small and large MBCs, and centrocytoid and transformed large lymphocytes (Figure 2A–D). Expansion of the IF was more frequent and more prominent than for the other nodal compartments. We named this subgroup the polymorphic subgroup.

In the second subgroup, comprising five (12%) of the 41 clonal lesions, the LN architecture was totally effaced by a nodular proliferation of monomorphic cells resembling small MBCs. The IF spaces in this subgroup were attenuated (Figure 2E,F). This subgroup was designated the monomorphic subgroup. Focally, two of the polymorphic neoplastic lesions had morphological areas that were similar to those in the monomorphic subgroup.

All of the reactive lesions showed the polymorphic pattern (Figure 3A–D). No morphological criteria were found for reliable differentiation of the neoplastic polymorphic lesions from the reactive lesions. However, some of the morphological patterns were seen more frequently in the neoplastic than in the reactive lesions, and vice versa. For example, the presence of many reactive secondary follicles, purely sinusoidal/parasinusoidal expansion of MBCs and microgranulomas occurred more frequently in the reactive lesions. Conversely, the absence or attenuation of germinal centres was more often observed in the neoplastic lesions. The morphological findings in polymorphic neoplastic and reactive lesions are compared in Table 3.

IMMUNOHISTOCHEMISTRY

Due to formalin fixation, kappa and lambda staining was evaluable only in plasma cells, except for two cases that showed surface membrane labelling of lymphocytes; both cases showed light chain restriction. Among the 36 polymorphic NMZL, plasma cells were found in 18 cases (50%) and in 12 out of 18 cases the plasma cells were monotypic for Ig light chain expression. In contrast, only two of the 5 (40%) monomorphic NMZL and 14 of the 19 reactive lesions contained plasma cells that all were polytypic. As mentioned above, in all of the three lesions with degraded DNA, kappa and lambda immunostaining revealed light chain restriction (i.e. two were kappa-positive, and one was lambda-positive). All the neoplastic lesions were negative for bcl-6, CD5, and cyclin D1, excluding the diagnosis of FL, CLL, and MCL. Only a single lesion expressed CD10

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in the absence of bcl-2 and bcl-6, in contrast to FL. CD23 was partially positive in four of the 41 neoplastic lesions and diffusely positive in three, all of which were negative for CD5. The annexin-A1 staining pattern, characterized by the presence of scattered positive cells in the IF in all investigated

Figure 2. Morphological spectrum of nodal marginal zone lymphoma (NMZL) (H and E staining). A. Expansion of the interfollicular area in a polymorphic NMZL. A residual germinal centre with a mantle zone is located at the centre. B. Marginal zone expansion in a polymorphic NMZL. C. Follicular colonization by a polymorphic NMZL. D. Cytological features of a polymorphic NMZL. Note the presence of blasts among the small lymphoid cells. E. Growth pattern of a monomorphic NMZL. F. Cytological features of the same case as in (E). Note the absence of blasts.
lesions, was compared with that of CD20, CD3 and T-bet/CD20 in 31 lesions. This revealed that the annexin-A1-positive cells were T cells. The result excluded the possibility that the T-bet-positive B cells represent HCL cells.

Immunohistochemical staining demonstrated positivity for IRTA1 in 15 of 19 (79%) reactive lesions, 24 of 36 (67%) polymorphic neoplastic lesions, and one of five (20%) monomorphic neoplastic lesions (Figure 4A–F).

The results of T-bet staining with polyclonal and monoclonal anti-T-bet antibodies showed that the polyclonal antibody is more sensitive. The number of positive cells labelled by the polyclonal antibody was always one scale higher (according to semiquantitative scoring) than the number of positive cells stained with the monoclonal antibody. Therefore, we included only the staining results obtained with the polyclonal antibody. T-bet positivity was seen in all reactive lesions, in 27 of 36 (75%) polymorphic and three of five (60%) monomorphic NMZL (Figure 5A–D). In seven of the NMZL, the intensity of T-bet expression was weak.

To determine how many of the T-bet-positive lymphocytes are B or T cells, double immunostaining was performed. T-bet/CD20 and T-bet/CD3 double staining revealed that, in all stained cases (nine of nine reactive lesions and 22 of 22 neoplastic lesions), either a majority or at least a significant number of T-bet-positive lymphocytes were B cells (Figure 6A–F). Comparison of double labelling results with H and E staining revealed that T-bet-positive B cells are
mainly located in the MBC distribution area. The investigation of T-bet/CD20 and T-bet/CD3 double staining in five FLs and five reactive follicular hyperplasias showed + to ++ T-bet expression; in all the FLs, the T-bet-positive cells were located only in the IF, whereas in the reactive lesions the T-bet-positive cells were present in the IF and sometimes also in the PF. The T-bet-positive cells consisted of a mixture of B and T cells, with varying ratios.

Expression of IRTA1 and/or T-bet was observed in all the reactive lesions and in 37 of 41 (90%) NMZLs: in 33 of 36 (92%) polymorphic and in four of five (80%) monomorphic lesions. Comparison of IRTA1 and T-bet immunostaining results is shown in Table 4. Comparing the distribution pattern of T-bet-positive and/or IRTA1-positive cells between reactive lesions and polymorphic NMZLs (the monomorphic subgroup could be morphologically differentiated from reactive lesions), IRTA1-positive and/or T-bet-positive cells were found in all four evaluated compartments of the LNs – the IF, PF, MZ, and S/PS (Table 5) – and in all cases they were distributed in more than one compartment. The density of positive cells in the above-mentioned locations varied between cases. Some of the distribution patterns were more common in reactive lesions and some were more frequent in neoplastic lesions (Table 5), which is in keeping with the morphological findings.

1 A distinct MZ distribution of IRTA1-positive and/or T-bet-positive cells with sharp margins with the mantle zone and IF was observed more frequently in neoplastic lesions than in reactive lesions [8/36 (22%) versus 1/19 (5%)]. The single reactive LN with clear IRTA1 and T-bet expression in the MZ was located in the mesocolon. IRTA1 positivity and/or T-bet positivity in the PF was also more frequent in polymorphic neoplastic lesions than in reactive lesions [20/36 (56%) versus 5/19 (26%)].

2 Follicular colonization by MBCs expressing IRTA1 and/or T-bet was detectable in 19 of 36 (53%) lesions of the polymorphic subgroup and in six of 19 (31%) reactive lesions. In some instances, the follicular colonization was so dominant that the lesion morphologically resembled a follicular lymphoma (Figures 4C and 5B).

Only four neoplastic lesions were negative for both IRTA1 and T-bet; all these lesions showed extensive infiltration by MBCs as seen with H and E staining.

The Ki67 index, with a mean value of 27%, varied from 5 to 60% in the polymorphic neoplastic lesions (Figure 7A). The mean value (9%) and its range (5–15%) were significantly lower in the monomorphic subgroup \( (P = 0.002) \) (Figure 7B). The mean value of the Ki67 index in MBC-containing areas of reactive lesions was 55%.

IgD staining was positive in the tumour cells of 15 of 36 (42%) polymorphic lesions. Among positive cases, five showed focal staining and six showed weak staining. None of the monomorphic neoplastic lesions expressed IgD. This enabled us to clearly differentiate our type from the ‘spleenic type’ of NMZL described by Campo et al.,27 despite some architectural similarities between these two types of lesion.

Ten of 19 (53%) reactive lesions and two of 36 (5%) polymorphic neoplastic lesions showed ≥+++ expression of CD30-positive cells, a feature that may be a source of diagnostic error and confusion with high-grade CD30-positive lymphomas (Figure 7C,D). There was no significant difference in CD30++ positivity between reactive and polymorphic neoplastic lesions [6/19 (32%) versus 13/36 (36%)]. Note that none of the monomorphic neoplastic lesions contained any CD30-positive cells.

The investigation of PD1 staining revealed ≥++ expression in extrafollicular areas in 21 of 36 (58%) polymorphic neoplastic lesions (Figure 7E) and one of 19 (5%) reactive lesions. However, + PD1 positivity was seen in 11 of 19 (58%) reactive lesions.

Immunostaining for CD21, CD27, IgM, CD38, CD138 and MUM1/IRF4 did not contribute to better characterization of MZC and/or MBC expansion.
Figure 4. IRTA1 staining in nodal marginal zone lymphoma (NMZL) and reactive marginal zone (MZ) expansion. A, IRTA1 staining of a polymorphic NMZL. IRTA1-positive cells proliferate in the interfollicular area (IF). B, IRTA1 staining of a polymorphic NMZL. The IRTA1-positive cells colonize the MZ. C, IRTA1 staining of a polymorphic NMZL. The IRTA1-positive cells colonize a follicle. D, IRTA1 staining of a monomorphic NMZL. IRTA1-positive cells are only present at the periphery of the nodules. E, IRTA1 staining of a reactive lymph node with expression in the sinus/parasinus region. F, IRTA1 staining of a reactive lymph node with expansion of monocytoid B cells. The IRTA1-positive cells populate the IF and the MZ, and colonize some follicles.
EBV-ENCODED RNA IN-SITU HYBRIDIZATION

In-situ hybridization showed significant numbers of EBV-infected cells in three of 19 (16%) reactive lesions and two of 41 (5%) neoplastic lesions with features of the polymorphic subgroup.

FLUORESCENCE IN-SITU HYBRIDIZATION

Polysomy of chromosomes 3, 18 and 7 was demonstrable in 14 of 31 (45%), five of 31 (16%) and two of 31 (6%) polymorphic NMZLs respectively. Only one of the monomorphic NMZLs showed polysomy, of chromosomes 3 and 18. Rearrangement of BCL6 was not detected in any of the NMZLs. None of the 10 reactive lesions investigated by FISH showed numerical aberration of chromosomes 3, 7 and 18 and/or BCL6 rearrangement. The results of the FISH assays are summarized in Table 6.

Discussion

Despite being the subject of many studies, the diagnosis of NMZL is still made by exclusion. The criteria for differentiating reactive lesions with MZC and/or MBC expansion from NMZLs are not defined. In an attempt to overcome these limitations, we investigated 60 cases with differing degrees of MZC and/or MBC expansion. We excluded FL, MCL, CLL and HCL by the use of CD20, bcl-6, CD10, CD23, CD5, cyclin D1 and annexin-A1 immunohistochemistry. We characterized these lesions in more detail by recognition of subtle morphological features and immunostaining for IRTA1, T-bet, IgD, Ki67, kappa, lambda, CD30

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Figure 6. T-bet/CD20 and T-bet/CD3 double staining in nodal marginal zone lymphoma (NMZL). A, T-bet/CD20 double staining in a polymorphic NMZL with expansion of T-bet-positive cells in the marginal zone and interfollicular area; the insert shows a higher magnification. B, T-bet/CD3 double staining of the same case as in (A); the insert shows a higher magnification. The double stains in (A) and (B) confirm that most T-bet-positive cells are B cells and not T cells. C, T-bet/CD3 double staining of a polymorphic NMZL in which the T-bet-positive cells colonize follicles. D, Higher magnification of T-bet/CD3 double staining of the same case as in (C). E, T-bet/CD20 double staining of a monomorphic NMZL. F, T-bet/CD3 double staining of the same monomorphic NMZL case as in (E).

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and PD1 molecules, as well as by PCR for clonal rearrangement of IG genes and FISH assay for BCL6 rearrangement and polysomy of chromosomes 3, 7, and 18.

Our study led to three significant findings: (i) IRTA1 and T-bet proved to be valuable positive markers for identifying MBCs and for differentiating MZ expansions from other B-cell reactions via their MBC component; (ii) overlapping morphological and immunophenotypic features of reactive MZC/MBC expansion and NMZL make PCR clonality assays (for both the IGH and IGK loci) and light chain restriction the only reliable methods for differentiating reactive from neoplastic lesions; and (iii) there are two subgroups of NMZLs, designated as polymorphic and monomorphic subgroups.

The finding that IRTA1 and T-bet are expressed in NMZL is not new. Falini et al.19 demonstrated IRTA1 expression in MBCs and MZLs in 2003. Dorfman reported T-bet expression in MZL in 2004.28 Despite such reports, the evaluation of these two molecules has not been introduced for the diagnosis of NMZL in the WHO lymphoma classification. In a parallel study with our present investigation, Falini et al.23 investigated 2104 different B-cell lymphomas for IRTA1 expression, and found that IRTA1 is a positive marker for the diagnosis of MZL. In this study, IRTA1 positivity was found in 73% of NMZLs and in 93% of extranodal MZLs. These findings and the report of T-bet expression in B-NHL related to postgerminal centre/memory B-cell lymphomas and MBCs32 prompted us to analyse the expression of both IRTA1 and T-bet in lymph nodes with morphological criteria for MBC and/or MZC expansion and, as a control, in follicular hyperplasia and follicular lymphoma. We confirmed, by double staining for CD3/T-bet and CD20/T-bet, that the majority of the T-bet-positive lymphocytes in our cases were B cells, except for scattered IF T cells. We observed positivity for IRTA1 in 67% of cases (40/60) and for T-bet in 82% (49/60) of cases. Combination of these two markers increased the detection rate for MBCs to 93% (56/60), and highlighted monocytoid differentiation of B cells, as well as their distribution in the S/PS, IF, PF and MZ of LNs from different sites, including the mesocolon. Both markers also proved to be valuable for the detection of follicular colonization by MBCs/MZCs. Follicular colonization could not be reliably detected by morphology alone.

Due to heterogeneous architectural patterns and the presence of polymorphic cell populations in NMZLs, the definition of specific morphological criteria for this lymphoma type have proved to be difficult.12,33,34 The most precise architectural categorization was

### Table 4. Expression patterns of IRTA1 and T-bet in reactive and neoplastic lesions

<table>
<thead>
<tr>
<th></th>
<th>IRTA1 positivity (%)</th>
<th>T-bet positivity (%)</th>
<th>Positivity of both T-bet and IRTA1 (%)</th>
<th>Positivity of T-bet and/or IRTA1 (%)</th>
<th>Negativity of both T-bet and/or IRTA1 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neoplastic lesions</td>
<td>25/41 (61)</td>
<td>30/41 (73)</td>
<td>17/41 (41)</td>
<td>37/41 (90)</td>
<td>4/41 (10)</td>
</tr>
<tr>
<td>Polymorphic neoplastic lesions</td>
<td>24/36 (67)</td>
<td>27/36 (75)</td>
<td>17/36 (47)</td>
<td>33/36 (92)</td>
<td>3/36 (8)</td>
</tr>
<tr>
<td>Monomorphic neoplastic lesions</td>
<td>1/5 (20)</td>
<td>3/5 (60)</td>
<td>0/5 (0)</td>
<td>4/5 (80)</td>
<td>1/5 (20)</td>
</tr>
<tr>
<td>Reactive lesions</td>
<td>15/19 (79)</td>
<td>19/19 (100)</td>
<td>15/19 (79)</td>
<td>19/19 (100)</td>
<td>0/19 (0)</td>
</tr>
</tbody>
</table>

### Table 5. IRTA1 and T-bet expression pattern of reactive and neoplastic lesions in different nodal compartments

<table>
<thead>
<tr>
<th>Lymph node compartment</th>
<th>Reactive lesions</th>
<th>Polymorphic neoplastic lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IRTA1 (%)</td>
<td>T-bet (%)</td>
</tr>
<tr>
<td>Sinus</td>
<td>14/19 (74)</td>
<td>16/19 (84)</td>
</tr>
<tr>
<td>IF</td>
<td>6/19 (32)</td>
<td>9/19 (47)</td>
</tr>
<tr>
<td>PF/MZ</td>
<td>3/19 (16)</td>
<td>5/19 (26)</td>
</tr>
<tr>
<td>GC (follicular colonization)</td>
<td>2/19 (11)</td>
<td>5/19 (26)</td>
</tr>
</tbody>
</table>

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Figure 7. A, Ki67 staining of a polymorphic nodal marginal zone lymphoma (NMZL) with a large number of Ki67-positive cells in the interfollicular area (IF). B, Ki67 staining of a monomorphic NMZL with a much lower Ki67 index than that of the polymorphic NMZL shown in (A); the insert shows a higher magnification. C, CD30 staining shows positivity in the perifollicular area and IF, the areas where the monocyteid B cells (MBCs) are located in a polymorphic NMZL; the insert shows a higher magnification. D, The same phenomenon as shown in (C) in a reactive lymph node with hyperplasia of MBCs and increased CD30-positive blasts in the IF; the insert shows a higher magnification. E, Unusual strong expression of PD1 in extrafollicular spaces of a polymorphic NMZL. The follicular T cells inside the residual germinal centre constitute a positive internal control for the PD1 expression level. F, PD1 staining in a lymph node with reactive follicular hyperplasia. The expression level in the extrafollicular T cells (short arrow) is significantly lower than that in the follicular T cells of the germinal centre (long arrow).
provided by Salama et al.\textsuperscript{12} in a study of 51 NMZL cases; they described diffuse, follicular/nodular, PF/MZ and IF proliferation patterns in NMZL. However, owing to the overlap of these patterns in the majority of the cases, the described criteria could not be used for subtyping NMZL. Campo et al.\textsuperscript{27} provided a new subtyping for NMZL by comparing the architecture and immunophenotype of NMZL with those of splenic and extranodal MZL. They subdivided NMZLs into MALT type, splenic type, and polymorphic type. There are also a number of studies that have focused on reactive MBC/MZC expansion and have described different morphological patterns.\textsuperscript{8,32,35,36} However, none of the above-mentioned studies evaluated neoplastic and reactive lesions in parallel.

Our study also showed that evaluation of the MZC/MBC-expanding lesions identified only some morphological and immunohistochemical findings that were helpful, but not specific, to distinguish NMZLs from reactive lesions. The absence or extensive attenuation of reactive secondary follicles, as well as follicular colonization, favoured neoplastic lesions. In contrast, extensive reactive follicular hyperplasia and sinusoidal/parasinusoidal expansion with MBC infiltration were more common in reactive processes. The only differentiating immunophenotypic criterion was the monotypic immunostaining of kappa and lambda immunoglobulins, which was an indicator of clonality. It is also necessary to mention that the absence of immunoglobulin light chain restriction could not exclude a diagnosis of lymphoma, as many of our neoplastic lesions showed either polytypic light chain staining in their plasma cells or no light chain expression at all. In these lesions, only the PCR clonality assay of \textit{IG} loci was able to differentiate reliably between reactive and neoplastic lesions. Also, in the particular clinical setting of childhood reactive MZ hyperplasia, light chain restriction can be associated with polyclonal immunoglobulin gene rearrangement by PCR assay. Therefore, a PCR clonality assay should be always applied when possible. The rearrangement pattern of the \textit{IGH} locus failed to detect clonality in 12% of the neoplastic lesions; therefore, the \textit{IG} light chain gene rearrangement was required to reveal B-cell clonality in these cases. The reliability of the results of the PCR clonality assays was substantiated by FISH data. Polysomy of chromosome 3, 8 and/or 17 was demonstrable in 15 of 36 studied clonal lesions, but in none (0/10) of the studied non-clonal cases. However, FISH is not sufficiently sensitive or specific to be used for clonality demonstration for diagnostic purposes.

Morphological evaluation of our neoplastic cases revealed two distinct subgroups, namely polymorphic and monomorphic. The polymorphic subgroup was characterized by partially preserved LN architecture. In these cases, a polymorphic cell population containing centrocytoid lymphocytes, MBCs and transformed blasts infiltrated the S/PS, IF, PF/MZ and germinal centres. Expansion of the IF zone was most frequent and most prominent. In contrast to the traditional morphological description of NMZL, they may not always demonstrate MZ/PF expansion. The MBCs/MZCs frequently expand into the IF in a lymph node that may lack the MZ/PF. This subgroup has some morphological similarities with the MALT-type and polymorphic-type NMZLs of Campo et al.\textsuperscript{27} All of our reactive lesions showed morphology similar to this subgroup.

In the monomorphic subgroup, the affected lymph nodes were totally effaced by back-to-back nodules containing a monomorphic population of small MBCs. Between the nodules, there were no or very small IF spaces. These cases are different from the NMZL splenic type defined by Campo et al.,\textsuperscript{27} despite some architectural similarity. The cell population in our monomorphic lesions was composed exclusively of small MBCs, whereas the splenic-type NMZL of Campo et al. contained a very heterogeneous cellular infiltrate. Moreover, IgD expression, the rationale behind the designation by Campo et al. of this group as NMZL splenic type, was lacking in all of our monomorphic cases.

In our study, all five monomorphic lesions proved to be clonal in the PCR assay; however, the number
of our cases is too small to exclude the occurrence of this pattern in reactive lesions. Furthermore, there is a study that reported on reactive nodal lesions, some of which showed similar morphological features to our monomorphic lesions. Also, to diagnose a monomorphic NMZL and to report that a lymph node is totally effaced by a monomorphic MBC population, indicates that complete excision of the lymph node is mandatory. A needle or incisional biopsy is not appropriate for this purpose. In two of the five monomorphic cases, T-bet staining was stronger in the periphery of nodules than in their centres, an appearance that is reminiscent of the MZ/PF. The focal presence of monomorphic morphology was observed in two of the polymorphic NMZL. These findings indicate an overlap between the two subgroups.

The morphological differences between our two subgroups were in keeping with the immunophenotypic findings. Polymorphic lesions were associated with a higher Ki67 index (mean value 27%) in MBC-containing areas than monomorphic NMZL (mean value 9%). Other differences were the presence of many CD30-positive lymphoid blasts and unexpected extrafollicular expansion of strongly PD1-positive T cells in polymorphic cases, which were both absent in the monomorphic subgroup. The low Ki67 index and the absence of CD30-positive blasts in monomorphic NMZLs are in keeping with the lack of large MBCs in these cases. The lack of PD1-positive cells in monomorphic lesions was compatible with the absence of the IF, the region that contained PD1-positive cells in polymorphic lesions. Thus far, strong PD1 expression is known only for intrafollicular Th2 cells. In extrafollicular spaces, T cells weakly express PD1 (Figure 7F). The strong extrafollicular expression of PD1 in our cases favours the view that these cells represent displaced follicular Th2 cells or non-follicular T cells with up-regulated PD1 expression resulting from unknown stimuli. The biological importance of this finding is unknown, and warrants further investigation. Muenst et al. recently reported that an increased number of PD1-positive T cells is associated with a favourable prognosis in follicular lymphoma. However, tissue microarrays were used in this study, preventing the evaluation of PD1-positive cell localization in all lymph node compartments.

In conclusion, we have identified IRTA1 and T-bet as molecules that positively characterize reactive and neoplastic MBC/MZC expansions. Their combined application increases the diagnostic sensitivity. The NMZLs studied show a wide morphological spectrum, ranging from remarkable interfollicular expansion by a polymorphic cell population to nodular proliferation of small monomorphic cells. This spectrum provided a basis for the distinction of two subgroups: monomorphic and polymorphic lesions. Reliable differentiation of reactive MBC/MZC expansions from NMZL can only be achieved by using molecular clonality assays of the immunoglobulin heavy and light chain gene loci.

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Monocytoid/marginal zone B-cell lesions


