Original Article
IgM expression in paraffin sections distinguishes follicular lymphoma from reactive follicular hyperplasia

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Abstract: The trapping of IgM-containing immune complexes (ICs) by follicular dendritic cells (FDCs) serves as an important step in promoting germinal center (GC) formation. Thus, the deposition of IgM-containing ICs on FDCs can be detected by antibodies recognizing IgM. The present investigation provides the first comprehensive report on the IgM staining pattern in follicular lymphoma (FL, n = 60), with comparisons to reactive follicular hyperplasias (RFH, n = 25), demonstrating that immunohistochemical staining for IgM in paraffin-embedded sections seems to be an additional tool for differentiating between FL and RFH. In RFH, IgM highlighted processes of FDCs, with stronger and more compact staining in light than in dark zones, with occasional very dim staining of GC B cells. In FL, IgM expression patterns were of three types. Pattern I (38 cases) stained tumor cells within neoplastic follicles, with no staining of FDCs. Pattern II (15 cases) stained neither tumor cells nor FDCs. Pattern III (7 cases) stained tumor cells with (3 cases) or without (4 cases) IgM expression; however, variable and attenuated IgM expression was observed on FDCs in each case. Interestingly, significant numbers of IgD+ mantle cells were preserved around the neoplastic follicles in these 7 cases. The data suggested that a complete or considerable loss of IgM expression in FDCs, reflecting the loss of IgM-containing ICs in FDCs, is a typical feature of FL. Increased IgM expression by GC B cells can also serve as an indicator of immunophenotypic abnormality in FL.

Keywords: Follicular lymphoma, reactive follicular hyperplasia, follicular dendritic cells, IgM, immunohistochemistry

Introduction

In routine pathology practice, follicular lymphoma (FL) and reactive follicular hyperplasia (RFH) are common biopsy findings with fundamentally different characteristics and prognoses, but both of which are sometimes difficult to distinguish. FL is one of the most common types of B cell lymphoma, and is characterized by a partial follicular growth pattern in the majority of cases, a morphological resemblance of the tumor cells to follicle centrocytes and centroblasts, and overexpression of Bcl-2 protein resulting from (14;18) translocation [1]. Distinct histological and immunophenotypic features have been used to differentially diagnose FL from RFH [2-4]; however each of these has its own limitations, often leading to difficulties in diagnosis. Bcl-2 expression in paraffin sections is the single most useful marker, being consistently negative in RFH [5, 6], but approximately 10-15% of FL cases will be negative by immunohistochemical analysis, especially in high grade cases [7-9]. Furthermore, diagnostic difficulties occur when there is prolonged antigenic stimulation and the RFH becomes florid, producing numerous enlarged follicles, these cases are difficult to differentiate from Bcl-2-negative, high grade FL.

Mature naïve B lymphocytes develop continuously from pluripotent progenitors in the bone marrow, subsequently migrating to secondary lymphoid tissue. Following activation of B lymphocytes by antigens in T cell areas, two distinct developmental paths are followed by B cells [10, 11]. In the early stage, a subset of activated B cells proliferates and differentiates into short-lived plasma cells that produce IgM antibodies. This process proceeds outside of germinal centers (GCs). Secreted IgM antibodies opsonize free antigens and form immune

Table 1. Antibodies used in this study

Antibody	Clone	Source	Dilution
CD20	L26	Labvision, Fremont, USA	1:100
CD3	SP7	Labvision	1:100
CD21	2G9	Labvision	1:100
CD35	KuN241	Labvision	1:100
CD10	56c6	Novocastra, Newcastle, UK	1:100
Bcl-6	LN22	Leica, Milton Keynes, UK	1:100
IgM	Polyclonal	DAKO, Capinteria, USA	1:200
IgD	Polyclonal	DAKO	1:100
Ki-67	MIB-1	DAKO	1:100
Bcl-2	Sp66	Spring Bioscience, Pleasanton, USA	1:100

mation on the primary antibodies used in this study (CD20, CD3, CD21, CD35, CD10, Bcl-6, IgM, IgD, Bcl-2, and Ki-67), including the source, manufacturer, and working dilution, is summarized in **Table 1**. Deparaffinized sections were treated with each of the above antibodies with the exception of anti-CD35 antibody, which was used only for eight RFH and 15 FL cases. Antigen retrieval was performed by pressure-cooking the slides in EDTA/Tris-HCl buffer (pH 9.0) for 5 min for the anti-IgM antibody, and 2.5 min for the other antibodies. Tissue was stained using a two-step EliVision amplification method (EliVision plus kit, Fuzhou Maixin Biotechnology Development Co., Ltd., Fuzhou, China) according to the manufacturer's instructions. The slides were then incubated with DAB+ chromogen (K-3468; DAKO, Carpinteria, USA) for 10 min. All sections were counterstained with hematoxylin with the exception of those labeled with anti-IgM antibody.

complexes (ICs), some of which will be trapped on the surface of follicular dendritic cells (FDCs). The alternate developmental choice for activated B cells is entry into a lymphoid follicle to establish a GC. During this process, the trapping of IgM-containing ICs by FDCs in a complement/complement receptor-dependent manner serves as an important step in promoting GC formation [12-14]. Thus, the deposition of IgM-containing ICs on FDCs can be detected by antibodies recognizing IgM [15-17].

Studies examining IgM expression on FDCs in FL cases have yielded conflicting findings [15, 17]. The present investigation provides the first comprehensive report on the IgM staining pattern in FL, with comparisons made to RFH. The results demonstrate that immunohistochemical staining for IgM in paraffin-embedded sections seems to be an additional tool for differentiating between FL and RFH.

Materials and methods

Case selection

Twenty-five samples of RFH and sixty FL specimens were collected from the Department of Pathology, Beijing Friendship Hospital (Capital Medical University, Beijing, China). All were reevaluated and the initial diagnosis was confirmed. As part of a laboratory validation study, twenty-two FL specimens, including all the Bcl-2-negative cases, had been previously examined for IgH gene rearrangements by PCR analysis with positive results.

Immunohistochemistry

Sections from formalin-fixed, paraffin-embedded tissue specimens were examined. Infor-

Evaluation of mantle zones in FL specimens

Mantle zones were identified by immunostaining for IgD. The degree of preservation of mantle zones was graded as follows: 0, few or no mantle cells surrounding GCs; ++, thin but relatively intact mantle zones, or those that disappeared focally; +, intermediate between 0 and ++.

Results

Histological diagnosis and immunophenotypic features of RFH and FL are summarized in **Table 2**. The relationship between IgM expression pattern III of tumor and mantle zone preservation grade in FL is summarized in **Table 3**.

Clinical features and histopathology

The 25 RFH specimens consisted of palatine tonsils (n = 10; seven male, three female; age range, 10-19 years), lymph nodes (n = 12; six male, six female; age range, 34-67 years) from patients with chronic lymphadenitis, and spleens (n = 3; two male, one female; age range, 42-57 years) from patients with congestive splenomegaly resulting from liver cirrhosis. Hyperplastic follicles had well-formed GCs and mantle zones. GCs at different developmental

Table 2. Immunophenotypic features of RFH and FL

Diagnosis	Total no.	IgM		IgD		CD10+	Bcl-6+	Bcl-2+
		FDC+	GC B cells+	FDC+	GC B cells+			
RFH	25	25 (100%)	0 (0%)*	0 (0%)	0 (0%)	25 (100%)	25 (100%)	0 (0%)
FL	60	7 (12%)#	41 (68%)	0 (0%)	17 (28%)	47 (78%)	60 (100%)	46 (77%)

The number (%) of cases is indicated. GC, germinal center; FDC, follicular dendritic cell; FL, follicular lymphoma; RFH, reactive follicular hyperplasia. *GC B cells are typically negative for IgM, but weak immunoreactivity was occasionally observed. #In these cases, a significant number of IgD-positive mantle cells clustered around the neoplastic follicles, and attenuated IgM expression was observed in FDCs.

stages could be seen in most specimens, including those consisting of centroblasts and centrocytes, with obvious polarization (light and dark zones), as well as GCs consisting primarily of centroblasts and tingible body macrophages, which may have resulted from oblique sectioning of the basal site of a secondary follicle. Atrophic GCs were also observed.

The 60 confirmed FL cases (38 male, 22 female; age range, 31-82 years) comprised lymph node (n = 53), tonsil (n = 3), spleen (n = 3), and soft tissue (n = 1). Most cases presented with disseminated disease. Based on the number of centroblasts in 10 randomly selected high magnification fields, and in accordance with the recommendations of the new World Health Organization classification [1], 19 cases were classified as grade I, 10 as grade II, 25 as grade IIIa, and six as grade IIIb. In all cases, a follicular growth pattern was observed in at least 25% of the infiltrated area. Varying degrees of mantle zone preservation was observed in 17 cases.

Immunophenotypic features

Conventional immunostaining: In RFH specimens, reactive GCs were positive for the follicular center markers CD10 and Bcl-6, but negative for Bcl-2. Ki-67 immunoreactivity revealed a high proliferation index (> 60%). The FDC meshworks in the GCs were sharply defined by CD21 expression, with a weaker, less dense staining pattern in dark than in light zones. CD21 was also weakly expressed by mantle cells. CD35 expression was detected in eight cases, and the pattern was similar to, albeit weaker than, that of CD21. CD21 and CD35 staining also revealed delicate FDC meshworks in the mantle zones.

All FL cases were Bcl-6-positive, with CD10 expression detected in 47 cases. However, Bcl-6- and/or CD10-positive tumor cells were seen in both follicular and interfollicular areas in most of the cases. The proliferative fraction, as determined by Ki-67 labeling, was variable in neoplastic follicles. There were 14 cases that were negative for Bcl-2, including two grade II, nine grade IIIa, and three grade IIIb cases. In a majority of cases (n = 34), dense, sharply defined FDC networks with CD21 and CD35 staining were observed in neoplastic follicles. Slightly or moderately decreased FDC networks were seen in 20 cases. A significant decrease in FDCs was only observed in six grade III cases, in which loosely distributed, disintegrated FDC meshworks or small clusters of FDCs were present in the follicular structures.

Immunostaining for IgM and IgD: In RFH specimens, IgM immunostaining reproducibly highlighted interwoven processes of FDCs, with absent or occasional very dim staining of GC B cells, which could be distinguished by a round or oval contour. GCs exhibiting polarization had densely meshed FDCs in the light zone, and much less compact meshworks in the dark zone (**Figure 1A**). There was sometimes a near-complete absence of IgM immunoreactivity in the dark zone and in individual follicles composed predominantly of centroblasts. A narrow, unstained rim separating FDCs from the mantle zones was occasionally present (**Figure 1B**). In addition, mantle zone cells were positive for IgM as well as for IgD, which was absent in FDCs and GC B cells (**Figure 1C**).

IgD-positive mantle cells were prominent in 17 of the 60 FL cases, including eight cases graded as +, and nine as ++. IgM expression patterns were of three types. In pattern I (n = 38), IgM immunoreactivity was observed in tumor cell membranes, appearing as evenly distributed round or oval contours, with no staining of intermeshed FDC processes (**Figure 2A-C**). In pattern II (n = 15), neither FDCs nor tumor cells

Table 3. Relationship between IgM expression pattern III and degree of mantle zone preservation in FL

Grade*	No. of FL cases	Pattern III of IgM expression# (no. of cases)
0	43	0
+	8	2
++	9	5
Total	60	7

FL, follicular lymphoma. *Grading criteria for mantle zone preservation: 0, absent or sparse mantle cells; ++, thin but relatively intact mantle zones, or focal disappearance; +, intermediate level between 0 and ++. #IgM expression pattern III: attenuated IgM expression by FDCs in neoplastic follicles.

within neoplastic follicles expressed IgM, although CD21-positive FDC networks were present (**Figure 2D**, **2E**). In contrast, FDCs in residual follicles showed strong IgM expression. Therefore, patterns I and II were characterized by an absence of IgM expression on FDCs. In pattern III (n = 7), a significant number of IgD-positive mantle cells were preserved around neoplastic follicles, including two cases graded as +, and five as ++. Whether tumor cells expressed IgM (n = 3) or not (n = 4), reduced IgM expression on FDCs was observed to variable degrees. In three cases, IgM staining showed only focal, but strongly positive FDC networks in the background of IgM-positive tumor cells in neoplastic follicles (**Figure 2F**, **2G**). In an additional three cases in which tumor cells did not express IgM, IgM-positive FDC networks were seen throughout neoplastic follicles, although the staining intensity was much weaker than in adjacent residual follicles (**Figure 2H-J**). In one case in which tumor cells were IgM-negative, no IgM expression was seen in FDC networks in most of the neoplastic follicles, whereas diffusely or focally distributed, IgM-positive FDCs were present in a small subset of Bcl-2-positive follicles. In contrast, IgM-expressing FDCs were never observed in FL cases in which there were few or no mantle cells surrounding GCs (graded as 0). Coexpression of IgD, mostly at a low level, was detected in 17 of the cases in which tumor cells were IgM-positive.

Discussion

FDCs trap antigens on their surface in the form of ICs via Fc fragment-binding receptors (CD23, CD32), complement receptors (CD21, CD35), and adhesion molecules (CD54, CD106, and CD44) [18, 19]. Accordingly, these functional molecules, particularly CD21, CD23, and CD35,

are highly expressed by FDCs [20-22]. In addition, FDCs are recognized by antibodies against IgM, reflecting the deposition of IgM-containing ICs on FDCs [15-17]. Six subsets of FDC have been distinguished within secondary follicles [15], based on their localization, morphology, and immunophenotype, showing only FDCs present in basal and apical light zones express IgM, whereas FDCs in the dark and outer zones of GCs have no IgM expression. In the current study, CD21 and CD35, which are associated with IC binding, were more weakly expressed by FDCs in GC dark zones than those in light zones. Similarly, IgM was also found to be expressed at a lower level on FDCs in the dark zone in most GCs, consistent with the suggestion that dark zone FDCs have a low capacity to trap ICs [23]; indeed, there were some individual follicles devoid of IgM-positive FDCs in the dark zone. The present findings thus provide evidence that FDC function and IC presentation is polarized within GCs. Although the exact function of dark zone FDCs is not well-defined, they may be involved predominantly in the stimulation of proliferation and in the survival of expanding B cells, rather than ICs presentation [23, 24].

Previous studies have reported a reduced expression of markers for mature FDCs in FL cases [20, 25, 26]. In this study, significant numbers of FDCs were seen in all low grade cases and a majority of grade III cases with CD21 and CD35 immunoreactivity, in accordance with the marked reduction of FDCs observed in six grade III cases.

The few studies that have examined IgM expression on FDCs in FL cases have yielded contradictory results, partly due to small sample sizes, but also due to differences in methodology. An absence of IgM expression on FDCs was reported in all five cases of FL evaluated by immunofluorescence on cryostat sections [15]. In contrast, FDCs isolated from two cases of centroblastic-centrocytic lymphoma presented immunoglobulin μ chains, the heavy chain found in IgM [17].

This study provides the first comprehensive analysis of a relatively large number of FL cases for which IgM expression was evaluated in paraffin sections. Although FDCs in neoplastic fol-
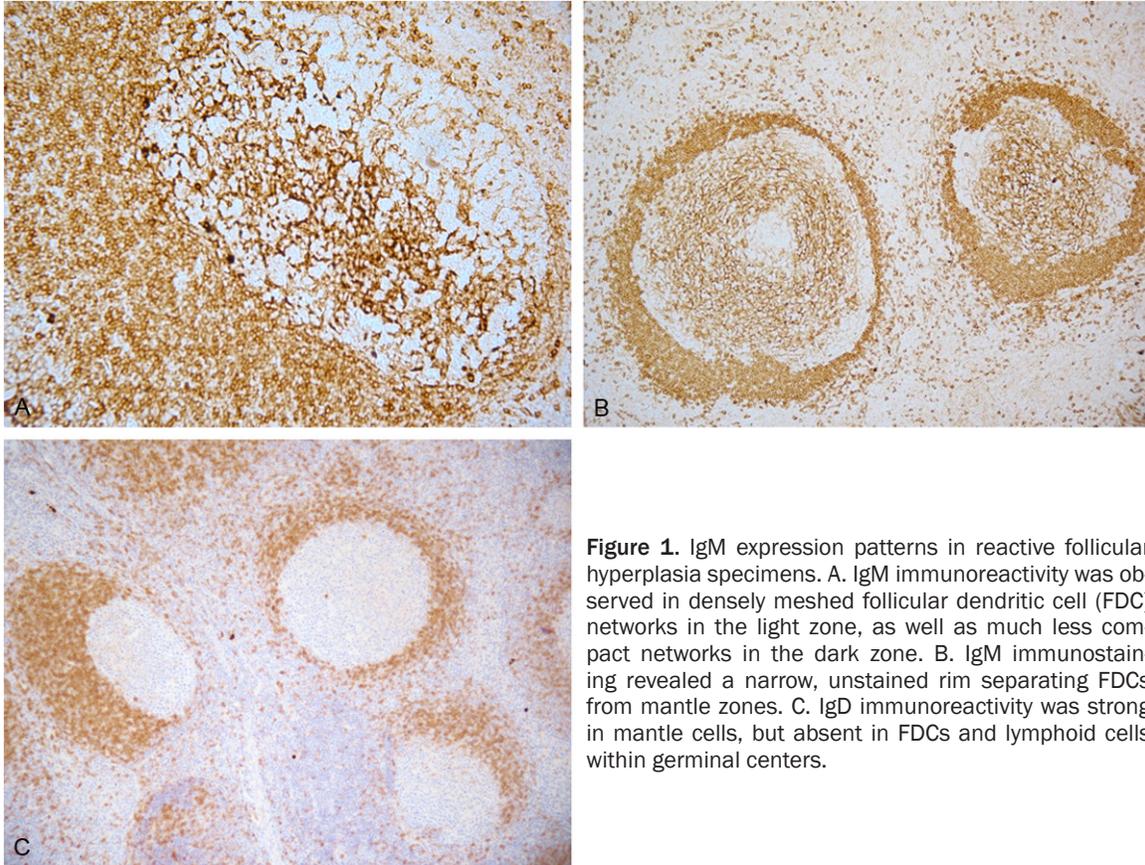
Figure 1. IgM expression patterns in reactive follicular hyperplasia specimens. A. IgM immunoreactivity was observed in densely meshed follicular dendritic cell (FDC) networks in the light zone, as well as much less compact networks in the dark zone. B. IgM immunostaining revealed a narrow, unstained rim separating FDCs from mantle zones. C. IgD immunoreactivity was strong in mantle cells, but absent in FDCs and lymphoid cells within germinal centers.

licles express molecules that participate in antigen binding (such as CD21 and CD35), in the majority of cases (53/60), they were found not to be IC-trapping. Varying degrees of IgM expression on FDCs were observed only in seven cases. Interestingly, all of these showed significant mantle zone preservation around the neoplastic follicles. One study demonstrated that follicular lymphoma may spread by specific colonization of reactive GCs, rather than by de novo production of GC-like structures (including a mantle cell zone) [27]. Moreover, FDCs in normal GCs have the ability to retain ICs for long periods of time, varying from a few weeks to months [28]. Therefore, although FDCs in neoplastic follicles generally express high levels of antigen-binding molecules such as CD21 and CD35, they fail to trap ICs, possibly due to a defect in the transfer of newly-formed ICs to the FDC surface. The present results revealed that the persistence of IgM expression in FDCs was highly associated with mantle zone preservation; it can therefore be speculated that in the early invasion of follicles by tumor cells, pre-existing ICs are still visible on the FDC surface to varying degrees. Over time, ICs decrease until eventually they are completely lost. Concurrently, profound morphological changes occur in the follicle, including the disappearance of the mantle zone. Thus, in reactive lymphoid tissues, IgM staining was only occasionally observed in GC B cells with very weak intensity, whereas upregulation of IgM expression was seen in tumor cells in 41 of 60 FL cases, suggesting that IgM can be a useful marker for the neoplastic process.

Two points about immunohistochemical staining should be emphasized. First, although it is notorious that immunoglobulin antibodies usually showed varying degrees of background staining, in our experience, prolongation of antigen retrieval time can effectively reduce the background, producing reliable and interpretable results for IgM antigen. In the current study, antigen retrieval was performed by pressure-cooking the slides in EDTA/Tris-HCl buffer (pH 9.0) for 5 min for the anti-IgM antibody, and 2.5 min for the other antibodies. Second, hematoxylin counterstaining should not been
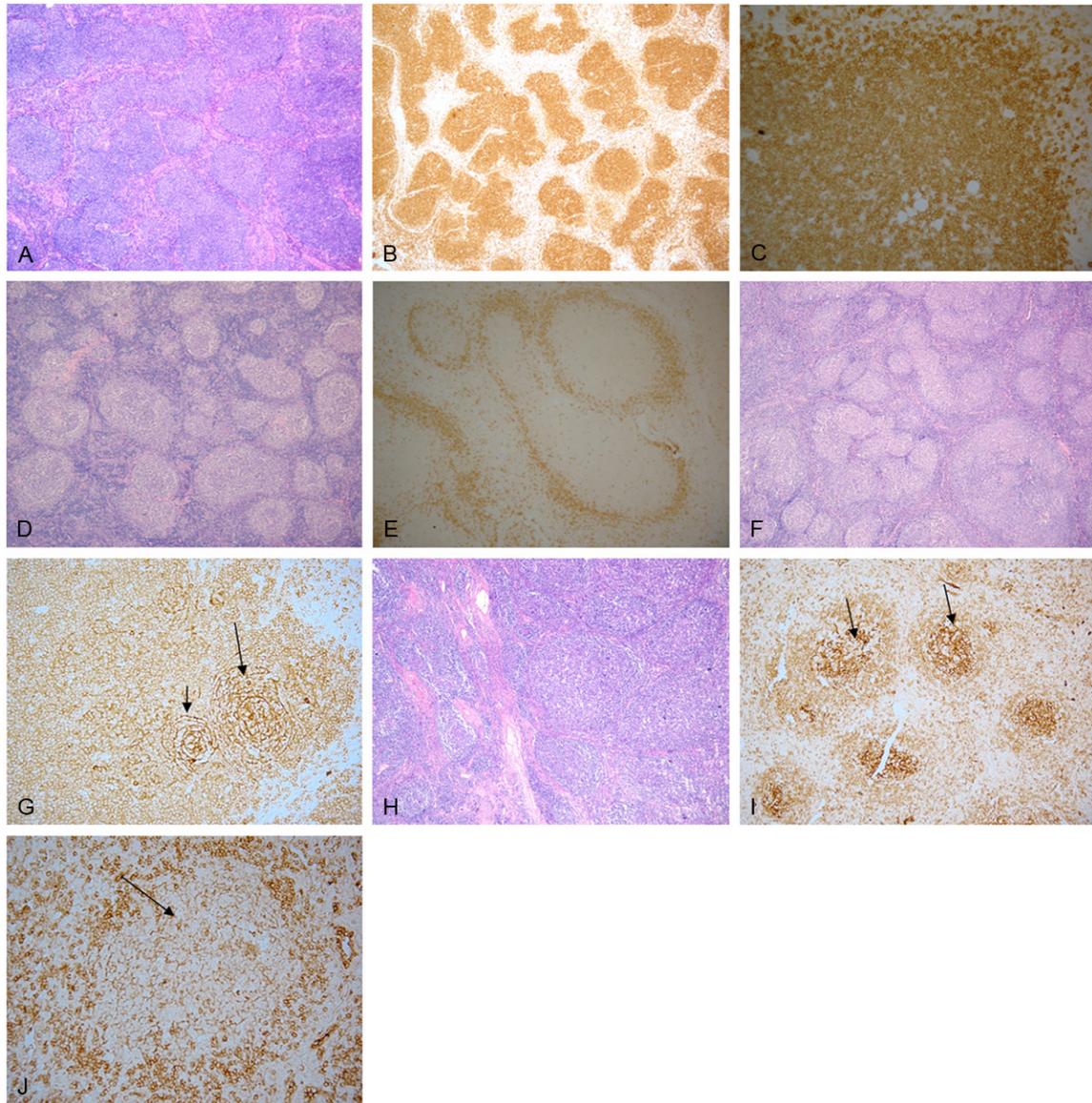
Figure 2. IgM expression patterns in follicular lymphoma (FL). Pattern I (A-C): (A) Low magnification view of a hematoxylin and eosin (H&E)-stained FL grade I specimen with a back-to-back arrangement of follicles. (B) Low and (C) high magnification views showing membrane IgM immunoreactivity in tumor cells, with no staining in the intermeshed processes of follicular dendritic cells (FDCs). Pattern II (D, E): (D) Low magnification view of an H&E-stained FL grade I specimen with closely packed neoplastic follicles. (E) Neither FDCs nor tumor cells within follicles expressed IgM, but mantle cells showed strong IgM expression. Pattern III (F, G): (F) Low magnification view of an H&E-stained FL grade II specimen with numerous large, irregular-shaped, closely packed follicles. Thin mantle zone around the follicles were visible. (G) Strong focal IgM immunoreactivity was observed in FDC networks (arrows) in the background of IgM-positive tumor cells in neoplastic follicles. Pattern III (H-J): (H) Low magnification view of an H&E-stained FL grade III specimen with a back-to-back arrangement of neoplastic follicles (right), along with numerous small reactive follicles (left). (I) High magnification view of reactive follicles with IgM-positive, densely woven FDC networks occupying the germinal centers (arrows). IgM was also expressed by mantle cells. (J) High magnification view of a neoplastic follicle showing loosely arranged FDC meshworks weakly expressing IgM (arrow). In addition, significant numbers of mantle cells around the neoplastic follicle expressing IgM were visible.

performed in conjunction with IgM staining, which would prevent the long, fine processes of FDCs from being easily distinguished from the round or oval-shaped B cells.

In conclusion, the results presented here demonstrate that immunohistochemical staining for IgM in paraffin-embedded sections seems to be an additional tool for differentiating

between FL and RFH in clinical pathology practice. IgM staining can assist in the diagnosis of FL, even in Bcl-2-negative cases. The data suggest that a considerable or complete loss of IgM expression in FDCs, reflecting the loss of IgM-containing ICs in these cells, is a typical feature of FL. Enhanced IgM expression by GC B cells can also serve as an indicator of immunophenotypic abnormality in FL.

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Disclosure of conflict of interest

None.

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