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Stratification of Patients with Follicular Lymphoma

Hasan A. Abd El-Ghaffar et al.*

*Clinical Pathology Department, Faculty of Medicine, Mansoura University, Mansoura
Oncology Center, Mansoura University, Mansoura
Egypt*

1. Introduction

Follicular lymphoma (FL) is an indolent lymphoid neoplasm that is derived from mutated germinal center B cells and exhibits a nodular or follicular histologic pattern. It is typically composed of a mixture of small, cleaved follicle center cells referred to as centrocytes and large noncleaved follicular center cells referred to as centroblasts. FL accounts for about 20 % of all lymphomas with the highest incidence in the USA and Western Europe. In Asia and in the developing countries the incidence is much lower [1].

2. The cell of origin of follicular lymphoma

The current theory that tumors are derived from mutated stem cells called cancer stem cells was suggested because stem cells and some cancer cells share self-renewal and differentiation capacities [2-4]. Although this hypothesis was postulated in early reports [5-7], definite proof of their existence came from recent studies in leukemia, where among the complete tumor cell population only a small subset of cells could initiate, regenerate and maintain the leukemia after transplantation into immunocompromised mice [8,9]. Using similar functional approaches, a variety of cancer stem cells have been identified in an increasing number of epithelial tumors, including breast, prostate, pancreatic and head and neck carcinomas [10]. Despite these outstanding discoveries in leukemias and solid tumors, the existence of lymphoma-originating cells with stem cell properties that may similarly generate lymphoma upon mutation remains a controversial and largely unexplored issue [11]. Recently, it has been proposed that committed lymphoid progenitors/precursor cells with an active V-D-J recombination program are the initiating cells of follicular lymphoma when targeted by immunoglobulin-gene translocations in the bone marrow. However, these pre-malignant lymphoma initiating cells cannot drive complete malignant transformation,

* Sameh Shamaa^{2,4}, Nadia Attwan³, Tarek E. Selim¹, Nashwa K. Abosamra^{1,4}, Dalia Salem^{1,4}, Sherin M. Abd El-Aziz^{1,4} and Layla M. Tharwat^{1,4}

¹Clinical Pathology Department, Faculty of Medicine, Mansoura University, Mansoura, Egypt

²Internal Medicine Department, Faculty of Medicine, Mansoura University, Mansoura, Egypt

³Pathology Department, Faculty of Medicine, Mansoura University, Mansoura, Egypt

⁴Oncology Center, Mansoura University, Mansoura, Egypt

requiring additional cooperating mutations in specific stem cell programs to be converted into the lymphoma originating cells able to generate and sustain lymphoma development [12].

3. Clinical aspects of follicular lymphoma

Signs and Symptom

Follicular lymphoma mainly affects older adults. The average age at diagnosis is about 55. Men and women are nearly equally affected. Follicular lymphoma is a slow-growing disease with minor warning signs that often go unnoticed for a long time before it is diagnosed. The disease is often advanced before a diagnosis is made. Most individuals are diagnosed in stage III or IV. However, even in advanced stages there is no immediate threat to life. The disease has a "waxing and waning" course, meaning that it flares up and regresses a number of times over years.

The first sign of the condition is often a painless swelling in the neck, armpit or groin that is caused by enlarged lymph nodes. However the majority of patients initially present with disseminated disease that follows a relatively indolent clinical course. Patients with follicular lymphoma typically present with superficial lymph nodes of small to medium size. All common superficial territories can be involved by the disease.

In some patients, the first symptoms are more insidious and related to the slow growth of lymph nodes in deep areas, usually in the infradiaphragmatic territories such as the retroperitoneum, the mesenteric, or the iliac areas. In those cases, patients may complain of atypical symptoms while the tumor bulk can be important, with single or confluent lymph nodes. Primary mediastinal involvement is uncommon, as well as isolated splenic enlargement[13]. Other symptoms may include loss of appetite and tiredness. The general status of the patient is usually preserved, Some people have night sweats, unexplained high temperatures and weight loss. These are known as B symptoms. Others have an altered performance status.

Primary involvement of extranodal areas is also very uncommon[14]. The bone marrow is involved in 50% to 60% of the cases. Follicular lymphoma can arise in the gastrointestinal (GI) tract, predominantly in the duodenum or the small intestine, [15,16]. where it can eventually represent the unique site of disease. Lymphoma infiltration may be unifocal or multifocal[13]. The new World Health Organization-European Organization for Research on Treatment of Cancer (WHO-EORTC) cutaneous lymphoma classification recognizes an entity called "primary cutaneous follicle center cell lymphoma" that includes what was previously known as "cutaneous follicular lymphoma variant" in the WHO classification. [17]. Other follicular lymphomas that may be considered as peculiar entities with a distinct behavior are those involving the testis [18] and the rare cases of follicular lymphoma encountered in children [19].

Follicular lymphoma is characterised by response to treatment with disease-free or asymptomatic disease intervals, alternating with recurrence/progression and may transform to aggressive lymphoma at a rate of around 3% per year [13]. This feature is usually—but not systematically—associated with a poor outcome [20]. The clinical factors associated with the risk of transformation (as well as the biology underlying this

phenomenon) are not fully characterized. Some reports indicated that early treatment and achievement of a complete response after the first-line therapy were associated with a lower risk of transformation in patients with follicular lymphoma [21].

4. Clinical prognostic factors

Several prognostic parameters for follicular lymphomas were identified in the last two decades, that led to the development of some prognostic indexes. The International Prognostic Index developed for aggressive lymphomas was also found to be able of predicting the outcome for patients with follicular lymphoma, but the proportion of patients in the higher-risk categories was usually limited. [13]

The first prognostic system specific to follicular lymphoma was developed by the Italian Lymphoma Intergroup (ILI) in the late 1990s [22]. Currently, the Follicular Lymphoma International Prognostic Index (FLIPI) [23] is deemed to be more applicable across a range of clinical settings. Both systems were developed prior to the introduction of monoclonal antibody therapy, which has profoundly changed the treatment and outcome of follicular lymphoma [24]. Hence, the FLIPI-2 was recently developed, in a prospective series of patients needing treatment, using parameters which were not previously amenable to retrospective analysis, and may represent a promising new tool for the identification of follicular lymphoma patients with different risk profiles in the era of immunochemotherapy [25].

The description of the Follicular Lymphoma International Prognostic Index (FLIPI) represents an important step in identifying patient subgroups with predictable outcome and comparing the results of clinical trials. Analyses of gene expression profiles or constitutive gene variations may also provide additional insights for prognostication in the near future. Furthermore, these data underline the complex interactions between the tumor cells and their microenvironment; recent attempts to translate these findings with immunohistochemical studies remain unable to robustly predict patient outcome. The therapeutic strategies in follicular lymphoma have been transformed by monoclonal antibodies, used alone or in combination with chemotherapy. Treatment options should be adapted to the clinical features at diagnosis and appear to be able to modify the overall survival of some subgroups of patients. Further efforts may focus on strategies that can alter the natural history of this disease [13].

5. Treatment

There is a general consensus that the natural history of advanced stages of low-grade or indolent lymphoma has not changed for the last 30 years. Patients with advanced stages of indolent non-Hodgkin's lymphoma have been treated for many years with various approaches, including deferred initial therapy (watch and wait), single-agent alkylating agents, radiation therapy, combination chemotherapy, and autologous stem-cell transplantation. Unfortunately, it has been impossible to demonstrate that the long-term prognosis for these patients has significantly changed with any these treatment options [26].

The decision to start first-line treatment depends not only on the stage but also on the symptoms of the disease [27]. According to The Swiss Group for Clinical Cancer Research

(SAKK) trials at least one of the following signs is present in order to start treatment: B symptoms; symptomatic enlarged lymph nodes or spleen; steady, clinically significant progression of lymphadenopathy, splenomegaly or other follicular lymphoma lesions documented by a 50% increase in size over a period of at least 6 months; involvement of at least 3 nodal sites (>3 cm), bulky disease (>7 cm), haemoglobin $<10\text{g/dL}$, and platelets $<100 \times 10^9/\text{L}$ due to bone marrow infiltration or splenomegaly [28].

In spite of encouraging early results [29], there is still no solid data to confirm that early treatment with rituximab significantly delays the need for new therapy, and whether this approach may alter the natural history of the disease and there is also conflicting results. Whether early treatment of follicular lymphoma is associated with a decreased risk of transformation [27].

Horning [30], reported the results of sequential treatment studies conducted at Stanford University from 1960 to 1991. Median survival times have ranged from 7 to 10 years, and the OS for each group of studies was overlapping. Indolent lymphoma is generally considered incurable because no plateau in the survival curve has been demonstrable. However, the development of monoclonal antibodies (MoAbs) has revolutionized the treatment of patients with follicular lymphoma [26].

Many trials utilizing different protocols of combination chemotherapy plus rituximab, eg. CVP [31], CHOP [32,33] concluded that OS for patients with follicular lymphoma has improved over time and that the choice of initial therapy may matter. Sacchi et al [34], concluded that FFS and OS have significantly improved in advanced-stage follicular lymphoma patients treated on GISSL (Gruppo Italiano Studio Linfomi) protocols during the last 18 years. These improvements are related to evolving front-line and salvage therapies, particularly the introduction of rituximab in combination with chemotherapy.

For patients needing therapy, most patients are treated with chemotherapy plus rituximab, which has improved response rates, duration of response, and overall survival. Randomized studies have shown additional benefit for maintenance rituximab both following chemotherapy-rituximab and single agent rituximab [35]. Rituximab maintenance for up to 2 years has a favorable side effect profile and, based on a systematic meta-analysis, substantially prolongs PFS and OS in relapsed disease even after antibody-containing induction in patients who have not received antibody as first-line therapy [36].

Stem cell transplantation (SCT) including both autologous and allogeneic SCT or experimental agent therapy is considered for recurrent disease [35].

In the small proportion of patients with limited non-bulky stages I-II, radiotherapy (involved or extended field, 30–36 Gy) is the preferred treatment having a curative potential [37]. However, in selected cases a watchful waiting may be discussed to avoid the side effects of radiation [38]. Radioimmunotherapy or chlorambucil plus rituximab remains an alternative in patients with low risk profile or contraindications for a more intensive chemoimmunotherapy [39,40].

Therefore, the therapeutic strategies in follicular lymphoma have been transformed by monoclonal antibodies, used alone or in combination with chemotherapy. Treatment options should be adapted to the clinical features at diagnosis and appear to be able to modify the overall survival of some subgroups of patients. Further efforts may focus on strategies that can alter the natural history of this disease [13].

6. Cytomorphology of follicular lymphoma

Follicular NHLs are characterized by a follicular growth pattern, but a diffuse area may also be present. The follicles of follicular lymphoma were more closely packed together, more monotonous in size and shape, and frequently lacked an obvious mantle zone [41].

These follicles contained a comparatively monomorphic cellular phenotype, mostly of centrocytes (small cells with cleaved nuclei) and occasional centroblasts (large cells with multilobated nuclei and multiple nucleoli). Mitotic figures were fewer than in reactive follicular hyperplasia (RFH) and tingible body macrophages were sparse or absent. Polykaryocytes were not observed in RFH or follicular lymphoma and sclerosis was only focally present to a minimal degree [42].

The distinction of FL from RFH is essential, as the latter represents a benign condition [43]. A number of morphological features are of value in making this distinction; in particular, a low density of follicles per unit area. The follicles are separated by wide interfollicular areas with prominent mantle zones. The variably sized follicles were composed of a polymorphic population of lymphocytes, dendritic cells, and tingible body macrophages that imparted a "starry sky" pattern. Mitotic figures were conspicuous. The presence of polarity within follicles and the lack of a monomorphic appearance within the follicles all favor RFH [42].

7. Immunophenotype

Being a mature B-cell lymphoma, FL express a large spectrum of B-cell markers, such as CD20, CD19, CD22, CD79a, and Pax5¹ [44].

FL cells express antigens of the germinal center including CD10 and Bcl-6. Most cases of FL express Bcl-2 protein which is highly correlated with the presence of the t(14;18) but may be expressed in cases with a clonal karyotype lacking the t(14;18) [45].

The follicles of RFH and follicular lymphoma stained positively for the B cell marker CD20 which highlight the difference in the width of the interfollicular region between the follicles. In RFH, T cells were predominantly located in the interfollicular zones but also lightly percolated throughout the center of the follicles, demonstrated by T cell marker CD3. CD5 stained T cells are in a nearly identical pattern. T cells stained positively for CD3 and CD5 in follicular lymphoma located within the interfollicular zones. The latter also displaying a more prominent spillover of CD20-positive cells than in RFH [42].

Follicles in both RFH and follicular lymphoma were positive for BCL-6. BCL-2 staining revealed that the follicular centers in RFH were negative and those in follicular lymphoma were positive. CD10 staining was positive for both RFH and follicular lymphoma but showed a greater interfollicular staining in follicular lymphoma [41].

Some cases of both RFH and follicular lymphoma manifested a vague follicular architecture that is difficult to detect. In these cases, CD23, a dendritic cell marker, outlined the follicles of RFH more clearly by disclosing a scaffolding or a cluster of interconnecting cells with elongated cytoplasmic processes. Follicular lymphoma manifested the same pattern of CD23 staining. Ki-67, a marker for cellular proliferation, showed dense positivity that was polarized (unevenly distributed or centered at one edge of the follicle) within RFH follicles. In contrast, follicular lymphoma demonstrated a more diffuse, evenly distributed staining

pattern within the follicular centers. Polyclonality was evident in RFH, with in situ hybridization revealing roughly equal populations of both kappa and lambda light chains. In follicular lymphoma rare cells stained positive for kappa or lambda [42].

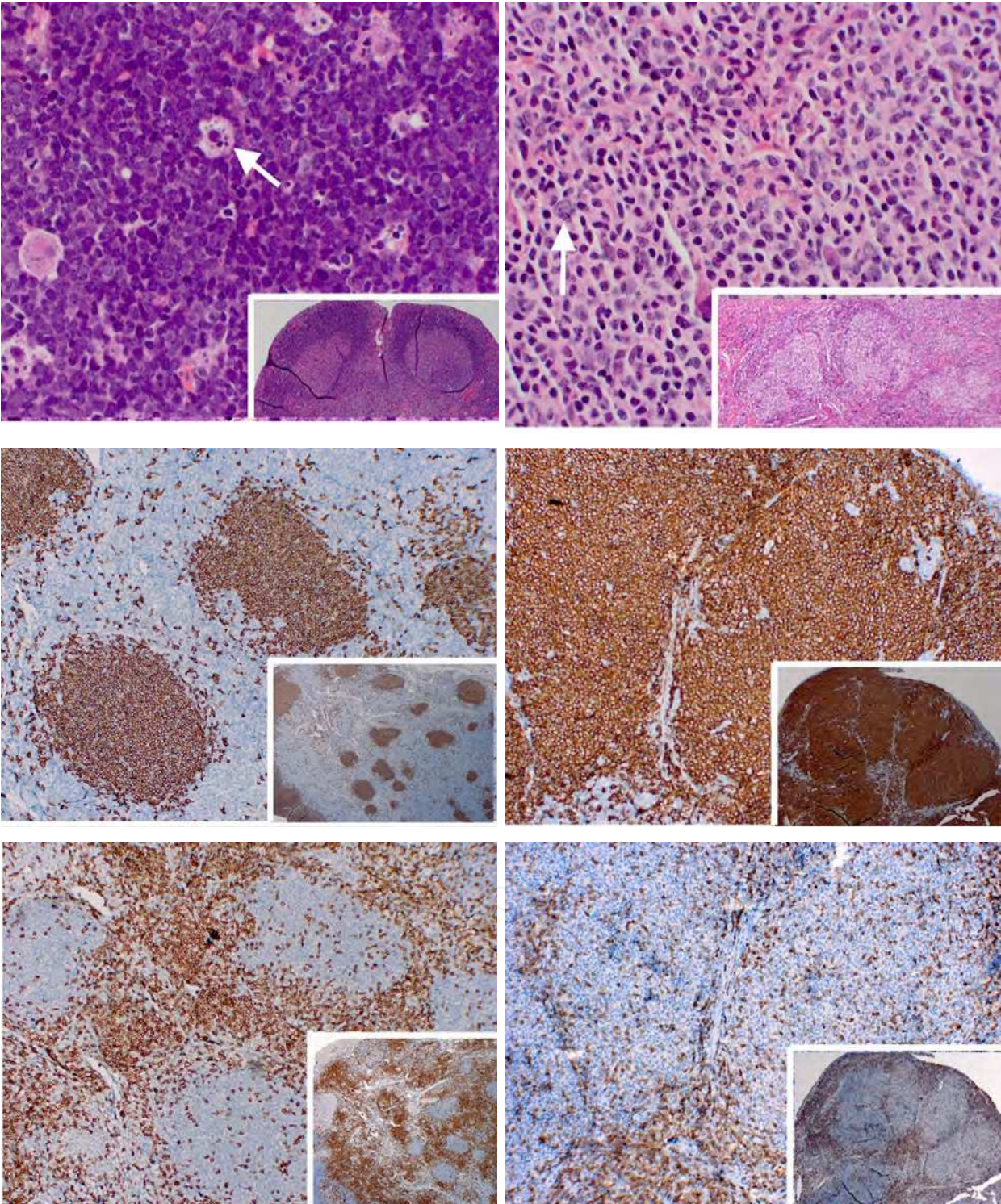
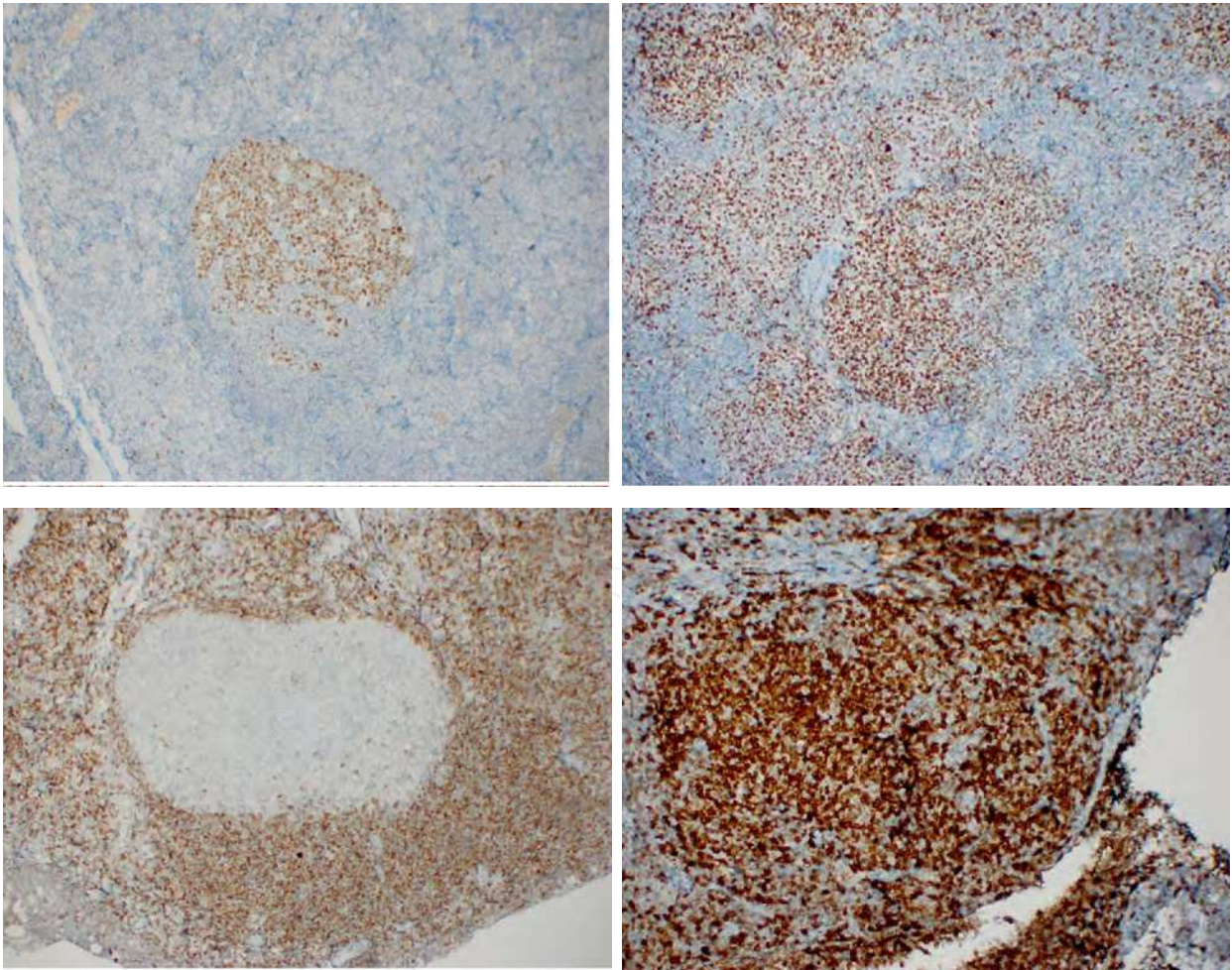


Fig. 1. Histopathological and immunohistochemical characterization of reactive lymphoid hyperplasia and follicular lymphoma.(Top left) Hematoxylin-eosin staining demonstrates follicles in reactive lymphoid hyperplasia (RLH) (inset). The follicles are composed of small

cells with mitotic figures and tingible body macrophages (arrow). (Top right) Hematoxylin-eosin-stained section of follicular lymphoma shows mostly small centrocytes with cleaved nuclei and an occasional centroblast (arrow) with a large nucleus and peripheral nucleoli. Inset shows the closer, less well-defined arrangement of several follicles. (Middle left) CD20 stains B cells within the follicles of RLH. The inset indicates the substantial width of the interfollicular zones. (Middle right) CD20 also stains the B cells in the follicles of follicular lymphoma; the close arrangement of the follicles can again be appreciated in the inset. (Bottom left) CD3 stains T cells in the interfollicular zones (inset); the T cells can also be seen percolating throughout the follicles of RLH. (Bottom right) Follicular lymphoma manifests nearly identical CD3 staining, with CD3⁺ cells prominent in the interfollicular zones (inset) as well as scattered within the follicles. (Top left and top right, hematoxylin-eosin, 400, insets, 25; Middle left through Bottom right, immunoperoxidase reactions, diaminobenzidine chromogen, 200, insets 25) [42].



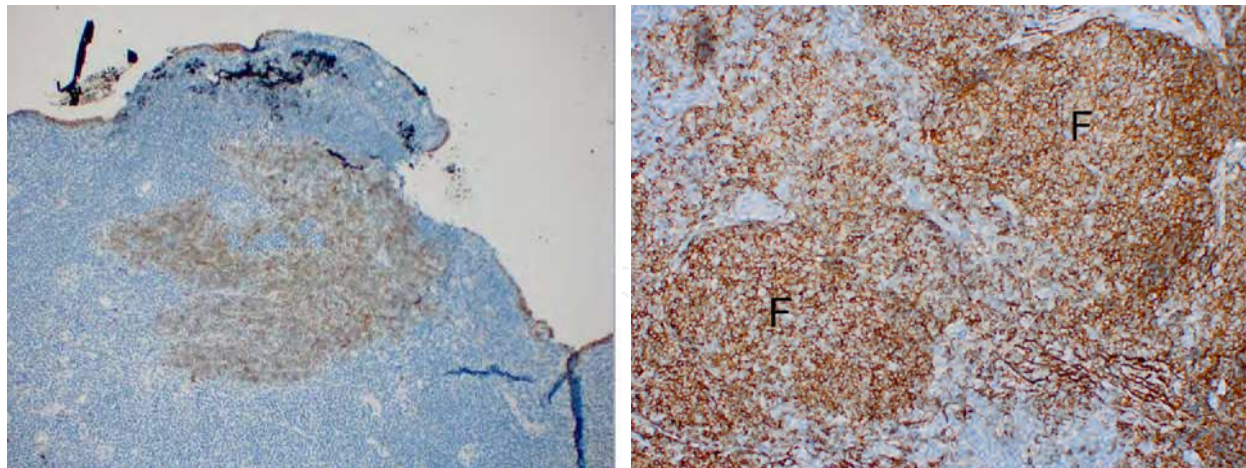


Fig. 2. Immunohistochemical characterization of reactive lymphoid hyperplasia and follicular lymphoma. (Top left) BCL-6 stains B cells within follicles of reactive lymphoid hyperplasia (RLH). (Top right) Follicles of follicular lymphoma are also positive for BCL-6. (Middle left) The follicles of RLH are negative for BCL-2. (Middle right) Follicles of follicular lymphoma are positive for BCL-2. (Bottom left) Follicles in RLH are positive for CD10. (Bottom right) Follicles (F) of follicular lymphoma are also positive for CD10 but with more interfollicular staining than RLH. (Immunoperoxidase reactions, diaminobenzidine chromogen, 200) [42].

8. Follicular lymphoma—How many grades?

The grading of FL was the subject of spirited discussion, both among the authors and the participants in the Clinical Advisory Committee. FL has traditionally been graded according to the proportion of centroblasts and into three Grades, 1-3 as detailed:

World Health Organization classification of follicular lymphoma (FL).

Follicular Lymphoma: Grading & Variants

Grade 1: 0-5 centroblasts/HPF

Grade 2: 6-15 centroblasts/HPF

Grade 3: > 15 centroblasts/HPF

3a: > 15 centroblasts, but centrocytes are still present

3b: centroblasts form solid sheets with no residual centrocytes [46].

Variants:

Primary cutaneous follicle center lymphoma

Pediatric follicle center lymphoma

Intestinal follicle center lymphoma

Diffuse follicle center lymphoma

Grade 1, 0-5 centroblasts/HPF

Grade 2, 6-15 centroblasts/HPF [43]

Pediatric FL lacking an association with the t(14;18). Primary cutaneous follicle center lymphoma (PCFCL) may contain a high proportion of large B-cells including large

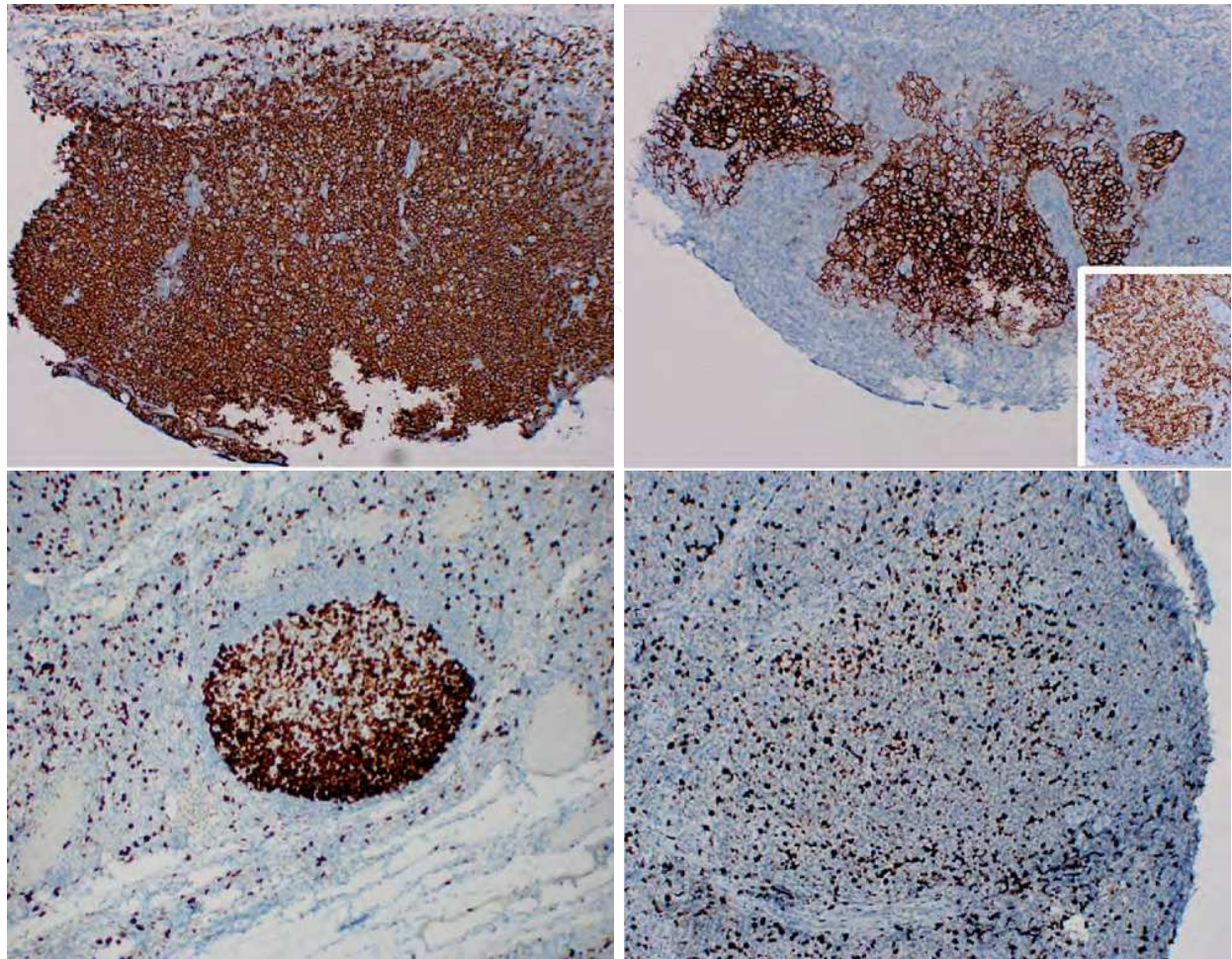


Fig. 3. Further immunohistochemical characterization of reactive lymphoid hyperplasia (RLH) and follicular lymphoma. (Topleft) CD20 densely stains this region of RLH without obvious follicular architecture. (Top right) CD23 highlights the dendritic cell scaffolding of the follicles in an adjacent section. The inset shows a similar dendritic architecture brought out by CD23 in follicular lymphoma. (Bottom left) Ki-67 stains cells within follicles of RLH in an uneven distribution; staining is crescentic and more dense at the bottom of the follicle. (Bottom right) Follicular cells in follicular lymphoma are more evenly and diffusely positive for Ki-67. (Immunoperoxidase reactions, diaminobenzidine chromogen, 200, inset, 25) [42].

centrocytes and centroblasts. Evidence of the t(14;18) is uncommon and most cases are BCL2 negative. Dissemination beyond the skin is rare, and the prognosis is usually excellent [47].

However, most studies have shown poor interobserver and intraobserver reproducibility. Moreover, the clinical significance of the separation of Grades 1 and 2 has been questioned, with minimal differences seen in long term outcome. Thus, the 2008 WHO classification lumps cases with few centroblasts as "FL Grade 1-2 (low grade)" and does not require or recommend further separation. FL Grade 3 is divided into Grades 3A and 3B, based on the absence of centrocytes in the latter category. Several studies have identified biological differences between these two subtypes, with most cases of FL Grade 3B being more closely related to DLBCL at the molecular level [47].

However, in clinical practice the separation of Grades 3A and 3B can be challenging. Diffuse areas in any Grade 3 FL should be designated as DLBCL (with FL) and are more commonly observed in Grade 3B. Further studies are likely to lead to more precise delineation of the Grade 3 cases truly belonging within FL and those representing an intrafollicular variant of the GCB (germinal center B cell) type of DLBCL.

The presence of diffuse areas within a FL appears to confer more aggressive clinical behavior. In addition, unusual cytological variants can be encountered that are not included in the World Health Organization classification, including cases with large centrocytes and others with small centroblasts; in some cases the latter may resemble the cytomorphology of Burkitt lymphoma and may be associated with MYC oncogene translocations [43].

A truly diffuse form of FL may be rarely encountered, but on closer inspection, most cases demonstrate vaguely follicular architecture that is underappreciated without the routine use of immunostains for follicular dendritic cells. When a diagnosis of true diffuse follicle center lymphoma is considered, the pathologist is encouraged to demonstrate co-expression of CD10 and Bcl-6 as well as presumptive evidence of the t(14;18). Approximately 10% of FL cases reveal the presence of a zone of cells resembling marginal zone B cells, immediately surrounding neoplastic follicles. Importantly, a residual benign mantle zone is not seen, helping to distinguish FL from a marginal zone lymphoma. The area of marginal zone differentiation within a FL takes on a distinct morphology with cells having moderate amount of pale cytoplasm; moreover, the immunophenotype is also different, with downregulation of CD10 and Bcl-6 expression by cells within the marginal zone compartment [48].

Unresolved issues in FL pathology remain and to some extent contribute to problems with reproducibility. Diffuse areas in FL are often not identified, due to underutilization of follicular dendritic cell stains. Similarly, follicular areas in suspected de novo DLBCL are underappreciated. Variability in the cytology of cells within follicles contributes to inconsistent reporting of FL grade. Follicular dendritic are commonly misidentified as centroblasts, resulting in higher grade and, in general, there is poor interobserver reproducibility for the counting of centroblasts. All of these factors lead to inconsistencies in the diagnosis and grading of FL biopsies, and to some extent raise questions regarding the validity of grading and clinical decision making based on grade [43].

Finally, a rare form of FL may be seen referred to as “in-situ” FL. In these uncommon cases, scattered malignant follicles are identified within lymph nodes revealing mostly benign features. The malignant follicles show typically more monomorphic germinal centres (GCs). GCs involved in intrafollicular neoplasia/in situ follicular lymphoma are composed mainly of centrocytes with no evident atypia. A feature of these centres is the relative absence of macrophages with tingible bodies and the absence of polarization into light and dark zones. The paucity of large centroblasts reflects their low proliferation rate in insitu FLs. Intrafollicular neoplasia/in situ follicular lymphoma is characterized by strong co-expression of Bcl-2 and CD10 in the involved GCs [49].

9. Fine needle aspiration cytology

FNA is a useful tool for staging as well as evaluating recurrences in lymph nodes and extra nodal sites without subjecting patients to multiple excisional biopsies. Transformation can

occur in some lymph nodes while low-grade lymphoma persists in others. An advantage of FNA is the ability to sample multiple lymph nodes, whereas open biopsy of different anatomic sites is not feasible

Grading FL is based on the proportion of centroblasts in neoplastic follicles; therefore, the ability to identify the various cellular components in a fine-needle aspirate is the first essential step to the grading process. In Papanicolaou stained preparations, centrocytes or cleaved follicular center cells are small to medium-sized cells with angulated, elongated, twisted, or cleaved nuclei with inconspicuous nucleoli and scant, pale cytoplasm.

Centroblasts are at least two times larger than a lymphocyte and usually are round or oval but occasionally have indented, irregular, or even lobulated nuclei. There is a narrow rim of cytoplasm, often basophilic to amphophilic. A large and central nucleolus with chromatin clearing around it is characteristic of immunoblasts. In more typical centroblasts, the chromatin generally is vesicular with one to three prominent, often peripherally located nucleoli. The cells also may be hyperchromatic. Centroblasts tend to be more fragile and, in some preparations, may not be preserved well [50].

It is important not to confuse centroblasts with follicular dendritic reticulum cells, which tend to aggregate within the center of the neoplastic follicles. Although dendritic cells have nuclei that are similar in size to centroblasts, the nuclei of dendritic cells are somewhat coffee bean-shaped with one side typically flattened and with fine, smooth nuclear membranes. The cytoplasm is indistinct, not basophilic, in contrast to that of centroblasts. In Papanicolaou-stained preparations, the chromatin is pale gray and finely granular with small, central, eosinophilic nucleoli. The cytoplasm of dendritic cells form long, dendritic processes that can be appreciated in cell blocks by IHC staining for CD21. Large cleaved cells also must be distinguished from centroblasts. Although there is an overlap in size with centroblasts, large centrocytes are more irregular in shape and lack the prominent nucleoli and chromatin pattern of centroblasts [50]

When it comes to differentiating individual cells, cytologic preparations are superior to hematoxylin and eosin-stained histologic sections, although cytologic preparations typically are less informative about architecture.

Cell blocks are complimentary to smears and provide additional architectural clues. The FNA process often aspirates intact follicular structures that can be appreciated in the cell block. The presence of intact follicles may be proven by special stains on sections of the cell block [50].

10. Grade versus pattern

All three grades of lymphoma can have varying proportions of follicular and diffuse areas. Grade 1 and 2 FL generally have a predominantly follicular pattern. Because they are better differentiated, they have retained the ability to recapitulate follicles [51].

Grade 3 FL occurs less frequently than Grade 1 and 2 FL, and a pure follicular pattern in Grade 3 FL is even more unusual. In Grade 3 FL, the presence of diffuse areas is more common, and most (but not all) studies show that this finding is associated with a worse prognosis.

In Grade 1 and 2 FL, there is conflicting evidence regarding whether the presence of large, diffuse areas or the degree of nodularity may alter prognosis significantly.

SO the WHO classification system recommends estimating the proportion of follicular and diffuse components in the pathology report

- Follicular (75% follicular),
- Follicular and diffuse (25–75% follicular),
- Minimally follicular (25% follicular).

Note, however, that the proportion of centroblasts within the neoplastic follicles is what determines the grade of a FL, not the degree of nodularity. Furthermore, the grade of FL, in combination with other clinical factors, ultimately is what influences treatment decisions [50].

11. Cytogenetics of follicular lymphoma

The t(14;18)(q32;q21) chromosome translocation represents the defining cytogenetic hallmark of FL and is encountered in 80%-90% of cases. Its molecular consequence is the juxtaposition of the B-cell lymphoma/leukemia 2 (BCL2) proto-oncogene with enhancer sequences of the immunoglobulin heavy chain gene (IGH) promoter region, thereby deregulating its expression and resulting in an overexpression of the BCL2 protein in the neoplastic follicles [52,53]. However, 10–15% of cases do not harbor the t(14;18)(q32;q21) and in these t(14;18)-negative cases, other mechanisms are thought to be involved in the pathogenesis [54]. Moreover, t(14;18)-positive B cells can be identified in the blood and lymphoid tissues of healthy individuals, and the number of t(14;18)-positive cells is influenced by gender, personal lifestyle and exposure to toxic substances [55].

The BCL2 proto oncogene, a potent anti-apoptotic molecule, is expressed in resting B cells in the perifollicular mantle zone and in post-follicular B cells, thereby promoting long-lived follicular precursor and memory B cells. Germinal center B cells, however, physiologically lack BCL2 expression and undergo apoptosis unless they are selected by specific antigens that drive them into processes termed somatic hypermutation and class switching. Due to the lack of BCL2 expression, amongst other factors, the large bulk of B-cells entering the GC microenvironment will be removed by apoptosis. The constitutive overexpression of BCL2 in germinal center B cells inferred by the t(14;18)(q32;q21) leads to an accumulation of inappropriately rescued B cells with a prolonged life span, allowing for the development of additional genetic hits to occur, that are required for the establishment of overt FL. Variant translocations of the t(14;18), such as the t(2;18) or t(18;22), juxtapose BCL2 to the loci of the immunoglobulin light chains (k,l) and, likewise, result in inappropriate and sustained BCL2 expression in GC B cells [56].

The occurrence of the t(14;18) in a pre-FL B cell can be viewed as a first hit in a multistep process that results in the clonal dysregulation of cell cycle control and apoptosis of the tumor cells. During process of lymphomagenesis, a number of additional genetic or epigenetic events occur in a non-random fashion that lead to overt FL. For example, constitutive expression of activation-induced cytidine deaminase (AID) in the GC environment in B cells overexpressing BCL2 may propagate continuous somatic hypermutation and class switch recombination activity that results in increased genomic

instability. This may, in turn, foster the occurrence of secondary oncogenic hits and, finally, result in the malignant transformation to overt FL [57].

Cong and co-workers[58],described the phenomenon of what they termed follicular lymphoma in situ in otherwise reactive, hyperplastic lymph nodes possibly representing the morphological equivalent of early, pre-invasive FL.

12. Secondary chromosomal aberrations in follicular lymphoma

A number of secondary chromosomal alterations have been described in FL including: structural and numerical changes. The complexity of the secondary alterations correlates with the grade – the higher the grade, the more complex aberrations are usually encountered [59].

It has long been recognized that these alterations occur in a non-random fashion. Partial trisomies of chromosomes 1q, 7, 8 and 18q, and deletions in 1p and 6q have been described as the most common secondary alterations, and deletions in the long arm of chromosomes 1 and 6 and in the short arm of chromosome 17 have been associated with a worse prognosis [60]. Some of these alterations may occur early in the course of the disease, whereas others might represent late genetic events. In addition, some of the alterations are mutually exclusive, while alterations of other chromosomal regions frequently appear together possibly leading to a coordinated deregulation of genetic pathways [61].

Some of the secondary chromosomal alterations may cancel the effect of the t(14;18) that initially forms a low-grade neoplasia with a follicular growth pattern and subsequently enable the transformation to highgrade lymphoma. This process has been associated with three distinct secondary genetic alterations in FL that have a profound impact on the biological program and the clinical course in FL. These include an additional introduction of a t(8;14)/MYC rearrangement in the tumor cells [62], the inactivation of TP53 by mutation and deletion and, finally, the inactivation of p16, frequently occurring by biallelic deletion [63].

The occurrence of a secondary MYC rearrangement in FL deserves particular attention, because these cases frequently demonstrate a Burkitt-like appearance and may be detectable by virtue of this specific morphology in combination with an overexpression of the BCL2 protein caused by the t(14;18) that is usually not encountered in classical Burkitt's lymphomas. Some studies suggest that the detection of TP53 mutations in primary diagnostic specimens of FL without signs of transformation also characterizes a patient subgroup with worse prognosis [64].

13. BCL2-negative follicular lymphoma

From recent studies t(14;18)-negative FLs belong to the biologic spectrum of FL, but show distinct genetic features as well as gene expression and immunohistochemical profiles that differ from their t(14;18)-positive counterparts [65]. The t(14;18)-negative FL appears to harbor genetic rearrangements of the BCL6 gene in 3q27[66] or trisomy 3 [67] whereas others show BCL2 expression on the immunohistochemical level despite the lack of the t(14;18)[68]. Moreover, increased expression of IRF4/MUM1, a protein associated with plasma cell differentiation has been described in FL without BCL2 rearrangement [69].

14. Molecular genetics of follicular lymphoma

Immunoglobulin heavy and light chains are rearranged in FL with the variable region genes showing extensive and ongoing somatic hypermutation [70, 71]. As a result of these mutations in the CDR-regions, PCR primer annealing may be hampered and depending on the primers used, immunoglobulin-PCR may not yield monoclonal products in a proportion of FL cases (10-40%). Multiplex PCR reactions using BIOMED-2 expanded primer sets detect closer to 90% of complete *IGH* (VH-JH) gene rearrangements, and clonality detection approximates 100% when primers detecting incomplete *IGH* (DH-JH) and light chain gene rearrangements are included [72].

For amplification of complete *IGH* (VH-JH) gene rearrangement, BIOMED-2 developed three sets of VH primers corresponding to the three VH FR regions (FR1, FR2, and FR3). Each set of primers consisted of six or seven oligonucleotides capable of annealing to their corresponding VH segments (VH1-VH7) with no mismatches for most VH segments. These VH primer sets were used in conjunction with a single JH consensus primer. The JH primer is fluorescently labeled to allow the detection of PCR products by Gene Scanning [73].

For incomplete *IGH* (DH-JH) rearrangements, seven family-specific DH primers were designed based on the high degree of homology within each DH family in combination with the consensus JH primer. Primers were designed such that crossannealing to other DH family segments would be minimal or preferably absent [73].

Six family-specific Vk primers were designed by van Dongen et al [73], to recognize the various Vk gene segments of the seven Vk families. The family-specific Vk primers were designed to be used in combination with either a set of two Jk primers or a Kde primer. A single consensus primer recognizing both Vλ1 and Vλ2 gene segments, as well as a Vλ3 primer, were designed by van Dongen et al [73], in combination to a single consensus primer for the Jλ1, Jλ2, and Jλ3 gene segments.

The *t*(14;18) and *BCL2* gene rearrangements is one of the best characterized recurrent cytogenetic abnormalities in peripheral B-cell lymphoproliferative disease [74]. FL is genetically characterized by this translocation which is present in up to 90% of the grade 1-2 FL cases [75,76] but the proportion depends on the technique used [77, 78, 79]. *BCL2* rearrangements are much less frequent in grade 3B FL [80]. As a consequence of the translocation, the *BCL2* gene (anti-apoptotic) from 18q21 is placed under the control of the strong enhancers of the *IGH* locus resulting in the deregulation of its normal pattern of expression [81,82]. The *BCL2/IGH* rearrangement is found in the PB of 25-75% of healthy donors, and also in reactive nodes, particularly if using sensitive nested or RT-PCR assays [83,84,85]. A recent study suggests that rather than being naive B-cells, these *BCL2*-rearranged cells are memory B-cells [86].

There is no single gold standard detection strategy for the *t*(14;18), and a combination of cytogenetics and southern blotting have been generally used [87,88]. Interphase FISH detection strategies offer an applicable alternative that have the potential to pick up more translocations [89]. For molecular diagnostic laboratories PCR-based detection strategies offer rapid results, are generally applicable, and can be used for residual disease monitoring. However, the primers commonly used have not been designed to take into account recent information on the molecular anatomy of the breakpoints. As a consequence when

compared to gold standard approaches, PCR-based techniques only detect up to 60% of translocations, which seriously impairs the diagnostic capability of PCR. However, BIOMED-2 primers have been developed using three multiplex tubes for detection of MBR-JH, 3'MBR- JH, and mcr-JH to maximize the detection of t(14;18) [73].). These data are supported by previous report from our molecular hematology laboratory. We found that FISH was superior to PCR in the detection of t(14'18) (q32'q21)-IGH-BCL-2 in formalin-fixed, paraffin-embedded tissue samples. Moreover, strong correlations between the FLIPI score and each of interphase FISH and CD10 expression were demonstrated [90].

Molecular profiling of many types of lymphoma using RQ-PCR and cDNA microarray has been used to predict survival by many researchers [91-94]. Genes involved in cell cycle control and DNA synthesis and metabolism (e.g. CXCL12, which is involved in signaling transduction and NEK2, which is involved in mitotic regulation, and MAPK1) are significantly up-regulated in the aggressive phase of FL[3]. MYC, as a known oncogene, and MYC-target genes (SFRS7, LDHA, MTHFD1, NME1, MSH2, and CKS2) are upregulated on transformation and may be implicated as a direct transforming factor [95-102].On the other hand, there is higher density of the T-cell infiltrate in low-grade FL as compared to high-grade disease and this is reflected by several T cell-related genes (CD3, CD2, CD69). However, genes related to T-cell and macrophage activation including several chemokine receptors (CCR1, CCL3, CCL5, CCL8, AKAP12, ILF3, GEM) are significantly upregulated on transformation, suggesting an important biologic role. Notably, specific antagonists to several of the above-mentioned chemokine receptors are available and offer an attractive possibility for therapeutic interventions [103].

15. Proposed algorithm for stratification of follicular lymphoma

The National Comprehensive Cancer Network(NCCN) has recently launched an algorithm for stratification of follicular lymphoma (Figure 4).

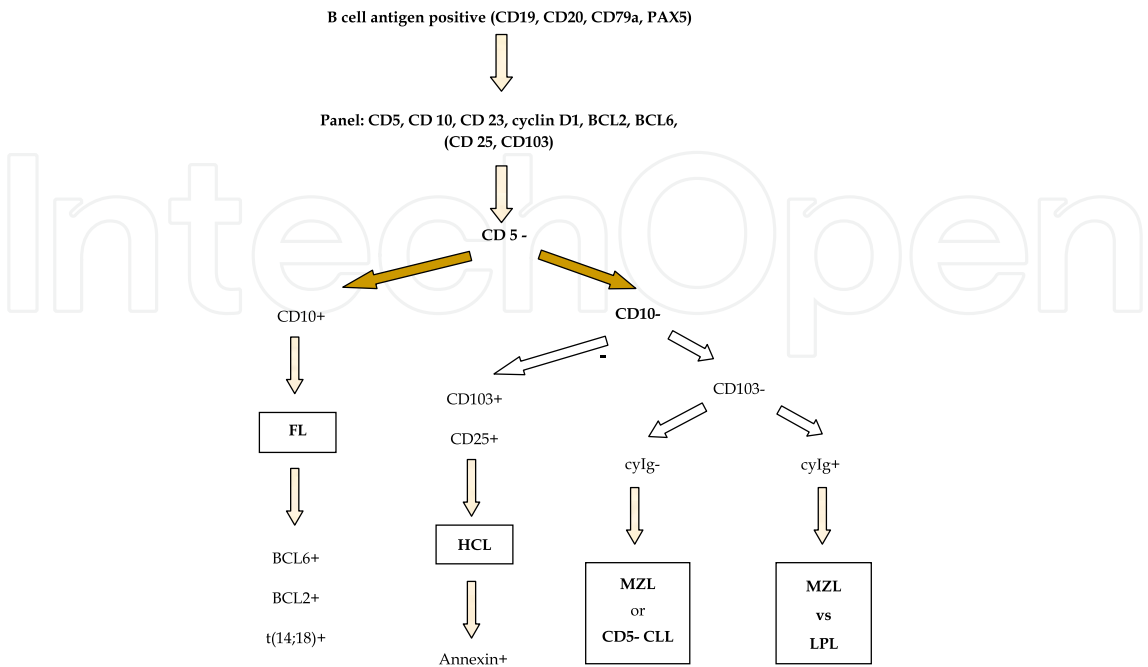


Fig. 4. Proposed algorithm for stratification of follicular lymphoma (NCCN)

16. References

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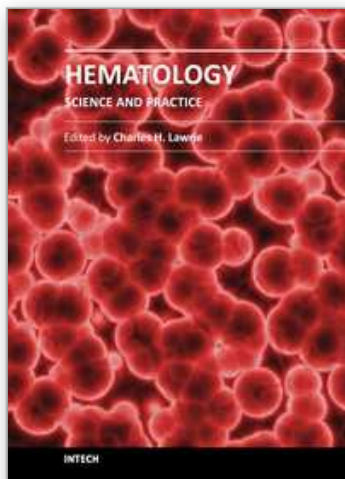
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Hematology encompasses the physiology and pathology of blood and of the blood-forming organs. In common with other areas of medicine, the pace of change in hematology has been breathtaking over recent years. There are now many treatment options available to the modern hematologist and, happily, a greatly improved outlook for the vast majority of patients with blood disorders and malignancies. Improvements in the clinic reflect, and in many respects are driven by, advances in our scientific understanding of hematological processes under both normal and disease conditions. Hematology - Science and Practice consists of a selection of essays which aim to inform both specialist and non-specialist readers about some of the latest advances in hematology, in both laboratory and clinic.

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Unit 405, Office Block, Hotel Equatorial Shanghai
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中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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