[Downloaded free from http://www.saudiannals.net on Sunday, May 09, 2010]

Grading of follicular lymphoma using flow cytometry

Walid A. Mourad, Faisal Rawas, Mohamed Shoukri, Abdelghani Tbakhi, Mohamed Al Omari, Asma Tulbah, Fouad Al Dayel

BACKGROUND: The treatment and prognosis of follicular lymphoma (FL) is dependant on the grade of the disease. In the World Health Organization classification of lymphoma, grading of FL into low grade (1 and 2) and high grade (3) is recommended. Grading of FL is possible in excision biopsy; histological grading is subjective and inconsistent. Grading is extremely difficult in needle core biopsies and fine needle aspirates. We attempted to grade FL using flow cytometry (FCM) and CD19/ forward scatter.

MATERIALS AND METHODS: Cases of FL seen in our institution and submitted for FCM were evaluated for the percentage of cells detected beyond the 500-channel mark (on a 1024 scale) on a CD19/forward scatter dot plot. We hypothesized that these cells most likely represent centroblasts and their percentage would reflect the grade of the disease. Histological grading of the lymphoma on the open biopsies constituted the reference for FL grade.

RESULTS: Thirty-six cases of FL, including 22 males and 14 females, ranging in age from 19 to 92 years (median, 42 years), were studied. There were 17 cases of low grade (grade 1; n=10 and grade 2; n=7) and 19 cases of high grade (grade 3) FL. The percentage of cells identified beyond the 500-channel mark on CD19/forward scatter dot plot ranged from 0.12% to 12.55% (median, 4.9%) in low grade (grade 1 and 2) whereas the percentage of those cells in high grade FL ranged from 6.22% to 51.95% (median, 21%; *P*=0.00001).

CONCLUSION: Our findings suggest that using a CD19/forward scatter dot plot can help identify centroblasts in FL making grading possible on FCM, especially in small biopsies and fine needle aspirates.

From the King Faisal Specialist Hospital & Research Center, Riyadh, Saudi Arabia

Correspondence and reprint requests:
Walid A. Mourad, MD
Pathology and Laboratory Department of Medicine, MBC 10
King Faisal Specialist Hospital & Research Centre
P.O.Box 3354
Riyadh 11211
Saudi Arabia
Tel: +966-1-442 4249
Fax: +966-1-442 4280
mourad@kfshrc.edu.sa

Accepted for publication
April 2006

Ann Saudi Med 2006;26(3):205-210

Follicular lymphoma is one of the most common lymphomas encountered in North America, representing approximately 30% of all lymphomas seen in tertiary care pathology practice.[1] This lymphoma is one of the few entities in the World Health Organization (WHO) classification that requires grading.[1] Low-grade follicular lymphoma usually follows a protracted course and is, for the most part incurable.[2] It usually requires single-agent chemotherapy or immunotherapy using monoclonal antibodies.[3] High-grade follicular lymphoma on the other hand is an aggressive lymphoma that requires multi-agent chemotherapy and can have a higher cure rate.[4] This suggests that grading of follicular lymphoma is one of the prerequisites for diagnosis of the disease. Grading of follicular lymphoma has, for many years, followed the grading system of Mann and Berrard.[5] The method depends on counting the number of centroblasts per high power field. Grade 1 follicular lymphoma would have less than 5 centroblasts per high power field, grade 2 would have 5-15 centroblasts per high power

[Downloaded free from http://www.saudiannals.net on Sunday, May 09, 2010]

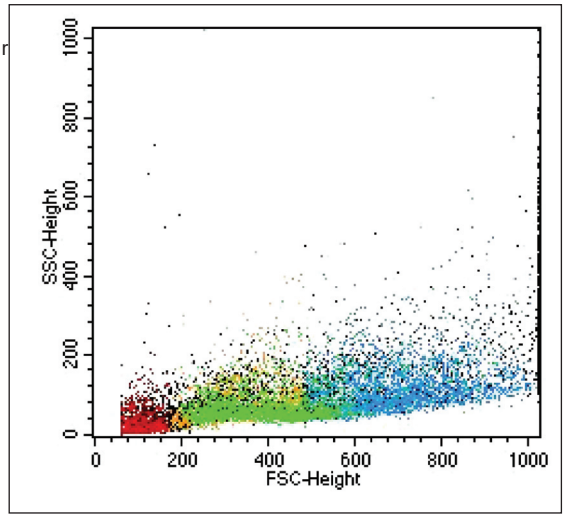

Figure 1: Flow cytometric side scatter (SS)/forward scatter (FS) dot plot showing debris (red), possible T-lymphocytes and centrocytes (green and yellow) and possible centroblasts (blue)

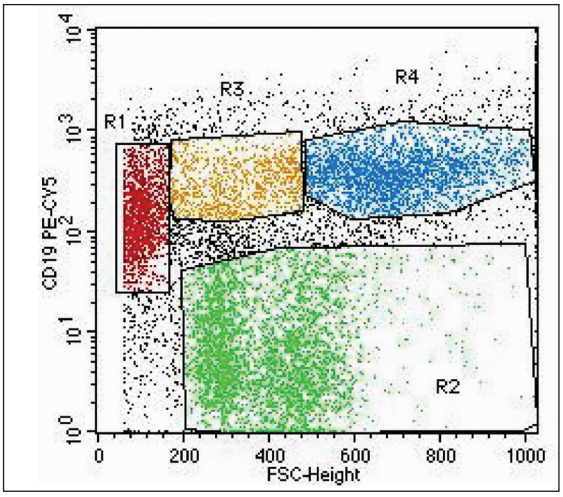

Figure 2: Flow cytometric CD19 / forward scatter showing debris (R1; red), putative T-lymphocytes (R2; green), putative centrocytes (R3; yellow) and putative centroblasts (R4; blue)

field, grade 3A would have more than 15 centroblasts per high power field and grade 3B would have sheets of centroblasts. The WHO recommends that grades 1 and 2 follicular lymphoma be identified as low-grade follicular lymphoma and grade 3 follicular lymphoma be given a high-grade designation.[6] The Mann-Berrard grading system is highly subjective, shows inter-observer variability and can only be implemented in open biopsies.[7,8,9] Clinical situations where open biopsy cannot be obtained may lead to the inability to grade follicular lymphoma.[10] This has led to the need for a more objective and standardized method of grading of follicular lymphoma.

Centroblasts are usually three to four times the size of normal T-lymphocytes and most of the time twice the size of a centrocyte.[11] Additionally, a study has shown that large cells can be identified by forward scatter on flow cytometry.[12] This suggests that on forward scatter in flow cytometry, centroblasts would migrate farther than centrocytes. Centrocytes would, however, be in the same location as reactive T lymphocytes on a flow cytometric forward scatter (Figure 1). To separate centrocytes, reactive T lymphocytes and centroblasts, CD19 forward scatter would help separate the putative three categories of lymphocytes (Figure 2). In the current study we hypothesized that the percentage of cells identified at or beyond the 500-channel mark represents the percentage of centroblasts and that would in turn represent the grade of the follicular lymphoma.

Materials and Methods

All cases of follicular lymphoma diagnosed in our institution and submitted for flow cytometry formed the basis for our study. All cases had to fulfill histologic and phenotypic criteria for follicular lymphoma. Histologically the cases had to show a follicular or follicular and diffuse growth pattern with characteristic centrocytes and centroblasts. Phenotypically all cases had to express CD10, CD20, Bcl-2 and Bcl-6.

Grading of the lymphomas

All cases were independently graded by two pathologists (AT and MAO) without knowledge of the flow cytometry result. The Mann and Berrard histological grading scheme was used. Grade 1 follicular lymphoma showed less than 5 centroblasts per high power field, grade 2 lymphoma showed 5-15 centroblasts per high power field and grade 3 disease showed more than 15 centroblasts per high power field. No attempt to subdivide grade 3 follicular lymphoma into grades 3A and 3B was made for the purpose of the study. Cases were subdivided into two main categories: low-grade follicular lymphoma encompassing grades 1 and 2 disease and high-grade follicular lymphoma encompassing grades 3A and 3B follicular lymphoma.

Flow cytometry

The following monoclonal antibodies were used: T-cell markers CD1a-PE (Coulter), CD2-PE (Becton-Dickinson; BD), CD3-FITC (BD), CD4-APC (BD), CD8-PE (BD), CD7-FITC (BD), CD5-FITC (BD) whereas B-cell markers included CD10-FITC (Coulter), CD19-APC (BD), CD20-PE (BD), CD22-APC (BD), CD23 APC (BD), CD79b PE (BD), FMC-7 FITC (BD), Kappa-FITC (BD), Lambda-PE (BD), CD19 PE (Coulter), Kappa FITC (Coulter) and Lambda FITC (coul-

[Downloaded free from http://www.saudiannals.net on Sunday, May 09, 2010]

ter). A monoclonal antibody against BCL-2 FITC (Pharmengin) was used. Additional antibodies used were CD45-PerCP (BD), HLA-DR-FITC (BD), CD11c-PE (BD), and 7-AAD (Coulter).

For immunophenotyping, lymph node biopsies were obtained and collected using RPMI media tube and delivered immediately to the flow cytometry section. Aliquots from single cell suspensions from the lymph node were washed twice using a PBS buffer with 1% BSA and 0.1% NaN_3. After the last wash the cells were filtered and the count adjusted to 0.5-1 × 10^9/L of which a 100 uL was placed in each tube and incubated with the appropriate combination of monoclonal antibodies for 15-20 minutes in the dark (room temperature). Once the incubation period was finished the red cells were lysed using ammonium chloride followed by a single washing step. The cells were resuspended in 0.5 mL of 1% paraformaldehyde and stored in the refrigerator until time of analysis.

Data acquisition was performed using Cell Quest software (BD) where a minimum of 10 000 events/tube were acquired during which the normal lymphocyte elements in the sample were positioned on the 200-channel mark to act as an internal reference point. Instruments were calibrated daily using CALIBRITE beads (BD) and two levels (normal and low) of controls were run daily to ensure consistency in the functions of the instruments.

Forward scatter (FSC) vs. side scatter (SSC) to gate around the abnormal cells was performed. For identifying the percentage of abnormal cells a dot plot of FSC vs. CD19 was created, and an electronic gate was created to detect the number of cells identified at or beyond the 500-channel mark on the FSC axis and an estimation of their percentage was done.

The Wilcoxon-signed-rank test was used to test the significance of the differences between the different grade lymphomas based on the percentage of cells identified at or beyond the 500-channel mark. The analyses were performed using the S-Plus commercial program, version 6.2, 2000 (Insightful Software, Seattle, Washington, USA).

Results

There were 36 cases of follicular lymphoma identified, which included 22 male and 14 female patients. The ages ranged from 19 to 97 years (median 42 years). There were 10 cases of grade 1, 7 cases of grade 2 and 19 cases of grade 3 follicular lymphoma.

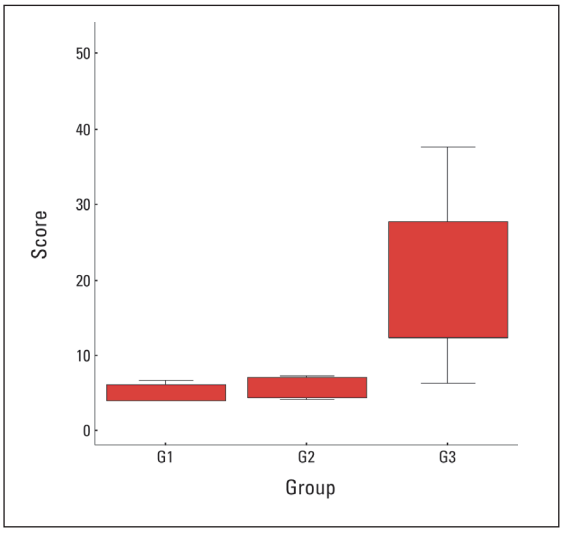

Figure 3: Box plot comparing follicular lymphoma grades 1 (G1), grade 2 (G2) and grade 3 (G3) follicular lymphoma.

Correlation of grade of lymphoma with flow cytometric CD19 forward scatter

Grade 1 follicular lymphomas had a percentage of cells at or beyond the 500-channel mark ranging from 0.12% to 6.6% (median, 4.6 %) whereas grade 2 follicular lymphomas had a percentage ranging from 4.12% to 12.55% (median, 7%). The difference between grade 1 and 2 lymphomas was not statistically significant (P=0.07). Grade 3 follicular lymphomas had a percentage of cells identified at or beyond the 500-channel mark ranging from 8.06% to 51.94% (median, 21%). The difference between grade 2 and 3 was statistically significant (P=0.0002). When grades 1 and 2 follicular lymphomas were combined they had a percentage of cells identified at or beyond the 500-channel mark ranging from 0.12% to 12.55% (median 4.9%). The difference between grades 1 and 2 combined compared with grade 3 was statistically significant (P=0.00001). Figure 3 shows the graphic representation of CD19 forward scatter of grades 1, 2 and 3 follicular lymphoma. Figure 4 shows grades 1 and 2 FL combined against grade 3 disease. Figures 5 to 7 show three different grade lymphomas and their corresponding CD19 forward scatter plots.

Discussion

Several prognostic indicators have been identified in follicular lymphoma.[13] Age has been used as a prognostic indicator. Patients older than the age of 70 years had a worse prognosis according to one study.[6] The presence of more than 6 chromosomal breaks

[Downloaded free from http://www.saudiannals.net on Sunday, May 09, 2010]

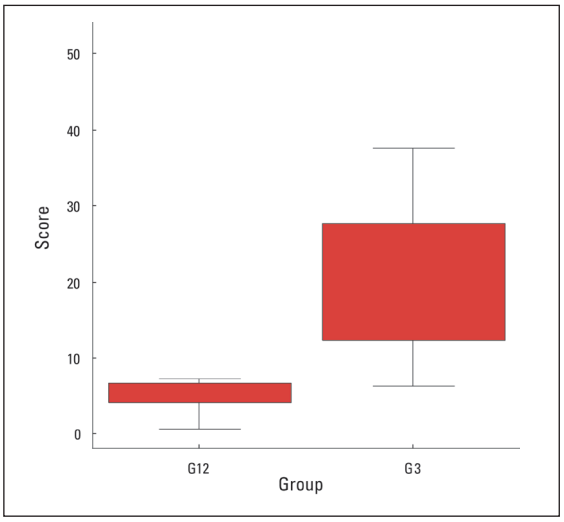

Figure 4: Box plot showing grades 1 and 2 (G12) follicular lymphomas combined in comparison with grade 3 (G3) follicular lymphoma.

has been associated with a poor outcome.[14,15] Other clinical prognostic factors include serum LDH levels and performance status.[16,17] The pattern of bone marrow involvement in follicular lymphoma has been shown to correlate with disease outcome, including the volume of bone marrow involvement, diffuse infiltrate and the number of lymphoma nodules.[18] More recently gene expression profiling has shown that follicular lymphoma subclasses can be identified leading to the identification of disease subgroups with different biological behaviors.[19,20] The technology needed to provide such gene profile at the clinical level is far from being implemented on a routine basis. Until this technology at the patient care level is available grading of follicular lymphoma has remained one of the most important aspects of predicting the behavior of the disease.[21-24] Grades 1 and 2 disease usually have a persistent prolonged course with a very low probability of cure. These two grades of the disease usually require monotherapy.[3,4] Grade 3 follicular lymphoma on the other hand shows a more aggressive course and shows potential for cure using multidrug chemotherapy. It thus seems that grading of follicular lymphoma is a necessity rather than an academic exercise in the management of follicular lymphoma.[13-15]

The most commonly used method of grading follicular lymphoma is that of Mann and Berrard and its modification.[5-8] The method has been repeatedly used to reflect the different behavioral patterns of follicular lymphoma with a certain degree of success. However, the method is for the most part, subjective and shows a great deal of inter-observer variability.[7]

Additionally the method has been primarily designed to evaluate several microscopic fields. This means that the grading method is only suitable for excision biopsies. Several situations arise where excision biopsy cannot be performed for the patient.[10] This means that needle core biopsies and fine needle aspirates may not be suitable for grading of follicular lymphoma. Studies have attempted to grade follicular lymphoma in fine needle aspirates with some success.[25] The studies still relied on visual counts of centroblasts and/or Ki-67 proliferative indices or DNA analysis. The incorporation of histologic grade with proliferative activity has also been attempted with some degree of success.[24,26]

There thus seems to be a need for a quantitative method to identify the exact percentage of centroblasts in follicular lymphoma using a reliable measuring device. We have shown that using CD19 forward scatter, three populations of lymphocytes can be identified in any given case of follicular lymphoma. The first population is that seen in the bottom left of the CD19 forward scatter plot and these are most likely T lymphocytes. The second population is that seen in the top left area of the CD19 forward scatter plot and these most likely are centrocytes. The third population is that seen in the top right portion of the CD19 forward scatter dot plot and these most likely are centroblasts. We have shown that the estimation of the percentage of these latter cells can be a reflection of the grade of the follicular lymphoma. We had a rare overlap between some of the cases. Overlap between different grades of follicular lymphoma does not necessarily mean a flaw in the flow cytometric method. Our patients should be clinically followed to see whether the flow cytometric or the histological methods are more biologically relevant. Additionally, the method can be fine-tuned to provide proper estimation of the number of centroblasts.

Although our method of estimating the grade of follicular lymphoma using CD19 forward scatter requires several studies for validation and for correlation with disease outcome, we believe that the method can be an accurate objective method of estimating the grade of follicular lymphoma and in time will replace the conventional method of grading follicular lymphoma.

[Downloaded free from http://www.saudiannals.net on Sunday, May 09, 2010]

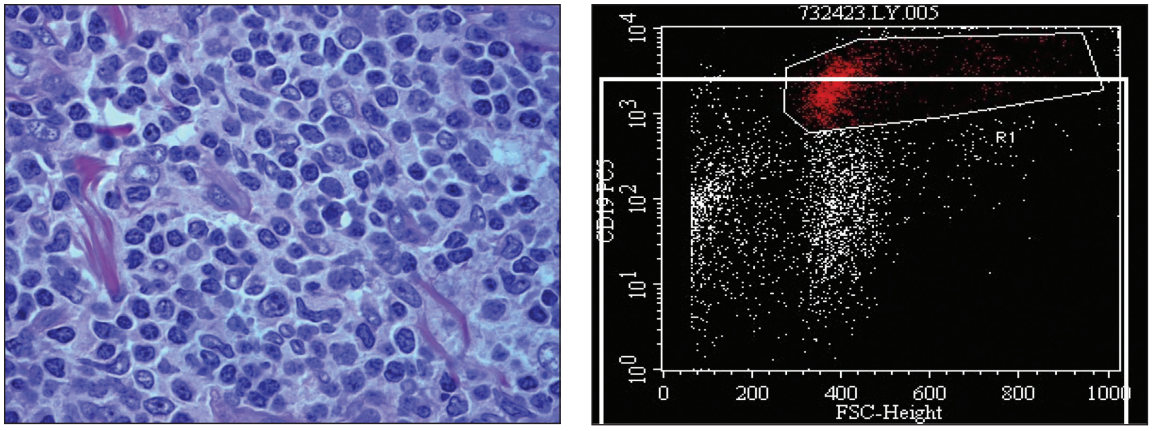

Figure 5: Photomicrograph of grade 1 follicular lymphoma (H and E X400) (left); Flow cytometric dot plot histogram of CD19 forward scatter of the same case showing the percentage of cells identified beyond the 500-channel mark at 3% (right).

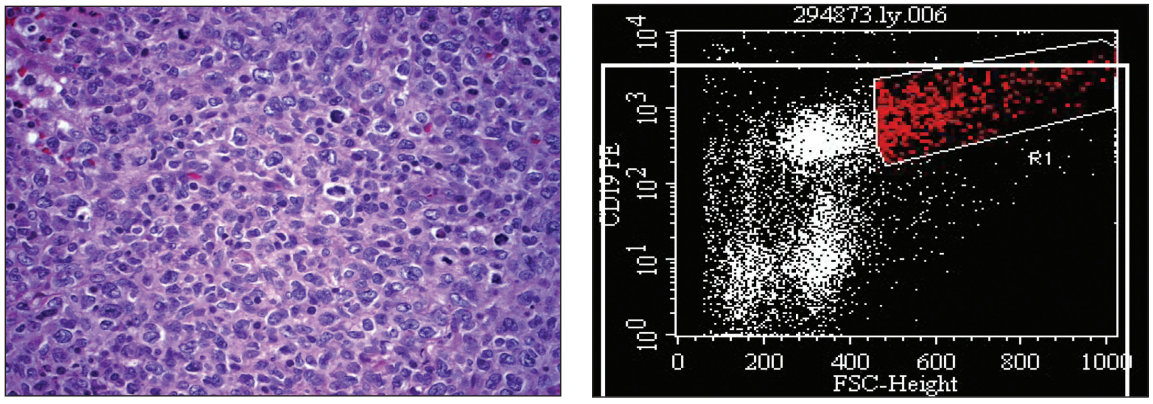

Figure 6: Photomicrograph of case of grade 2 follicular lymphoma (H and E X400); B) (left); Flow cytometric dot plot histogram of CD19 forward scatter of the same case showing the percentage of cells identified beyond the 500-channel mark of 7% (right)

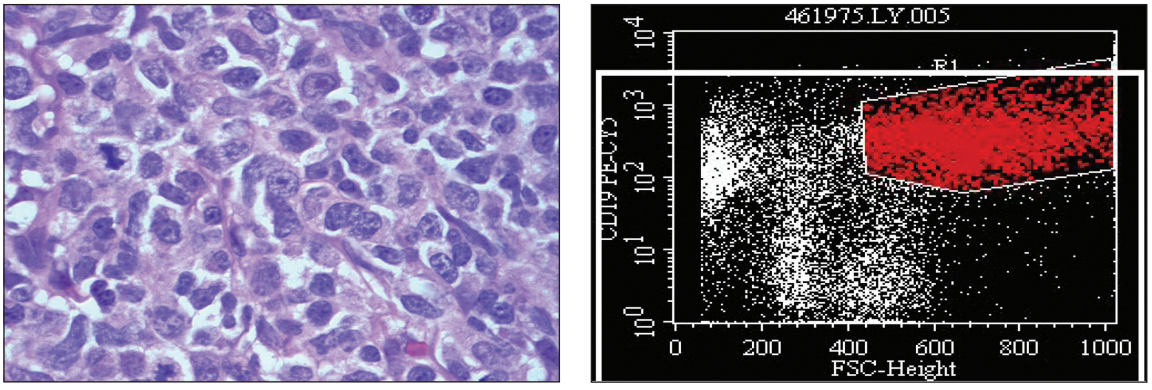

Figure 7: Photomicrograph of case of grade 3 follicular lymphoma (H and E X400) (left); Flow cytometric dot plot histogram of CD19 forward scatter of the same case showing the percentage of cells identified beyond the 500-channel mark at 37% (right)

[Downloaded free from http://www.saudiannals.net on Sunday, May 09, 2010]

References

1. Anonymous. A clinical evaluation of international lymphoma study group classification of non-Hodgkin's lymphoma. The non-Hodgkin lymphoma classification project. Blood 1997; 89:3909-3918

2. Longo DL, Young RC, Hubbard SM, Wesley M, Fisher RI, Jaffe E, Berrard C, DeVita VT, Jr Prolonged initial remission in patients with nodular mixed lymphoma. Ann Intern Med 1984;100:651-656

3. Bartlett NL, Rizeg M, Dorfman RF, Halpern J, Horning SJ. Follicular large-cell lymphoma: Intermediate or low grade? J Clin Oncol 1994; 12:1349-1357

4. McLaughlin P, Fuller LM, Velasquez WS, Butler JJ, Haegmeister FB, Sullivan-Haley JA, Dixon DO. Stage III follicular lymphoma: durable remissions with combined chemotherapy-radiotherapy regimen. J Clin Oncol 1987; 5:867-874

5. Mann RB, Berrard CW. Criteria for the cytologic subclassification of follicular lymphoma: a proposed alternative method. Haematol Oncol 1983; 1:187-192

6. Maartense E Le Cessie S, Kluin-Nelemans HC, Kluin PM, Snijder S, Wojermans PW, Noordijk EM. Age-related differences among patients with follicular lymphoma and the importance of prognostic scoring systems: analysis from a population-based non-Hodgkin's lymphoma registry. Ann Oncol 2002; 13:1275-1284

7. Nathwani BN, Metter GE, Miller TP, Burke JS, Mann RB, Barcos M, Kjeldesberg CR, Dixon DO, Winberg CD, Whitcomb CC. What should be the morphologic criteria for the subdivision of follicular lymphoma. Blood 1986; 68:837-845

8. Whitcomb CC, Dixon DO, Miller TP. Morphologic subclassification of follicular lymphoma: variability of diagnoses among hematopathologists, a collaborative study between the repository center and pathology panel for lymphoma clinical studies. J Clin Oncol 1985; 3:25-38

9. Metter GE, Nathwani BN, Burke JS, Winberg CD, Mann RB, Barcos M, Kjeldesberg CR, Whitcomb CC, Dixon DO, Miller TP. Morphologic subclassification of follicular lymphoma: variability of diagnosis among hematopathologists, a collaborative study between the repository center and pathology panel for lymphoma clinical studies. J Clin Oncol 1985; 3:25-38

10. Zhao X, Cherian S, Sargent R, Seethala R, Bonner H, Greenberg B, Bagg A. Expanded population of surface membrane immunoglobulin light chain-negative B cells in lymph nodes are not always indicative of B-cell lymphoma. Am J Clin Pathol 2005; 124:143-150

11. Knowels DM Ed. Neoplastic hematopatholgy. 2nd ed. Lippincott Williams Wilkins 2001: 824

12. Gong JZ, Williams DC, Liu K, Jones C. Fine-needle aspiration in non-Hodgkin lymphoma. Evaluation of cell size by cytomorphology and flow cytometry. Am J Clin Pathol 2002; 117:880-888

13. M Federico, U Vitolo, PL Zinzani, T Chisesi , V Clo, G Bellesi, M Magagnoli, M Liberati, C Boccomini, P Niscola, V Pavone, A Cuneo, G Santini, M Brugiatelli, L Baldini, L Rigacci, L Resegotti. Prognosis of follicular lymphoma; a predictive model based on a retrospective analysis of 987 cases: Inergruppo Italiano linfomi. Blood 2000; 95:783-9.

14. Tilly H, Rossi A, Stamatoulas A, Lenormand B, Bigorgne C, Kunlin A, Monconduit M, Bastard C. Prognostic value of chromosomal abnormalities in follicular lymphoma. Blood 1994; 84:1043-1049

15. Levine EG, Arthur DC, Frizzera G, Peterson BA, Hurd DD, Bloomfield CD. Cytogenetic abnormalities predict clinical outcome in non-Hodgkin lymphoma. Ann Int Med 1988; 108:14-20

16. Anonymous A predictive model for aggressive non-Hodgkin's lymphoma. The International non-Hodgkin's lymphoma prognostic factors project. N Engl J Med 1993; 329:987-994

17. Anonymous. A clinical evaluation of the International Lymphoma Study Group classification of non-Hodgkin's lymphoma. The non-Hodgkin's lymphoma Classification Project. Blood 1997; 89:3909-3918

18. Canioni D, Brice P, Lepage E, Chababi M, Meignin V, Salles B, Xerri L, Peaud PY, Rousselot P, Peuchmaur M, Solal-Celigny P, Brousse N, The group d'etude des lymphomas folliculaires. Bone marrow histologic patterns can predict survival of patients with grade 1 and follicular lymphoma: a study from the groupe d'etude des lymphomas folliculaires. Br J Haematol 2004; 126:364-371

19. Dybakaer K, Iqbal J, Zhou G, Chan WC. Molecular diagnosis and outcome prediction in diffuse large B-cell lymphoma and other subtypes of lymphoma. Clin Lymph 2004;5:19-28

20. Glas Am, Kersten MJ, Delahaye LJ, Witteveen AT, Kibbelaar RE, Velds A, Wessels LF, Joosten P, Kerkhoven RM, Bernards R, van Krieken JH, Kluin PM, van't Veer LJ, de Jong D. Gene expression profiling in follicular lymphoma to assess clinical aggressiveness and to guide the choice of treatment. Blood 2005; 105:301-307

21. Anderson T, Bender RA, fisher RI, DeVita VT, Chabner BA, Berrard CW, Norton L, Young RC. Combination chemotherapy in non-Hodgkin's lymphoma: results of long-term follow-up. Cancer Treat Rep 1977; 61:1057-1066

22. Glick JH, Barnes JM, Ezdinli EZ, Berard CW, Orlow EL, Bennett JM. Nodular mixed lymphoma: results of a randomized trial failing to confirm prolonged disease-free survival with COPP chemotherapy. Blood 1981; 58:920-925

23. McLaughlin P, Fuller L, Velasquez WS, Butler JJ, Hagemwister FB, Sullivan-Haley JA, Dixon DO. Stage III follicular lymphoma: durable remission with a combined chemotherapy-radiotherapy regimen. J Clin Oncol 1987; 5:867-874

24. Martin AR, Weisenburger DD, Chan WC, Ruby EL, Anderson JR, Vose JM, Bierman PJ, Bast MA, Daley DT, Armitage JO. Prognostic value of cellular proliferation and histologic grade in follicular lymphoma. Blood 1995; 85:3671-3678

25. Sun W, Caraway NP, Zhang HZ, Khanna A, Payne LG, Katz RL. Grading follicular lymphoma on fine needle aspiration. Comparison with proliferative index by DNA image analysis and Ki-67 labeling index. Acta Cytol 2004; 48:119-126.

26. Wang SA, Wang L, Hochberg EP, Muzikansky A, Harris NL, Hasserjian RP. Low histologic grade follicular lymphoma with high proliferation index: Morphologic and clinical features. Am J Surg Pathol 2005; 29:1490-1496