The prognostic significance of the intra-follicular tumor cell proliferative rate in follicular lymphoma

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Manuscript received June 8, 2006. Accepted January 4, 2006.

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ABSTRACT

Background and Objectives

In follicular lymphoma histological grading is used to predict clinical behavior and to stratify patients for treatment. However, the reproducibility of histological grading is poor and the clinical significance of the difference between grade 1 and grade 2 follicular lymphoma is unclear. Data on proliferation characteristics with respect to prognosis in follicular lymphoma are inconsistent.

Design and Methods

We assessed the Proliferation Index in follicles, using Mib-1 immunohistochemical staining in lymph node biopsies from 51 patients with follicular lymphoma who were receiving uniform first-line treatment consisting of cyclophosphamide, vincristine, prednisone and interferon α2b.

Results

The median Proliferation Index was 16.9 (range 3.1-49.2). In grades 1 and 2 follicular lymphoma (n=45) it was 16.1, compared to 24.2 in grade 3 (n=6; p=0.02). At a median follow-up of 71 months, patients with a Proliferation Index below the median had a significantly prolonged time to progression (median not reached vs. 15 months for those with a Proliferation Index above the median; p=0.0006) and improved overall survival (median not reached vs. 42 months, respectively; p=0.002). In multivariate analysis, the Proliferation Index retained its predictive value. Additional prognostic information was especially provided in patients with a low International Prognostic Index. Histological grade did not predict outcome.

Interpretation and Conclusions

The Proliferation Index is a biological marker that is strongly and independently predictive for outcome in follicular lymphoma, as shown even in this relatively small series of patients. It is easily applicable and reproducible and therefore superior to histological grading in identifying clinically aggressive follicular lymphoma, requiring other types of treatment.

Key words: follicular lymphoma, proliferation, mib-1, prognosis.

Haematologica 2007; 92:184-190

Follicular lymphoma is the second most common lymphoma and is usually characterized by an indolent course. Most patients present with advanced stage disease and at some point require therapy, which typically results in only a temporary remission. Follicular lymphoma is currently treated with chemotherapy, radiotherapy, interferon α and several forms of specific immunotherapy (using anti-CD 20 monoclonal antibodies), but no curative treatment, with the possible exception of allogeneic stem cell transplantation, is available.

One of the main issues in follicular lymphoma is to identify patients with an adverse prognosis who might benefit from more intensive treatment or experimental treatment modalities. Apart from clinical prognostic indices such as the International Prognostic Index and the Follicular Lymphoma International Prognostic Index,[1-3] stratification for treatment is often based on histological grading.[4,5] Given identical treatment, patients with grade 3 follicular lymphoma have a worse prognosis than those with grade 1 or 2 follicular lymphoma, but when treatment is intensified by anthracycline-containing regimens, this difference disappears.[6-8] A major problem in histological grading is the considerable intra- and inter-observer variability,[9,10] due to the subjective nature of the technique. Although the fraction of proliferating cells is prognostic in aggressive lymphomas,[11,12] its predictive value in follicular lymphoma is less clear. It is difficult to interpret data from studies on proliferation in follicular lymphoma because of differences in methodology and patient populations. To evaluate the prognostic significance of the proliferation rate in indolent follicular lymphoma, we studied the intra-follicular Proliferation Index in relation to histological grade and clinical outcome in 51 patients with indolent follicular lymphoma.

Design and Methods

Patients

Consecutive patients with previously untreated indolent follicular non-Hodgkin's lymphoma in Ann Arbor stages II bulky (larger than 5 cm), III or IV who started treatment between December 1994 and January 2000 were included in this study. All patients were uniformly treated according to local guidelines with eight 4-weekly CVP courses [cyclophosphamide 750 mg/m^2 intravenously on day 1, vincristine 1.4 mg/m^2 (to a maximum of 2 mg)] intravenously on day 1, and prednisone 60 mg/d orally on days 1-5) in combination with interferon α2b (5×10^6 IU subcutaneously 3 times a week) as induction therapy. Thereafter, responding patients received maintenance treatment with interferon α2b (5×10^6 IU subcutaneously 3 times a week) until intolerable toxicity, relapse or progressive disease, whichever came first.[13] An initial wait-and-see period was allowed before induction therapy was started. When relapse or progressive disease occurred, further treatment was left to the discretion of the treating physician. Staging was done according to the Ann Arbor criteria, by a thorough physical examination, computed tomography scanning of the thorax and abdomen and unilateral trephine bone marrow biopsy.

At the time of patient accrual, the Follicular Lymphoma International Prognostic Index had not been introduced and the exact number of involved nodal areas – one of the five components of this Index – was not documented. Therefore, for the purpose of this study the International Prognostic Index was used rather than the Follicular Lymphoma International Prognostic Index. The clinical data of the patients were collected prospectively. All patients visited the outpatient clinic at each cycle of chemotherapy and every 3 months during interferon maintenance therapy and follow-up.

Histological grading

All lymphoma specimens were centrally reviewed by two experienced hemato-pathologists (*JHJMvK and KH*) and were classified and graded according to the WHO criteria.[14] The absolute number of centroblasts in ten neoplastic follicles was counted using a 40x high-power microscopic field (hpf, 0.159 mm^2). Grade 1 cases had 0-5 centroblasts/hpf, grade 2 cases had 6-15 centroblasts/hpf and grade 3 cases had >15 centroblasts/hpf. In grade 3a follicular lymphoma centrocytes were still present whereas grade 3b was defined as follicles consisting of solid sheets of centroblasts only.

Immunohistochemistry

The fraction of proliferating cells (Proliferation Index) was assessed in samples of lymph node biopsies taken at the time of diagnosis of the lymphoma. The samples had been fixed in formalin and embedded in paraffin using routine methods. First, we identified the nature of proliferating cells by both CD20/mib-1 and CD3/mib-1 immunohistochemical double staining, thus visualizing proliferating B-cells and non-tumor T cells. Staining was performed by routine techniques; visualization was done with di-amino benzidine (DAB) for the anti-CD3 and the anti-CD20 antibody and with Fast Red for the mib-1 antibody. For the quantification of the Proliferation Index only the mib-1 antibody (a monoclonal mouse anti-human antibody directed against the Ki-67 antigen) (DAKO, Denmark) was used as a proliferation marker. For this quantification, 5μm thick paraffin sections were mounted onto 3-amino-propyltriethoxysilane (APS)-coated slides. After deparaffination, inhibition of endogenous peroxidase and microwave cooking in citrate buffer for 10 minutes, the sections were incubated for 1 hour at room temperature with the mib-1 antibody. Subsequently, after washing with phosphate-buffered saline, the sections were incubated with biotinylated horse anti-mouse and peroxidase-conjugated avidin. Visualization was performed with DAB substrate and counterstaining with methylene blue.

Intra- and extra-follicular proliferation

In general, the number of proliferating cells was greater in the follicles than in inter-follicular areas (Figure 1A). There were few intra-follicular T-cells (Figure 1B), whereas there were abundant inter-follicular T cells, including mib-1 positive T cells (Figure 1C-D). Thus, it essential to realize that inter-follicular proliferating cells do not solely represent tumor cells and should not be included in the Proliferation Index of the tumor B cells. Therefore, the Proliferation Index was analyzed on intra-follicular cells.

Assessment of the proliferation Index

In each section 200 cells per follicle were assessed in five randomly selected follicles using a 40x hpf, for a total of 1000 cells per section. A cell was considered mib-1 positive when any amount of brown staining was present, regardless of the intensity of the staining. The Proliferation Index was defined as (no. of mib-1 positive cells/ total no. of cells) x 100. All sections were assessed by one investigator (HAT), blinded to the clinical data of the patients. To establish inter-observer variability, a control series of 25 randomly selected sections (50%) was assessed by a second investigator (AK) blinded to the outcome of the first assessment and the clinical data of the patients.

Statistical analysis

The inter-observer variability was evaluated by calculating the coefficient of variation and Spearman's rank correlation. Differences between groups were analyzed using the Mann-Whitney U test or the Kruskal-Wallis test. Progression-free survival (PFS) and overall survival (OS) were measured from the start of induction therapy with CVP and interferon-α until the time of disease progression or death from any cause, respectively, or until the end of the observation period. In patients whose management included an initial wait-and-see policy, PFS and OS were also measured from the date the induction therapy was started. Survival curves were calculated according to the Kaplan-Meier method and the Cox proportional hazards model was used for the analysis. The level of statistical significance was set at 0.05 (two-sided).

Results

Patients

Between December 1994 and January 2000, 62 consecutive patients with follicular lymphoma started treatment according to the guidelines. Adequate biopsy material to perform immunohistochemical staining was available from 51. The clinical characteristics of these patients are summarized in Table 1. In eight patients an initial wait-and-see policy was adopted, which lasted a median of 11 months (range, 2-32 months). During the follow-up, which lasted a median of 71 months, 21 patients (41%) died: three patients in the group who achieved a complete remission after induction therapy, 11 in the group who had a partial remission and seven in the group with progressive disease. Sixteen patients did not receive all eight CVP courses, eight of whom because of progressive disease during induction therapy.

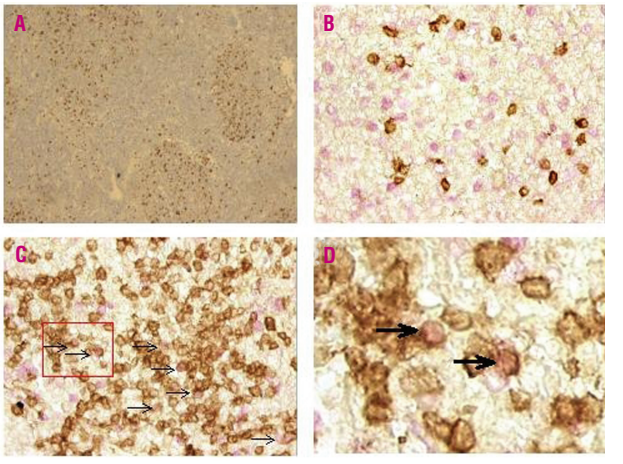

Figure 1. Sections of lymph nodes with follicular lymphoma; intra- and inter-follicular proliferation. (A) Mib-1 staining (brown) shown at 100× original magnification. Note the typically increased proliferation in the follicles. (B, C, D) Mib-1 (pink) and anti-CD3 staining (brown) shown at 400× original magnification. Note the abundant presence of T cells and double-stained proliferating T cells (arrows) in the inter-follicular region (C, D) compared to intra-follicularly (B).

Reproducibility of the Proliferation Index

The Proliferation Index ranged from 3.1 to 49.2 with a median of 16.9. The median value of the Proliferation Index in the series assessed by the second investigator was 17.6 (range 4.2-59.2). The coefficient of variation was 0.14 and the correlation coefficient of the two assessments was 0.81 (p<0.001).

The Proliferation Index in clinical and histological subgroups

The Proliferation Index in different clinical and histological subgroups is shown in Table 2. The Proliferation Index was significantly lower in patients with histological grade 1 or 2 lymphoma than in those with grade 3 lymphoma (Figure 2). Remarkably, the Proliferation Index was higher in patients in whom treatment was commenced some time after the diagnosis (i.e. following a wait-and-see policy) than in patients who were treated immediately at the time of diagnosis.

The Proliferation Index and patients' outcome

After a median follow-up of 71 months, the median PFS for all patients was 25 months, whereas the median OS was not reached. The median PFS and OS according to the Proliferation Index are shown in Table 3. Both PFS and OS were significantly shorter in patients with a high Proliferation Index, regardless of the cut-off point. When the six patients with grade 3 follicular lymphoma

Table 1. Patients' characteristics, histological grade, response to induction therapy and interferon use.

	All patients n=51
Age (years)	
Median	53.3
Sex	
Male	30
Female	21
Ann Arbor stage	
II	3
III	11
IV	37
Bone marrow involvement	
Present	35
Absent	13
Bulky disease (more than 5 cm)	
Present	20
Absent	29
WHO performance	
Not more than 1	42
More than 1	7
B-symptoms	
Present	9
Absent	41
Lactate dehydrogenase	
Normal	37
Above normal	11
Extranodal localization apart from bone marrow	
Present	3
Absent	46
International Prognostic Score	
0	3
1	24
2	17
3	3
4	1
Berard grade	
1	35
2	10
3	6
Response to induction therapy	
Complete remission	18
Partial remission	23
Stable disease	1
Progressive disease	9
Interferon stopped due to toxicity	14

Table 2. Median Proliferation Index (PI) in clinical and histological subgroups.

	n	*Median PI*	*p value*
Bulky disease present	19	18.2	NS
Bulky disease absent	29	16.4	
Histological transformation	8	16.9	NS
No transformation	43	18.3	
Wait-and-see policy	8	22	0.03
No wait-and-see policy	43	15	
Complete remission°	18	12.4	0.055
Partial remission°	23	19.4	
Progressive disease°	9	21.7	
Histological grade 1	35	16.4	0.02*
Histological grade 2	10	12.6	
Histological grade 3	6	24.2	

*°After induction therapy; NS: not statistically significant. *p-value when grades 1 and 2 combined are compared to grade 3. The total in the different characteristics can be less than 51 due to missing data.*

Table 3. Progression-free survival and overall survival according to the Proliferation Index and histological grade.

	n	*Median PFS (months)*	*p*	*Median OS (months)*	*p*
PI > median	25	15	0.0006	42	0.002
PI < median	26	NR		NR	
PI upper tertile	17	15	0.009	40	0.017
PI lower two tertiles	34	34		NR	
PI upper quartile	12	14	0.026	41	0.007
PI lower three quartiles	39	30		NR	
PI as a continuous variable		0.016		0.053	
Histological grade 1	35	24	NS	NR	NS
Histological grade 2	10	30		NR	
Histological grade 3	6	15		60	

PI: Proliferation Index; PFS: progression-free survival; OS: overall survival; NR: not reached; NS: not statistically significant.

were excluded from the analysis, the results remained significant. Importantly, the Proliferation Index also maintained its prognostic impact on PFS and OS when tested as a continuous variable. PFS and OS in patients with the Proliferation Index above and below the median are shown in Figure 3.

Other parameters that were associated with a worse OS in univariate analysis were male sex, the presence of bulky disease and an intermediate or high International Prognostic Index. When these parameters were tested in multivariate analysis the association of high Proliferation Index with a worse OS retained its significance (Table 4). In the subgroup of patients with a favorable clinical profile as indicated by a low International Prognostic Index, both PFS and OS were significantly worse in patients with a Proliferation Index above the median (n=17) than in those with a low Proliferation Index (n=12) (Figure 4A). This difference was absent in patients with an intermediate or high International Prognostic Index (Figure 4B), although the number of patients was too low to draw any firm conclusions for this subset of patients.

Histological grade did not significantly predict PFS or OS (p>0.4 for OS), probably due to the low number of grade 3 cases.

Discussion

Follicular lymphoma is a clinically heterogeneous dis-

ease with considerable differences in survival times. To improve outcome, the identification of patients at risk of early progression and death is as important as the development of new therapeutic modalities. We established the prognostic value of the intra-follicular Proliferation Index in patients with follicular lymphoma, treated with CVP chemotherapy plus interferon-α. The Proliferation Index is significantly associated with both PFS and OS, independently of other risk factors, including the clinically-based International Prognostic Index score. In contrast to the International Prognostic Index, the Proliferation Index is of predictive value for PFS and response rate to induction therapy. Importantly, we demonstrate that within the large group of patients who belong to the low-risk group according to the International Prognostic Index, the proliferation characteristic significantly identifies patients with a very poor prognosis.

In this study, as in most studies on the significance of proliferation in follicular lymphoma,[15-21] there was an association between large cell histology and a higher proliferative activity. However, a large overlap was present indicating an inherent difference between large cell morphology and proliferation characteristics. A major concern about histological grading is its lack of reproducibility,[9,10] which precludes its general application. In contrast, the reproducibility of the Proliferation Index was excellent. There was no statistically significant correlation between PFS or OS and histological grade, mainly due to the small number of patients with grade 3 follicular lymphoma. The median Proliferation Index of the patients in whom a wait-and-see policy was adopted was higher than that in those who were treated immediately after diagnosis. This may seem to be in contradiction with the observed prognostic value of the Proliferation Index. It should, however, be noted that eventually all patients received treatment. Apparently, the prognostic information of the Proliferation Index is valid in chemotherapy-treated patients; it may be less suitable for selecting those patients in whom starting therapy can be delayed. Prospective studies in which patients are stratified according to the Proliferation Index are needed to elucidate this aspect further.

Few studies have addressed proliferation in relation to outcome in follicular lymphoma. Cibull *et al.*[15] investigated lymph node biopsies from 33 patients with follicular lymphoma by performing Ki-67 immunostaining and silver nuclear organizer region counting (AgNOR). The proliferation rate did not predict survival. Llanos *et al.*[16] studied 49 patients with follicular lymphoma and used a semi-quantitative grading system to quantify Ki-67 positivity in areas of highest Ki-67 expression. No correlation was found between Ki-67 expression and OS. Czader *et al.*[22] manually quantified the mib-1 positive fraction in the follicles of lymph node sections from 49 patients with follicular lymphoma. Although the range of the Proliferation Index in this study was identical to that in our study, they found that achievement of a complete remission was associated with a high proliferation fraction. The proliferation rate did not correlate with survival times. A large study on proliferation in follicular lymphoma was conducted by Martin *et al.*,[17] who examined 106 cases. They reported a positive correlation between the Proliferation Index and OS, but this failed to be statistically significant when tested in multivariate analysis together with the histological grade, age and the International Prognostic Index. As in our study, the proliferative rate was correlated with the histological grade of follicular lymphoma. Importantly, 60% of the patients in Martin's study were classified as having follicular large cell lymphoma (i.e. grade 3 follicular lymphoma). Of the remaining 40% only five patients (4.7%) had follicular small cell lymphoma (grade 1 follicular lymphoma). In our study only 12% of the cases were classified as having grade 3 follicular lymphoma whereas 69% had grade 1 disease. Our study should, therefore, be considered as a study addressing really indolent follicular lymphoma, whereas Martin's

Table 4. Factors prognostic for overall survival.

Variable	*n*	*Hazard ratio (95%-confidence interval) univariate*	*multivariate*
Male vs. female	29/21	2.31 (0.90-5.97)	1.56 (0.56-4.32)
Bulky disease: present vs. absent	19/29	3.39 (1.39-8.30)*	2.76 (0.99-7.69)
Proliferation Index above vs. below the median	25/26	3.88 (1.49-10.10)*	2.92 (1.08-7.94)*
IPI not low vs. low	20/29	4.05 (1.64-9.98)*	4.31 (1.65-11.25)*

*The total in the different characteristics can be less than 51 due to missing data. *statistically significant ($p<0.05$).*

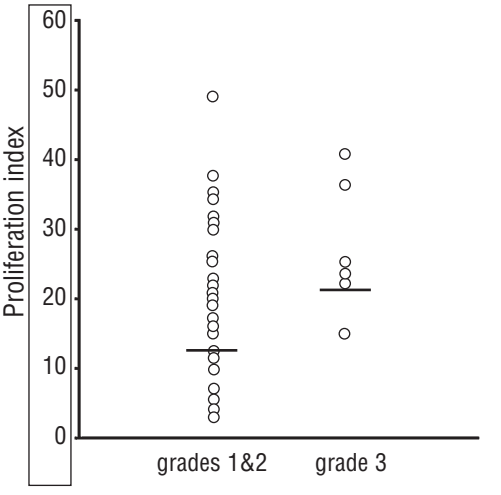

Figure 2. Proliferation indices of grades 1 and 2 FL combined, compared to grade 3 FL. The median value is indicated.

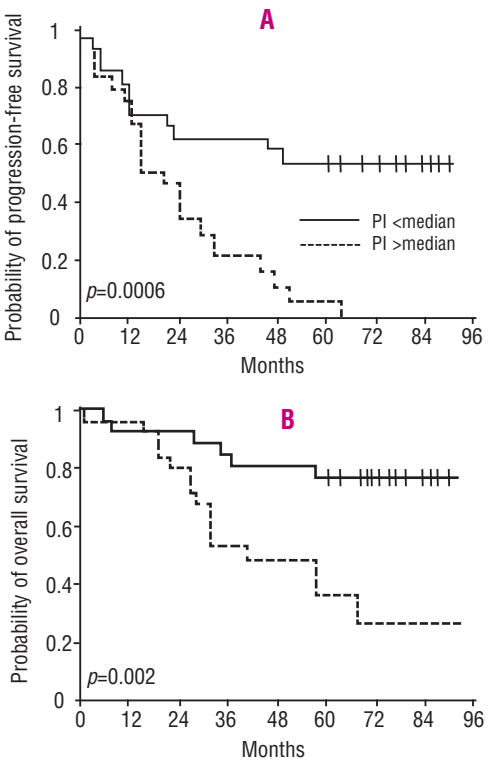

Figure 3. Progression-free survival (A) and overall survival (B) of patients with a Proliferation Index (PI) above or below the median.

study focused on more aggressive variants of follicular lymphoma. In addition, all patients in the study by Martin et al, including those with grade 1 or 2 follicular lymphoma, received anthracycline-containing chemotherapeutic regimens as primary treatment, whereas none of our patients was treated anthracyclines. A third point of difference concerns the methodology of assessing proliferation. Martin *et al*. used a quantitative image analysis system, whereas we chose to measure the Proliferation Index manually. Manual counting avoids over-estimation of the number of centroblasts, which are larger. In addition, measurement fields in the study by Martin *et al*. were not randomly chosen, thereby introducing a bias in the analysis that may have affected reproducibility.

Recently, in patients with low grade follicular lymphoma Wang *et al*. failed to show a significant difference in OS between those with a high Proliferation Index and those with a low Index.[23] The shape of the survival curve of patients with low grade follicular lymphoma with a high Proliferation Index was remarkably similar to that of patients with grade 3 follicular lymphoma. Also disease-specific survival was significantly better in patients with a low Proliferation Index, corroborating our observations. This study was, however, retrospective and patients were not treated uniformly. Moreover, the fraction of proliferating cells was estimated and not actually counted and no multivariate analysis with other predictors of outcome was performed.

The combination of CVP chemotherapy plus the monoclonal anti-CD20 antibody rituximab has been demonstrated to be superior to CVP alone and is now considered the new standard treatment for follicular lymphoma.[24] In a small series of patients with follicular lymphoma treated with rituximab plus chemotherapy, a significant correlation was found between inferior treatment response and high mib-1 expression.[25] This corroborates our observations, and indicates that proliferative rate may maintain its value as a prognosticator in the rituximab era. Nevertheless, the prognostic significance of the intra-follicular Proliferation Index should be validated in a prospectively followed cohort of patients treated with a rituximab-containing regimen.

In conclusion, we observed that the intra-follicular Proliferation Index is a strong and independent prognostic factor for PFS and OS in follicular lymphoma, while histological grading did not predict patients' outcome in our series of patients. Additional prognostic information is especially provided in patients classified as having a low risk of early progression according to accepted clinical parameters, even given the relatively small number of patients studied. Manual assessment of the Proliferation Index is easily applicable and reproducible and can serve as a method for stratifying patients in clinical trials to select patients needing more intensive treatment and to discriminate them from patients with an upfront good prognosis who might be over-treated with an intensive first-line approach.

Authors' Contributions

AK: contributed to the design of the study, acquired, analyzed and interpreted the data, wrote the paper, and approved its final version; HAT: contributed to the conception and design of the study, acquired the data, and approved its final version; JMMR: contributed to the design of the study, acquired, interpreted the data, revised the paper, and approved its final version; GFB: contributed to the design of the study, analyzed and interpreted the data, revised the paper, and approved its final version; KH: contributed to the design of the study, acquired, interpreted the data, revised the paper, and approved its final version; MMK: contributed to the design of the study, interpreted the data, revised the paper, and approved its final version; JHJMvK: contributed to the conception and design of the study, acquired and interpreted the data, revised the paper, and approved the final version.

Conflict of Interest

The authors reported no potential conflicts of interest.

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