DNA flow cytometry of follicular non-Hodgkin's lymphoma

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Abstract

S-phase fraction, an index of cellular proliferation, and DNA ploidy were measured by DNA flow cytometry in a retrospective study of lymph node biopsy specimens from 83 cases (before treatment) of follicular non-Hodgkin's lymphoma, Working Formulation categories B and C. The correlations between these measures and survival, clinical stage, symptoms and histopathological factors were investigated. Aneuploidy was rare ($n = 16$) and had no effect on length of survival or transformation to high grade lymphoma. The overall mean S-phase fraction was 3.6%; for the whole series increasing S-phase fraction was associated with decreased survival. A high S-phase fraction (more than 5%) in initial biopsy specimens was also associated with an increased risk of subsequent high grade transformation at relapse. There was no difference between the survival or proliferative activity of tumours composed of mainly small cleaved cells compared with those composed of mixed small and large cells. There was no difference in survival or proliferative activity between tumours showing a pure follicular growth pattern and those with a mixed follicular and diffuse growth pattern. Multifactorial analysis showed that an S-phase fraction of more than 5% and B symptoms were the most important factors determining survival in these follicular non-Hodgkin's lymphomas.

Since the early studies of Peckham and Cooper¹ it has been repeatedly shown that low grade non-Hodgkin's lymphomas (NHL) exhibit overall lower proliferative activity than high grade NHL.² In view of the different prognoses of high and low grade NHL it is hardly surprising, therefore, that the degree of proliferative activity is inversely related to the length of survival. If this effect is merely linked to histopathological grade, however, there is little reason to measure proliferation. The measurement of proliferation by either DNA flow cytometry, 3H-thymidine labelling index, or Ki-67 score becomes clinically relevant when it can be shown that the result has an independent prognostic effect within homogenous subtypes of NHL of similar grade.

In this study we examined the effect of

proliferative activity on the prognosis of follicular NHL. Characteristically follicular NHL are low grade indolent tumours of follicle centre B lymphocytes which are often widely disseminated at presentation, relapse frequently, and may undergo transformation to high grade NHL.³ Because of the unpredictable behaviour exhibited by some cases of follicular NHL it would be advantageous to select cases with a worse prognosis for more aggressive treatment. Using flow cytometry Griffin *et al* showed a significant effect of proliferative activity on the prognosis of follicular NHL⁴; and in our previous study⁵ of a small number of cases, high proliferative activity in initial biopsy specimens was associated with subsequent transformation at relapse. Neither study took into account clinical prognostic variables.

We have now examined a larger group of low grade follicular lymphomas using DNA flow cytometry of archival histopathological material and present evidence that proliferative activity has an independent effect on prognosis.

Methods

A retrospective DNA flow cytometric study of 83 patients with low grade follicular NHL diagnosed between 1961 and 1987 at St Thomas's Hospital was undertaken. In all 83 cases biopsy material before treatment was available for flow cytometry and histological classification. For 22 of the 83 cases biopsy material was also available from subsequent relapses. Information to determine clinical stage, treatment, remission, clinical relapse and overall survival were obtained from the clinical notes. The demographic data for the series are shown in table 1.

HISTOPATHOLOGY

Histological sections were stained with haematoxylin and eosin, Giemsa, and for reticulin. All cases were also stained immunohistochemically with MB1, MB2, and L26 monoclonal antibodies to confirm a B cell lineage.

Cases were subclassified cytologically (table 2) using the Working Formulation⁶ into those composed predominantly of small cleaved cells (category B in the Working Formulation) and a mixed small and large cell group (category C in the Working Formulation). Cases consisting of follicular lymphomas with either a clear predominance of large cells or composed wholly of large cells were specifi-

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Table 1 Clinical features of patients with follicular lymphomas

Men	n = 43
Women	n = 40
Mean age at presentation	55.5 years (21.4–80.3)
Median follow up	45 months
Clinical stage:	
1	n = 17
2	n = 9
3	n = 19
4	n = 21
Not known	n = 17
Clinical symptoms:	
A	n = 62
B	n = 21
Treatment:	
Observation	n = 12
Radiotherapy	n = 22
"Mild" chemotherapy	n = 34
"Aggressive" chemotherapy	n = 7
Not known	n = 8

Table 2 Comparison of S-phase fractions in various histological subtypes

	Mean S-phase fraction (95% confidence limits)	Probability
All cases (n = 83)	3.6 (3.1–4.0)	
Architectural subtype:		
follicular (n = 69)	3.5 (3.0–4.0)	NS
follicular and diffuse (n = 14)	4.2 (0.8–10)	
Cytological subtype:		
small cell (n = 61)	3.3 (2.9–3.8)	NS
mixed (n = 22)	4.4 (3.2–5.7)	
First biopsy:		
untransformed at relapse (n = 12)	2.5 (1.7–3.3)	0.01
transformed at relapse (n = 10)	5.0 (3.4–6.6)	

cally excluded from the series because they are generally considered to be of higher grade malignancy. Similarly, cases with a pure diffuse architecture or cases of centrocyclic lymphoma⁷ were also excluded from the series. Cases were also subdivided into those which showed either a pure follicular architecture or a combined follicular and diffuse architecture in the same biopsy specimen. The number of cases showing clinically important sclerosis was documented as this is reported to be of prognostic value.⁸

DNA FLOW CYTOMETRY

DNA flow cytometry was performed using samples prepared from archival paraffin wax blocks. The method for preparation was slightly modified from that of Hedley *et al*⁹ and has been described previously.¹⁰ We have shown that results obtained by this method correlate highly with results obtained from fresh lymphoid tissue.^{10,11} Briefly, 50 µm sections were dewaxed, rehydrated, and incubated in pepsin for 30 minutes at pH 1.5. The resulting suspension was centrifuged and the pellet resuspended in Isoton containing 1 µg/ml of 4,6-diamidino-2-phenylindol-hydrochloride. The suspension was then syringed through a narrow gauge needle and filtered through 35 µm pore size nylon gauze. Ten thousand cells per sample were analysed on a Beckton Dickinson FACS analyser.

Tumours were considered to be DNA aneuploid if two distinct G₁/G₀ peaks were present. A DNA index of 1.0 indicates the presence of only diploid cells which have a normal amount of DNA. A full peak coefficient of variation (CV) was calculated for

the G₁/G₀ peak. The mean CV for the whole series was 3.9. For reasons discussed previously,¹¹ cytometric readings with a high CV (more than 8.0) were considered unacceptable. The proportion of cells synthesising DNA (S-phase fraction) was calculated by the method of Baisch¹² for cases which were DNA diploid. In cases which were DNA aneuploid the S-phase fraction was calculated using the methods described by Camplejohn and Macartney.¹¹

Outcome was assessed in terms of overall survival and relapse-free survival. The effects of histological pattern, cytological group, clinical stage, A and B symptoms, DNA ploidy and S-phase fraction on survival were analysed separately using life tables. Histological subtypes were compared for survival using the log rank test¹³ and S-phase fractions in different groups were compared using the Wilcoxon rank sum test. To examine the independent effect of these factors on survival and to allow for varying lengths of follow up, Cox's proportional hazards model was applied.¹⁴

Results

Tables 1 and 2 show general characteristics for the whole series of 83 cases and the numbers of cases falling into various histological subtypes. The projected median survival for the whole series was 7.6 years. Complete remission was achieved in 51% of cases and the median remission duration was 1.7 years. Forty four per cent of those achieving remission, however, subsequently relapsed. Because there was no consistent policy of taking biopsy specimens from patients to document relapse, histological material was available for only 22 of the patients who relapsed. At rebiopsy, 10 of these patients had transformed into high grade NHL while the remainder still showed low grade follicular NHL. In the main series there was no difference in survival between the cases falling into the various histological subtypes or clinical stages, although there was a trend for cases with mixed small and large cell histology to do slightly worse. Only eight cases showed clinically important sclerosis and there was no difference in either their survival or S-phase fraction compared with the main group. The survival of patients with B symptoms (median 21 months) was significantly (p = 0.02) worse than those with A symptoms.

PLOIDY

DNA aneuploidy was found in 16 of the 83 cases and the most common aneuploid DNA index was 1.1 (n = 7). There was no difference in survival between DNA diploid and aneuploid cases and there was no difference in the incidence of aneuploidy between various histological subtypes or clinical stages.

PROLIFERATIVE ACTIVITY

The mean S-phase fraction for the whole series of 83 patients was 3.6% with a median of 3.0% (table 2). There was no difference between the S-phase fractions seen in various histological

Survival curves for follicular lymphomas with S-phase fractions below ($n = 67$) and above ($n = 16$) 5%.

subtypes (table 2). Because of this, cases were grouped together for further analysis. This showed a significant ($p = <0.01$) difference in the survival of cases with S-phase fractions of at least 5% compared with less proliferative cases (figure). This difference in survival was seen when comparisons were made between tumours with proliferative activity above and below 5% or when S-phase was treated as a continuous variable. Multifactorial analysis showed that only S-phase fraction and the presence of B symptoms significantly affected the overall survival. Analysis was also stratified by decade of diagnosis, but this did not affect the observations reported for S-phase or ploidy. As in our previous study, cases which subsequently underwent a documented high grade transformation had a significantly higher S-phase fraction in their initial biopsy specimens at the time of first presentation (table 2), reflecting the important effect of S-phase on survival.

Discussion

Several factors are reported to have a prognostic effect in follicular NHL. They include the presence of anaemia, abnormal liver function tests, B symptoms, increasing age, advanced stage, increasing tumour bulk and poor response to treatment.^{3 15-17} Histologically, the proportion of large cells, mitotic index, and the presence or absence of a mantle zone have also been claimed to have a prognostic effect.^{15 18-22} In general, growth pattern has no significant effect, although some authors report a trend to poorer survival in diffuse tumours. To circumvent some of the problems associated with the morphological heterogeneity of follicular lymphomas and achieve a more homogenous group of cases we excluded from our study cases with a diffuse architecture or those with a high content of large cells.

Because this was a retrospective study patients did not receive uniform pretreatment assessments or standard treatment protocols. We were therefore unable to consider certain prognostic factors such as disease bulk in our analysis, and clinical staging data were only available for 80% of cases. This also probably accounts for the rather high percentage of stage 1 cases in the series. The inclusion of cases

spread over a 25 year period would be unacceptable if high grade NHL were under consideration because of changes in treatment during this time. Therapeutic options for low grade follicular NHL, however, have not changed substantially, and we were unable to detect any difference in survival between cases diagnosed during the first and second half of the period studied.

Several studies have previously suggested that proliferative activity, measured by flow cytometry, Ki67 index, or mitotic index, affects the prognosis of follicular NHL. In these studies, however, the grouping of cases for analysis has often included a mixture of low grade lymphomas and results are not specific for follicular lymphomas.²³⁻²⁵ Alternatively, the effects of all the other factors such as symptoms, stage, histological subtype and architecture have not been studied or numbers are small.^{4 5 20 26 27}

The major finding of our study is that proliferative activity seems to be the most important factor governing survival in follicular NHL, confirming previous more limited studies.^{4 5 27} DNA aneuploidy was rare and had no prognostic effect. We also confirmed our previous finding that tumours with high proliferative activity in the initial biopsy specimen (more than 5%) were more likely to sustain high grade relapse.

Our results have implications for the treatment of follicular NHL. Although the decision to treat this disease must be based on consideration of factors such as symptoms, nevertheless the results presented here do provide an argument for treating follicular NHL with high proliferative activity with more aggressive therapeutic regimens.

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Eponyms in pathology . . .

RICKETTS, Howard Taylor (1871–1910) was an American microbiologist and epidemiologist who qualified from Northwestern university in 1897, later working in the department of pathology at the university of Chicago. He carried out important field studies on Rocky Mountain Spotted Fever and showed that the causative organism is transmitted to man by the bite of a wood tick. These small bacillus-like parasites were grouped together under the name rickettsia by Da Rocha-Lima in honour of Ricketts. Ironically, he died of typhus fever (another rickettsial infection) while studying it in Mexico City in 1910.

ASKANAZY, Max (1865–1940) was a Swiss pathologist, born in East Prussia, and educated in Königsberg, West Germany. He became professor of general pathology in Geneva in 1905, and his major contributions were in the fields of haematology, parasitology, and oncology. His description of the large granular eosinophilic cell derived from the thyroid follicular epithelium is the same as that of Hürthle (a German histologist, 1860–1945) and the two eponyms are synonymous with the term “oxyphil cell”.