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Prognostic indicators in centroblastic-centrocytic lymphoma

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SUMMARY The prognostic importance of ploidy and proliferative index (%S+G2) assessed by flow cytometry, mitotic and centroblast counts, and histological growth pattern were evaluated in biopsy specimens taken before treatment from 60 cases of centroblastic-centrocytic non-Hodgkin's lymphoma. Cases with a high proliferative index ($\geq 18\%$) or DNA aneuploidy showed significantly poorer survival than those with a low proliferative index ($< 18\%$). A high mitotic count was also associated with a poor prognosis. On multiple regression analysis the flow cytometric assessments and mitotic counts were significant predictors of survival.

Assessments of proliferative activity clearly have prognostic potential in centroblastic-centrocytic lymphoma and may permit more accurate characterisation of individual tumours.

Centroblastic-centrocytic (CB-CC) non-Hodgkin's lymphoma comprises a heterogeneous group of tumours with a variable prognosis and response to treatment. They present a clear histological continuum with respect to the extent of follicular and diffuse growth patterns and proportion of centroblasts which is reflected to some degree by differences in clinical behaviour.[1-4] While accepted treatment is commonly based on histological assessment together with clinical variables such as age, disease bulk, and history,[5] our experience shows that these are unreliable predictors of response in any one patient. The problem is compounded by the lack of reproducibility of pathological diagnosis.[6 7] There is clearly a need for more objective and reproducible means of assessing these patients.

Several studies over recent years have examined proliferative activity in non-Hodgkin's lymphoma by thymidine labelling,[8 9] mitosis counts,[10] Ki-67 immunostaining,[11 12] and flow cytometry on both fresh[13] and paraffin wax embedded tissue.[14 15] Taken as a whole, these studies indicate that classically defined "high grade" tumours have a higher proliferative activity and a higher prevalence of DNA aneuploidy than those of "low grade". Perhaps not surprisingly, high proliferative activity has been shown to impart a significantly worse prognosis in non-Hodgkin's lymphomas in general.[9 10 12 16]

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The principal aim of this study was to determine whether these generalised observations hold true within the group of CB-CC non-Hodgkin's lymphoma where there are difficulties in clinical assessment and management. In particular, we wished to ascertain whether estimation of proliferative activity would provide prognostic information of value in refining treatment options. Flow cytometry was chosen as an objective, reproducible technique together with simple counting techniques which could be routinely applied in non-specialist centres.

Material and methods

One hundred and eleven blocks of pre-treatment biopsy specimens from 60 patients received in the pathology departments at the University of Leeds and Pinderfields Hospital, Wakefield, between 1976 and 1986 were studied (mean n = 1·9 blocks per case). The patients comprised 34 women and 26 men with an age range of 33–88 years (median 56·0 years).

HISTOLOGICAL ASSESSMENT

Sections from each case were stained by haematoxylin and eosin and Gordon and Sweet's reticulin methods. The growth pattern (follicular, follicular and diffuse, or diffuse) was noted. For each slide a mitotic count was carried out in 10 high power fields of 0·159 mm^2 each by a single observer. In the same fields a differential cell count was performed along an intercept line to provide an estimate of the percentage of

centroblasts. In follicular tumours counts were restricted to within the neoplastic follicles. In diffuse and follicular and diffuse tumours counts were carried out in random fields within the tumour.

FLOW CYTOMETRY

Nuclear DNA content was estimated using a modification of the method of Hedley *et al.*[17] Fifty μm sections were cut from each block and transferred to glass slides. The sections were dewaxed in xylene and rehydrated through graded alcohols. Sections were then washed in distilled water, transferred from the slide to a test tube containing 0·05% pepsin (Sigma, Poole, Dorset) in 0·9% sodium chloride adjusted to pH 1·5 with 2N hydrochloric acid, and incubated for 30 minutes at 37°C. Cells were centrifuged at 2000 rpm and washed twice in distilled water before staining in a solution (1 μg/ml) of 4′, 6′-diamidino-2-phenylindole dihydrochloride (Boehringer, Mannheim, West Germany) in RPMI 1640 tissue culture medium at 20°C for 30 minutes. This was followed by filtration through 16 layers of butter muslin and syringing through a 23 gauge needle to prevent clumping. Samples were analysed on an EPICS V flow cytometer (Coulter Electronics, Hialeh, Florida, USA). For excitation a Coherent Innova-90 5W UV-enhanced argon ion laser was used at 50 mW at a wavelength of 350 nm. A 408 nm interference filter removed scattered ultraviolet fluorescence. Ten thousand nuclei were counted. DNA aneuploidy was defined as the presence of more than one G_0/G_1 peak.[18] The proliferative index (%S+G2) was determined using the PARA 1 cell cycle analysis program (commercial software, Coulter Electronics, Hialeh, Florida, USA). Calculation of proliferative index was not attempted in DNA aneuploid tumours due to overlapping cell populations. The median half peak coefficient of variation of the G_0/G_1 peak was 7% (range 3·2–10·2%).

SURVIVAL DATA AND STATISTICS

Survival data for each case were obtained from the Yorkshire Regional Cancer Registry. Statistical analysis was carried out using life table analysis, the log rank test,[19] and Cox's multiple regression model[20] using the BMDP statistics packages BMDP1L and BMDP2L (University of California).

Mitotic counts and proliferative indices were compared using Spearman's rank correlation coefficient.

Results

Histological assessment showed that 20 cases of each histological growth pattern were present. Mitotic count was possible on 58 cases and showed a wide range of four to 107 mitoses/10 high power fields (HPF) (median 21·0/10 HPF). The percentage of centroblasts varied from 1–18% (median 4·5%).

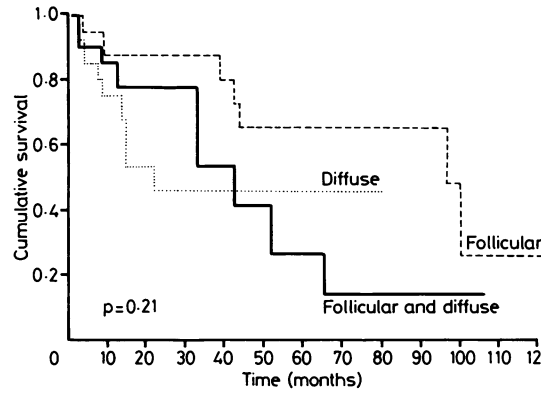

Fig 1 *Survival curves for follicular, follicular and diffuse, and diffuse cases of CB-CC non-Hodgkin's lymphoma.*

Flow cytometry showed that 11 cases were DNA aneuploid (18%) and 49 diploid (82%). In the diploid cases the proliferative index (%S+G2) ranged from 6–40% (median 21%). No significant correlation was found between the mitotic count and proliferative index (r_s = 0·12; p > 0·1).

RELATION TO SURVIVAL

Growth pattern The survival curves according to growth pattern are shown in fig 1. Tumours with diffuse components had a worse survival than those with a purely follicular growth pattern. The differences between the three groups did not reach significance (p = 0·21).

Mitotic count The mitotic count was significantly related to survival when an arbitary value of 50 mitoses/10HPF was used to separate cases into high and low mitotic count groups (p = 0·01). Any value from 25 to 80 mitoses/10HPF gave significant separation of the groups but the value of 50 mitoses/10HPF identified a small group (n = 8) with an extremely poor survival (fig 2).

Centroblast count Cases with a centroblast count above 5% tended to have a worse survival but the difference did not reach significance (p = 0·28).

Ploidy and proliferative index Comparison of DNA aneuploid with diploid cases showed no difference in survival patterns. When diploid cases alone were considered, however, those with a proliferative index above 18% showed a significantly worse survival (p = 0·02) (fig 3). Comparison of DNA aneuploid and diploid cases with a low proliferative index (< 18%) showed that DNA aneuploid cases had a significantly

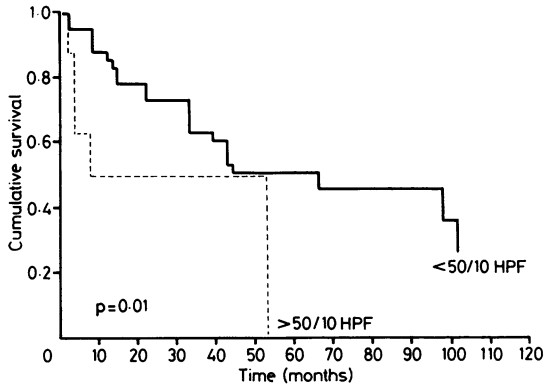

Fig 2 *Survival curves for cases with mitotic counts of < 50 mitoses/10HPF and ≥ 50 mitoses/10HPF.*

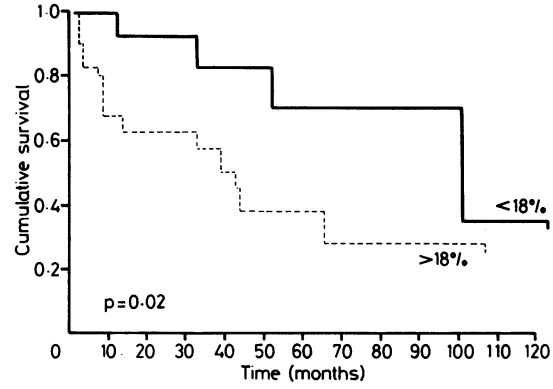

Fig 3 *Survival curves for patients with diploid tumours with proliferation indices of < 18% and ≥ 18%.*

worse survival (p = 0·05). This suggests that the lack of difference in the survival patterns when DNA aneuploid and diploid cases as a whole are compared is due to the poor survival of high proliferative index (≥ 18%) diploid cases. Comparing a combined group of DNA aneuploid and diploid cases with a high proliferative index (≥ 18%) with diploid cases showing a low proliferative index (< 18%) confirmed a significant difference in survival (fig 4). It was also possible to identify by flow cytometry a small group (n = 8) of diploid cases with a low proliferative index of less than 15% that had an excellent survival (p = 0·05) with no deaths over a 100 month follow up period.

MULTIPLE REGRESSION ANALYSIS

A Cox's multiple regression analysis was carried out on the factors outlined above by inserting the most significant values from the life table analysis into the model. Flow cytometric grouping (DNA aneuploid + diploid high (≥ 18%) proliferative index *v* diploid low (< 18%) proliferative index) and mitotic count (≥ 50 mitoses/10HPF *v* < 50mitoses/10HPF) were found to be significant and independent predictors of survival. The high risk flow cytometric group confirmed a relative risk of death of 3·6 and the high risk mitotic count group a relative risk of 4·4. Growth pattern and centroblast count failed to improve the model when flow cytometric results and mitotic count had been entered.

Discussion

This study has extended previous work on proliferative activity in non-Hodgkin's lymphoma by showing that measurement of proliferative activity has prognostic importance within the group of CB-CC non-Hodgkin's lymphoma. Proliferative activity, estimated by flow cytometry or by mitosis counting, was more important than histological growth pattern or proportion of centroblasts, both cited previously as prognostic indicators in assessing the likely outcome in CB-CC non-Hodgkin's lymphoma.[13 21 22]

Flow cytometry has been widely applied to non-Hodgkin's lymphoma.[13–15 23] It has been shown that classically defined high grade tumours have a higher proportion of proliferating cells than those of low grade. Not surprisingly, therefore, proliferative activity assessed by flow cytometry has been shown to have prognostic importance across the broad range of non-Hodgkin's lymphoma.[16]

The median coefficient of variation of 7% is slightly higher than that found in previous studies.[14] This could be expected to lead to an underestimate of the incidence of DNA aneuploidy. The incidence of 18% found in this study, however, compares favourably with figures for low grade lymphoma in other series,[16 23] and the finding that ploidy in itself is of no

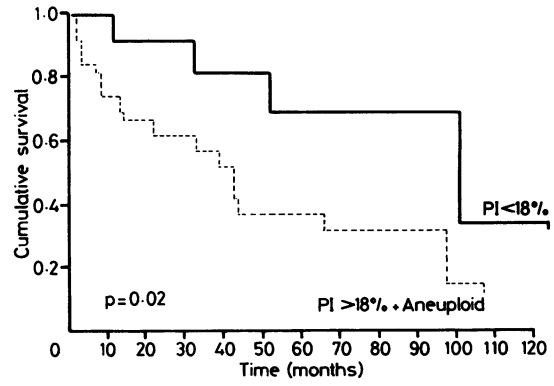

Fig 4 *Survival curves comparing cases with diploid tumours and a low proliferative index (< 18%) with DNA aneuploid or diploid high proliferative index cases (≥ 18%).*

prognostic value is in keeping with previous findings in non-Hodgkin's lymphoma.[15 24 25] The %S + G2 derived using the PARA1 program yields higher values than other studies of comparable tumours using the method of Baisch *et al.*[14]

The true level of cell proliferation in these tumours is unknown as there is no "gold standard" and cell cycle analysis programs yield varying figures for the same DNA histogram. Interestingly, DNA aneuploid and high proliferative index diploid cases could be combined and showed a significantly worse prognosis than low proliferative index diploid cases. This is similar to findings for rectal adenocarcinomas[26] and suggests that aneuploidy itself may be associated with a higher proliferative activity, as suggested in renal adenocarcinoma.[27] The finding that proliferative index is of prognostic importance is interesting in the light of previous results[14] showing that cases of CB-CC non-Hodgkin's lymphoma which progress to high grade centroblastic lymphoma have a significantly higher S phase fraction than cases which are histologically stable. S phase values above 10% have also been shown to be associated with a worse survival in a mixed group of "poor histology" non-Hodgkin's lymphoma.[25]

Mitosis counting is an attractive technique by virtue of its simplicity and the lack of specialised equipment required. Previous work on non-Hodgkin's lymphoma has shown that satisfactory inter- and intraobserver variation can be obtained.[10 28 29] It is encouraging, therefore, to find that mitotic counts can give useful prognostic information in CB-CC non-Hodgkin's lymphoma. Mitotic activity has previously been shown to have prognostic importance in non-Hodgkin's lymphoma as a whole[10 30] and in several subtypes of diffuse non-Hodgkin's lymphoma.[31 32] Akerman *et al*[10] examined a group of 32 CB-CC lymphomas from within their larger series of mixed non-Hodgkin's lymphoma and showed on multivariate analysis that mitotic count was an independent prognostic variable. In contrast, studies of large cell lymphoma[33] and follicular lymphoma of several subtypes[29] have failed to show a correlation between mitotic count and survival.

The reasons for such differences are probably complex. The mitotic count depends on both the proportion of cycling cells and the duration of mitosis. Both parameters vary considerably in different neoplasms so that mitotic count should not be expected to have a simple relation to proliferative activity when comparing different tumours. This probably accounts for the lack of correlation found in this study between mitotic count and proliferative index assessed by flow cytometry, a finding previously recorded in large cell lymphoma,[24] and their apparent independence as prognostic indicators. Chemotherapeutic and radiotherapeutic responses may also be related to cell kinetic variables, and in some tumours this may confound the importance of mitotic counts. For example, tumours with a long mitotic phase and a relatively high mitotic count may be more responsive to treatment and therefore show a better survival.

The identification of different prognostic groups within a single subtype of non-Hodgkin's lymphoma, albeit one with known heterogeneity, serves to highlight the necessarily artificial nature of classification systems based principally on assessment of morphological rather than biological criteria. While such systems are broadly successful,[34] the underlying continuum of clinical behaviour limits the usefulness of the classification for the individual patient. Quantitative examination of basic biological variables, such as proliferative activity, may lead to more accurate characterisation of individual tumours and therefore to more appropriate treatment.

Before this can take place, there is a need for further studies comparing proliferative activity assessed by various means with other factors known to influence survival in non-Hodgkin's lymphoma such as stage, age, and symptoms.[34-37] There is evidence that labelling index[9] and presence of DNA aneuploidy[24] are not related to stage and symptoms but these associations remain to be fully characterised. Prospective studies are necessary to elucidate further the clinical importance of estimates of proliferative activity in the diagnosis and treatment of lymphoma.

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