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SIGNIFICANCE OF CELL PROLIFERATION INDEX IN ASSESSING HISTOLOGICAL PROGNOSTIC CATEGORIES IN HODGKIN'S DISEASE

An immunohistochemical study with Ki67 and MIB-1 monoclonal antibodies

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ABSTRACT

Background and Objective. In their review of the Rye histopathological classification of Hodgkin's disease, Bennett and coworkers have proposed that the nodular sclerosis (NS) type should be divided into two diagnostic categories on the basis of their clinical behaviour. In order to evaluate whether the proliferative activity of HD cells might correlate with histology in NS subtypes, we reviewed and re-evaluated cryostat and paraffin-embedded sections from 80 cases sent to our centre from 1986 to 1991.

Methods. In the present study, we investigated the growth cell fraction of 53 cases of Hodgkin's disease with nodular sclerosis by using Ki67 and MIB1 monoclonal antibodies to determine whether proliferative activity is associated with different pathological subtypes and prognostic categories. Eight cases with an interfollicular pattern and 19 with mixed cellularity were also investigated. The results in each group were compared to the others.

Results. The values of Ki67 and MIB1 were highly correlated (r = 0.88). In Hodgkin's disease with nodular sclerosis, two groups with significantly different growth fractions were morphologically identified: one with lymphocyte predominance and mixed cellularity subtypes, another composed of cases with variously extensive lymphocyte depletion. The figures were compared with those of interfollicular subtype, which fell into the first group, and of mixed cellularity type, in which the proliferative cell activity was significantly higher than in the second nodular sclerosis group. In all cases, Reed-Sternberg and Hodgkin cells accounted for the majority of the cell growth fraction, although a variable percentage of T-lymphocytes were also Ki67- or MIB1-positive. Taking the median value (15%) of MIB1 positive cells as a cut-off, a significant correlation (p= 0.05) was observed between MIB1 positivity and bulky disease, and a good trend (but not a significant relationship) between MIB1 and overall survival, disease-free survival, staging and the clinical response to therapy.

Interpretation and Conclusions. Assessment of the growth cell fraction in Hodgkin's disease with different nodular sclerosis patterns provides biological support for the morphological reclassification of their degree of malignancy into two main groups with different impacts on the clinical parameters and a possible relation with the outcome of treatment.

Key words: Hodgkin's disease, growth fraction, immunohistochemistry, Ki67, MIB1

The clinical and therapeutical approach to Hodgkin's disease (HD) has undergone marked changes in the last twenty years, mainly due to improved diagnostic radiological procedures and first-choice or salvage chemo-radiotherapeutical protocols. By contrast, the influence of the conventional Rye histological classification has progressively declined in prognosis, particularly in view of the heterogeneity of the nodular sclerosis (NS) type. Several attempts have thus been made to further divide this histological type into prognostically distinct groups. Reviewing a large series of HD patients randomized into the clinical trials of *British National Lymphoma Investigation* (BNLI), Bennett *et al.* suggested that subdivision of NS into grade I (encompassing lymphocyte predominance and mixed cellularity patterns) and grade II (mixed cellularity with lymphocyte depletion and lymphocyte depletion patterns) is of critical predictive value.[1]

Since the rate at which a tumor proliferates has long been considered to bear a relationship to its clinical course, assessment of the growth fraction by cell-cycle-related antigens could contribute to

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explore the biological characters of the NS subsets proposed. However, few reports have dealt with the proliferative activity of HD and its correlation with histology. Hodgkin and Reed-Sternberg cells (HRSCs) have been identified as the proliferating elements,[2-5] but a relationship between the growth fraction and the clinical and pathological parameters, has not been established. Furthermore, most of these studies have been carried out on fixed tissue using monoclonal antibodies against proliferating cell nuclear antigen (PCNA), which gives a high background[6] and is possibly associated with DNA repair other than proliferation.[7] Thus we reviewed and re-evaluated cryostat and paraffin-embedded sections stained with Ki67 and MIB1 monoclonal antibodies, respectively, from 80 HD cases sent to our centre from 1986 to 1991, to evaluate whether the growth fraction in NS subtypes, recognized according to Bennett *et al.*'s modification of the Rye scheme[1] correlated with histology and clinical parameters.

Materials and Methods

Patients

Eighty cases of HD selected from the files of the Department of Pathology, University of Turin, were included in this study. All the original slides were reviewed by one of us (GP). NS was found in 53 cases, mixed cellularity (MC) in 19 cases; 8 cases fell into the interfollicular (IF) subtype, which is considered as a variety of NS.[8] The NS cases were divided according to Bennett et al's modification of Rye classification[1] into the following subtypes: 7 NS with lymphocyte predominance (NS-LP), 19 NS with mixed cellularity (NS-MC), 7 NS-MC with lymphocyte depletion (NS-MC-LD), and 20 NS with lymphocyte depletion (NS-LD) (Figure 1).

Immunohistochemistry

Cryostat sections from liquid-nitrogen-frozen slices stored at –80°C were cut and processed with an ABC immunohistochemical method,[9] using Ki67 MoAb diluted 1/40 (Dakopatts, Denmark) and tetrachloride diaminobenzidine (Sigma Chemical) as chromogen. In 20 cases, double staining with Ki67/CD3 Moab and Ki67/CD20 Moab was carried out (CD3: Leu 4, CD20: Leu 16, Becton Dickinson, Mountain View, CA, USA) using the same ABC procedure and immunoalkaline phosphatase with fuchsin as chromogen for CD3 and CD20 staining.[10] Serial sections were cut and stained with hematoxylin-eosin to distinguish HRSCs from the other populations.

Paraffin sections from all cases were also microwave-treated as previously reported,[11] stained with MIB1 MoAb using the ABC procedure and compared with the corresponding Ki67 stained sections.

Assessment of immunohistochemical positivity

Stained cells were evaluated under a x40 lens. Two different counts were carried out: in the former, HRSCs were counted separately from the other positive cells (mostly lymphocytes), in the second one HRSC and the other positive cells were considered together. Nuclei from at least 200 HRSCs in the first count, and at least 1,000 cells in the second one were counted on each slide, and the percentage of Ki67 or MIB1 positive cells was evaluated. Because of differences in cell crowding, the positive cells were previously evaluated at low power in separate, randomly chosen, fields. Forty cases were counted by two observers, and forty cases by the same observer on three separate occasions: the inter- and intra-observer variation was below 10%. The percentages of the double-stained cases were evaluated with the same criteria.

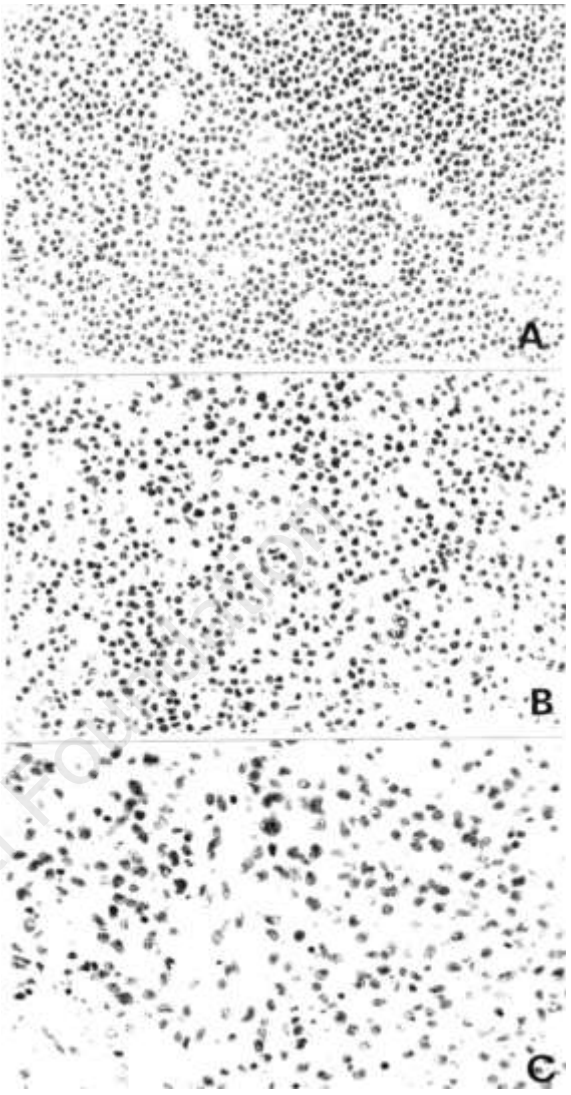

Figure 1. Examples of lymphoid and HRSC densities in the nodules of three varieties of HD with NS. (A) Lymphocyte predominance (270×); (B) Mixed cellularity (270×); (C) Lymphocyte depletion (350×).

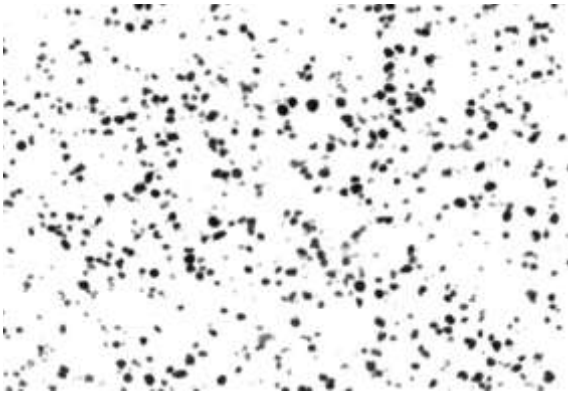

Figure 2. LD Hodgkin's disease: numerous HRSCs and small lymphoid cells are strongly MIB1- positive. ABC procedure on paraffin section (375×).

Table 1. Mean percentages of MIB1 overall positive cells (both HRSCs and lymphocytes) in 80 cases of HD.

Histological type	*No. of cases*	*MIB1 %*
NS-LP	7	17.4±5.3
NS-MC	19	15.9±7.7
NS-MC-LD	7	26.6±7.7
NS-LD	20	28.8±6.7
MC	19	36.7±9.1
IF	8	16.0±8.3
Total	80	

Legend: NS-LP: nodular sclerosis with lymphocyte predominance; NS-MC: nodular sclerosis with mixed cellularity; NS-CM-LD: nodular sclerosis with mixed cellularity and fields of lymphocyte depletion. NS-LD: nodular sclerosis with lymphocyte depletion; MC: mixed cellularity; IF: interfollicular.

Mitotic index

Mitosis were counted in ten high power fields for each case. The mean value of each NS subgroup was calculated.

Statistical analysis

The mean percentages of cycling cells were compared by using ANOVA (BMPD Statistical Software, Los Angeles, CA, USA). They were correlated with the following clinical and pathological parameters: overall survival, disease-free survival, clinical stage, presence of bulkiness, response to therapy (response not determined, partial response, complete response, no response). The mitotic index of the NS I and II subgroup was statistically compared with the t test of Student.

Results

Ki67 immunostaining in Hodgkin's disease

The staining pattern of Ki67 and MIB1 antibodies consisted of intranuclear brown granules corresponding to the chromatin distribution (Figure 2). The two markers showed high correlation when the values obtained on the same cases were compared (r= 0.88). In the interfollicular variety, obvious residual germinal centers showed a high number of Ki67-positive cells. These did not interfere with the cell count. Table 1 shows the mean percentages of overall stained cells. Because of the high correlation between Ki67 and MIB1 immunostaining, our results refer to MIB1 expression on paraffin sections, which allows a better cytological identification. The percentage of MIB1-positive HRSCs (Table 2) ranged from 75.1 to 81.2%, while that of MIB1-positive lymphocytes ranged from 9.6 to 20.5% (interobserver difference 5-8%). These values did not correlate with the six histological types.

A significant difference in the percentage of the whole growth fraction (both HRSCs and lymphocytes) was observed between cases encompassed in the NS grade I group [NS-LP (17.42%) and NS-MC (15.9%)], and those of the NS grade II group [NS-CM-LD (26.6%) and NS-LD (28.8%)] (Table 3). The overall percentage of NS I cases (16.6%) was significantly different from that of NS II cases (27.7%) (p< 0.001). No significant difference was found between the two subtypes of NS I group nor between those of NS II group taken separately. Comparison with other types showed that the percentage of overall proliferating cells in both NS groups was significantly lower than that of the MC (36.7%), whereas that of the IF type (16%) was similar to that of the first NS group.

Table 2. Mean percentages of MIB1 positive HRSCs and lymphocytes in 80 cases of HD

Histological type	*No. of cases*	*HRSCs*	*Lymphocytes*
NS-LP	7	76.9±12.3	19.0±10.1
NS-MC	19	75.1±10.0	13.6±9.2
NS-MC-LD	7	77.8±14.7	13.4±6.8
NS-LD	20	79.5±12.6	20.5±9.6
MC	19	81.2±15.5	15.1±4.7
IF	8	77.5±10.5	9.6±6.0
Total	80		

Legend: NS-LP: nodular sclerosis with lymphocyte predominance; NS-MC: nodular sclerosis with mixed cellularity; NS-CM-LD: nodular sclerosis with mixed cellularity and fields of lymphocyte depletion. NS-LD: nodular sclerosis with lymphocyte depletion; MC: mixed cellularity; IF: interfollicular.

Table 3. Statistical comparison of the mean percentages of overall Ki67 positive cells in histological varieties of HD.

NS-LP (I)	vs	NS-MC-LD (II)	p=0.02
NS-LP (I)	vs	NS-LD (II)	p<0.001
NS-MC (I)	vs	NS-MC-LD (II)	p<0.001
NS-MC (I)	vs	NS-LD (II)	p<0.001
NS-MC (I)	vs	MC	p<0.0001
IF	vs	MC	p<0.0001
NS-MC-LD (II)	vs	MC	p=0.01
NS-MC-LD (II)	vs	IF	p=0.02
NS-LD (II)	vs	IF	p<0.001
NS-LD (II)	vs	MC	p=0.003

Legend: NS-LP: nodular sclerosis with lymphocyte predominance; NS-MC: nodular sclerosis with mixed cellularity; NS-CM-LD: nodular sclerosis with mixed cellularity and fields of lymphocyte depletion. NS-LD: nodular sclerosis with lymphocyte depletion; MC: mixed cellularity; IF: interfollicular; I: NS grade I; II: NS grade II; ns: not significant.

Double immunostaining of lymphocytes

Double immunostaining showed that most of the MIB1-positive lymphocytes were $CD3^+$, and CD20- and unevenly distributed in all 20 cases.

Mitotic index

The mean value of the NS I subgroup was 11.6±6.5, whereas that of the NS II subgroup was 17.1±9.8. They were not statistically correlated (p=0.09).

Table 4. Relationship between Ki67 immunostaining and clinical parameters.*

Bulky	p= 0.05
Overall survival	p= 0.14
Disease-free survival	p= 0.12
Staging	p= 0.16
Response to therapy	p= 0.20

** The median value of Ki67 positive cells (15%) was used as the cut-off.*

Correlation with clinical parameters (Table 4)

Taking the median MIB1-positive value as the cut-off (15%), a significant correlation was found between the percentage of stained cells and the presence of bulky disease ($p<0.05$). No significant correlation was found with overall survival ($p=0.14$), disease-free survival ($p= 0.12$), staging ($p= 0.16$) and response to therapy ($p= 0.09$).

Discussion

The main purpose of this retrospective study was to see whether the growth fraction in HD with NS correlated with its clinical and pathological reclassification proposed by Bennett *et al*.[1] We also included the IF subtype, which has been considered a variety of the NS group.[8] In Bennett *et al*.'s scheme, the NS subtypes I and II were related to actuarial survival. Ferry *et al*.[12] have recently emphasized the critical role of histologic parameters in predicting overall and disease-free survival in NSHD. We applied two monoclonal antibodies (Ki67 and MIB1), respectively on frozen sections and paraffin sections. However, because of the high correlation between the two markers ($r = 0.88$), we performed the statistical analysis using the MIB1 values in consideration of the better cytological identification on paraffin sections.

Our results indicated that the growth fraction of NS subtypes, as expressed by Ki67- and MIB-1 positivity, was closely related to Bennett *et al*'s two prognostic groups.[1] The overall growth fraction, including HRSCs and small lymphocytes, of these two groups, was significantly different ($p < 0.001$). As expected, no significant differences between NS-LP and NS-MC nor between NS-MC-LD and NS-LD were found. The growth fraction of the IF-type was similar to that of the first group. The highest MIB1 ratio was displayed by the MC type (36.7%). This was significantly higher than that of the second group ($p < 0.0001$). These results strengthen the hypothesis that in HD, as in non-Hodgkin's lymphomas,[13] there is a relationship between the histologic grade of malignancy and the growth fraction, and provide biological support for the differences between the two prognostically distinct NS groups.

With regard to the cell distribution of MIB1 positivity, our evaluation indicates that HRSCs, irrespective of histological type, were always the greatest part of the growth fraction, indicating that the absolute number of these cells accounts for the differences between the growth fraction values among the HD subgroups. Few MIB1-positive lymphocytes were also found in nearly all samples and double staining showed these cells to belong mostly to the T lymphoid lineage, as expected considering the cell composition of the Hodgkin's tissue. Although it is at present unclear whether or not these MIB1-positive lymphocytes actually participate in proliferation and expansion of Hodgkin's tissue, this finding indicates that various numbers of lymphocytes, mostly of T lineage, entered the cell cycle along with HRSCs.

In our series the percentage of cycling T cells was extremely variable, ranging from 5% to 27%, regardless of the histological classification. Unfortunately these data are difficult to compare with those reported in the literature mainly because of the different technical approaches. In a previous study Schmid *et al*.[3] reported higher numbers of PCNA-positive T-cells both in *classic* HD and in nodular paragranuloma, whereas only a low percentage was reported by Hell *et al*. and Freeman *et al*.[4-5] Similarly, some investigators found a higher number of cycling HRSCs by using PCNA instead of MIB1. These discrepancies may be at least partially explained by the association of MIB1 and PCNA with different phases of the cell cycle,[14] leading to a different selection of reactive cells.

Despite the high number of cycling HRSCs demonstrated in this study and in others by cell cycle-related antigens, the mitotic index calculated in our series was very low. This may be explained considering that simple count of the mitotic figures only reflects the M phase of the cell cycle, whereas MIB1 is an indicator of cell cycling but it does not provide information about cell cycle length nor about which phase of the cycle neoplastic cells are *frozen* at.[15] Therefore, it could be argued that in HD nearly all HRSCs are in cycle, but since they either spend a long time completing it or do not reach the mitotic phase, true proliferation rate would not be so high as indicated by the MIB1 cell reactivity. Moreover, the neoplastic cells of HD exhibit a high percentage of abortive mitoses and DNA fragmentation that may explain this discrepancy with the growth fraction results.[16]

Recently, several biological factors involving HD and possibly dealing with characteristic differences in its pathological and clinical pattern, and affecting the growth fraction, have been reported. Among these, the presence of Epstein-Barr virus and other herpesviruses[17-23] in a high percentage of HD specimens, could be regarded as a putative candidate for HRSCs and lymphocytes activation. The similar cell distribution of EBV and cell-cycle related markers in HD samples and the more exten-

sive expression of EBV-LMP in the more aggressive histological types of HD,[24] would support a possible interrelationship among cell growth fraction, viral infection and histological aggressiveness of HD. Additional factors include production of many cytokines by Hodgkin's tissue and its related cell lines,[25,26] expression of IL-2 receptor (CD25) by T-cells,[27] and effects of p53 and bcl-2 proteins on apoptosis.[28]

The lack of association of cell proliferative activity with survival in our series could be related to the improvement of therapy over the last 20 years. However, a significant correlation (p=0.05) was observed between MIB1-positivity and bulky disease, i.e. a clinical parameter determined before therapy and indicative of biological aggressiveness.

A good trend but not a significant correlation was found between MIB1-positivity and the clinical response to chemo- and radiotherapy, which mainly affect the cell cycle. This result is in keeping with that reported by Erdkamp *et al.*[29] using DNA flow cytometry. They showed that while aneuploidy was not related to complete remission rate, relapse-free and overall survival, a better relationship with these parameters was obtained when analyzing the S-phase fraction, although this did not prove to be an independent prognostic parameter.

As a whole our results indicate that: 1) growth fraction, as defined by Ki67 or MIB1 immunostaining, is a biological parameter of histological severity, since it is associated with the prognostic groups defined by Bennett *et al.* in their revision of the Rye classification; 2) anomalous T cells are part of the growth cell fraction; 3) although the comparison of the growth fraction with histological criteria alone does not indicate a clear relationship with some clinical parameters, the growth fraction may be taken as an indicator of expansion of the neoplastic and neoplastic-related cells, since it correlates with bulky disease.

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