

## RAPID COMMUNICATION

# Prognostic Significance of the Ki-67–Associated Proliferative Antigen in Aggressive Non-Hodgkin's Lymphomas: A Prospective Southwest Oncology Group Trial

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The growth fraction of tumors from patients with non-Hodgkin's lymphomas (NHL) has been shown to correlate with survival in retrospective studies. The growth fraction can be evaluated using immunohistochemical techniques employing the Ki-67 monoclonal antibody (MoAb) that marks a nuclear protein present in cycling cells. The purpose of this study was to evaluate the clinical utility of the Ki-67 MoAb for predicting survival. Using a prospective trial design in a multi-institutional cooperative trials group, the proliferative index, clinical outcome, and statistical correlations were independently assessed for previously untreated patients with advanced stages of intermediate- and high-grade histologies of NHL treated on Southwest Oncology Group study (SWOG 8516, Intergroup 0067). The proportion of Ki-67–positive cells was determined on

snap-frozen thin tissue sections. A proliferative index of 80% or greater, as determined from prior retrospective studies, identified a group of patients (18%) who had a poor outcome. Overall survival was significantly reduced in these patients with a high Ki-67–associated proliferative index compared with those with a low proliferative index ( $P = .001$ ). One-year survival estimates were 82% (low proliferative index) versus 18% (high proliferative index). A multivariate regression analysis incorporating commonly used clinical prognostic features confirmed the independent effect of proliferation on survival (relative risk estimate 5.9; 95% confidence interval, 2.2, 16.1). The Ki-67 MoAb identifies a group of patients with rapidly fatal NHL for whom currently available chemotherapy is inadequate. © 1994 by The American Society of Hematology.

OVER THE PAST 15 YEARS, attempts to improve therapy for patients with intermediate and high-grade non-Hodgkin's lymphomas (NHL) have been based on clinical observation and empiric changes in chemotherapy regimens. Unfortunately, these changes have not resulted in measurable improvement of outcome as gauged by randomized comparisons of new treatment regimens with standard CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone).<sup>1</sup> Although several clinical features of pa-

tients with NHL, including age, sex, stage, performance status, and symptom status, reproducibly predict relative outcome for patient subgroups, these clinically derived prognostic factors do not identify a subgroup of patients for whom currently available therapy is unacceptably inadequate.<sup>2-5</sup> Furthermore, these clinical characteristics do not provide insight into the biologic mechanisms of treatment failure and do not suggest changes in future treatment strategy to improve outcome. Consequently, we began a search for biologic variables that correlate with prognosis and provide testable hypotheses for future treatment-related studies. In the current study, we test the effect of the tumor proliferative rate on prognosis.

The growth fraction of NHL has previously been studied with a variety of techniques including assessment of DNA content using flow cytometry and uptake of thymidine using radioactive tritiated labeling.<sup>6,7</sup> Those methods have shown that high proliferative rates in lymphoma biopsies correlate with poor prognosis. More recently, immunohistochemical methods using the monoclonal antibody (MoAb) Ki-67 have been used with success in pilot studies.<sup>8-11</sup> Ki-67 detects a nuclear antigen associated with proliferation.<sup>12,13</sup> The proportion of malignant cells marked by Ki-67 has been previously shown to correlate with histologic grade and outcome.<sup>8-11</sup> However, these findings have been based on retrospective analyses of clinical outcome using patients with considerable heterogeneity with regards to histology, treatment, and clinical prognostic features.

We have previously reported that the growth fraction determined by the Ki-67 MoAb correlated with prognosis in a retrospective study of 105 consecutive patients with diffuse large-cell NHL (DLCL).<sup>8</sup> In that study, a Ki-67 value of 60% (proportion of Ki-67–positive cells to total number of malignant cells) was found to be the cutpoint dividing patients into prognostic groups with statistically different survival. Potential problems with that study included the retrospec-

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tive analysis, cutpoints established after multiple analyses, and heterogeneity of clinical features within the patient group. In the current study, we report that Ki-67 determination is a useful tool to identify patients having an extremely poor prognosis. The study incorporates a prospective design with predetermined cutpoints for testing Ki-67 and uses a patient population with advanced disease treated with doxorubicin-containing chemotherapy.

## MATERIALS AND METHODS

Patients studied for the effect of proliferation on outcome are a subset of eligible patients entered on Southwest Oncology Group (SWOG) 8516 (INT 0067), a therapeutic intergroup trial for NHL.<sup>1</sup> SWOG 8516 compared the efficacy and toxicity of four doxorubicin-containing chemotherapy regimens among previously untreated patients with advanced stages of intermediate- and high-grade NHL. Participating SWOG institutions were encouraged to register patients on this parallel laboratory-based study by submitting snap-frozen viable tumor specimens to the SWOG Lymphoma Repository in Tucson, AZ, where tissue section immunohistochemistry was performed. Results of immunohistochemistry were collected without knowledge of clinical outcome.

**Patient selection and treatment.** Between April 4, 1986 and June 15, 1991, 899 eligible patients were randomized to receive CHOP, m-BACOD (methotrexate with leucovorin rescue, bleomycin, doxorubicin, cyclophosphamide, vincristine, and dexamethasone), ProMACE-CytaBOM (prednisone, doxorubicin, cyclophosphamide, and etoposide followed by cytarabine, bleomycin, vincristine, and methotrexate with leucovorin rescue), or MACOP-B (methotrexate with leucovorin rescue, doxorubicin, cyclophosphamide, vincristine, prednisone, and bleomycin).<sup>1</sup> Eligibility criteria for SWOG 8516 have been recently described in detail. In brief, patients with biopsy proven, measurable intermediate- and high-grade histologies (Working Formulation categories D through H and J) with advanced stages of disease (bulky stage II, stage III, and stage IV) were eligible.<sup>14</sup> The histopathologic diagnoses were based on consensus review by expert hematopathologists (P.M.B., K.F., C.R.K., B.N.N., B.S., R.R.T.). Exclusion criteria included prior treatment, acquired immunodeficiency syndrome (AIDS)-associated NHL, a previous history of low-grade NHL, prior malignancy, central nervous system (CNS) lymphoma, heart disease, and impaired creatinine clearance. Patients from SWOG institutions meeting these criteria were eligible for simultaneous registration on this laboratory-based study.

**Tissue acquisition.** A portion of the initial biopsy specimen (>5 mm<sup>3</sup>) was snap-frozen within 15 minutes of biopsy in OCT compound (Miles Laboratories, Elkhart, IN) at -150°C in isopentane quenched in liquid nitrogen. Frozen OCT blocks were wrapped in aluminum foil, placed in an airtight ziplock plastic bag, and mailed in a sealed heavy-grade styrofoam container filled with dry ice and packed inside a cardboard box. The specimens were mailed via overnight express on Mondays through Thursdays. A standard paraffin-embedded histologic section stained with hematoxylin and eosin accompanied each specimen. Eligibility for this study required submission of an adequate specimen determined at the time of arrival at the Central Repository and registration on SWOG 8516.

**Immunohistochemistry.** We used a previously described immunohistochemical method employing a primary mouse antihuman MoAb (see below), a biotin-conjugated secondary antibody, avidin-horseradish peroxidase, and the chromagen diaminobenzidine tetrahydrochloride (DAB) as a detection agent on snap-frozen sections.<sup>8</sup> Specifically, we used the MoAb Ki-67 as the primary antibody (mouse antihuman, 1/40 dilution; Dakopatts, Copenhagen, Denmark) to detect nuclear proliferation antigen. The secondary

antibody was a biotin-conjugated F(ab<sub>2</sub>) (goat antimouse IgG, 1/200 dilution; Caltag, San Francisco, CA). Negative controls substituted isotype-matched irrelevant antibodies with 2% bovine serum albumin (BSA, Gamma Biologicals, Houston, TX) in phosphate-buffered saline (PBS).

Ki-67 was quantified by determining the number of positive cells expressing nuclear Ki-67 (brown coloration cells) among the total number of cells (blue coloration cells counterstained with methylene green) within high power (40 × objective) microscopic fields. Field selection sought areas of highest Ki-67 expression evident by lower power scanning. Typically, the total cell count (denominator) exceeded 400 cells. The cell counts were performed without knowledge of clinical outcome. Our previously published methods have been refined for this study after critical review of our initial report by including all cells with any degree of brown nuclear coloration as a positive result (including cells with stain limited to the nucleoli).<sup>8</sup>

**Statistical analyses.** Patient characteristics were compared using  $\chi^2$  tests. Survival was measured from the date of registration to death from any cause or to the date of last contact. Estimates of survival were made using the method of Kaplan and Meier.<sup>15</sup> Deaths from all causes are included in estimates of survival. Overall survival for the study groups was compared using log-rank tests.<sup>16</sup> The Cox regression model was used to estimate relative risk.<sup>17</sup> All significance tests were two-sided. Cutpoints for Ki-67 positivity used to compare outcome were determined before analysis based on our initial report and subsequent modification of counting technique (by counting all cells with any amount of brown stain, the proportion of proliferating cells increased).

## RESULTS

Between April 1986 and June 1991, 899 eligible patients were registered on SWOG 8516 to test the efficacy of four standard drug regimens. Patients from participating SWOG institutions were also eligible for this parallel prospective study to test the importance of the tumor proliferative index on outcome. Sixty patients were studied using the Ki-67 MoAb. The proportion of Ki-67-positive cells to tumor cells (proliferative index) varied from 4% to 95% (median, 50%). The distribution of patients with observed proliferative rates is shown on the histogram in Fig 1.

Clinical features of the study group (60 patients) and the remaining 839 eligible patients from the treatment protocol are compared in Table 1. There are no significant differences in the proportion of patients with known adverse prognostic factors, including older age, male sex, advanced stage, poor performance status, systemic symptoms, greater than one extranodal site of disease, high serum lactic dehydrogenase (LDH), or involved bone marrow. There were no differences by histologic subtype or randomized treatment arm received. Patients were also categorized by risk according to the new International Prognostic Index as recently proposed by Shipp et al.<sup>18</sup> There were no significant differences in prognostic risk distribution between patients having Ki-67 data available and all other eligible patients entered on SWOG 8516 ( $P = .58$ , Table 2). Furthermore, overall survival of the Ki-67 study group was similar to that of the remaining patients ( $P = .32$ , Fig 2). The 3-year survival estimate for patients studied with the Ki-67 was 49% and for the remaining patients was 52%.

Survival for the study group was determined after quantitating Ki-67 expression (percentage of Ki-67-positive

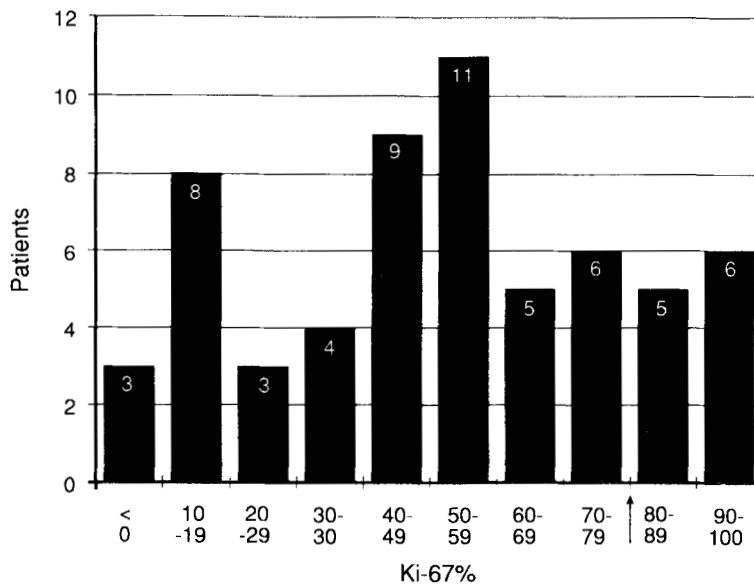


Fig 1. The proliferative index using Ki-67 ranged from 4% to 95%. The distribution of patients with observed proliferative indices is shown on the histogram (60 patients).

cells). Survival for patients having a Ki-67  $\geq 80\%$  (11 patients) was compared with survival for patients having a Ki-67 less than 80% (49 patients). Overall survival was significantly different (Fig 3,  $P = .0004$ ). Only 18% of patients having a Ki-67  $\geq 80\%$  survived 1 year; whereas, 82% of patients having a Ki-67 less than 80% survived 1 year. The 3-year estimates of survival were 18% for patients with a Ki-67  $\geq 80\%$  and 56% for patients with a Ki-67 less than 80%. Nine deaths occurred among 11 patients having a Ki-67  $\geq 80\%$ , and all deaths occurred within 1 year of registration.

Table 1. The Clinical Features of 60 Patients Studied With the Ki-67 MoAb Are Compared With All Other Eligible Patients Entered on SWOG 8516 (INT-0067)

Clinical Features	Ki-67 Data Available, No. (%) (n = 60)	Ki-67 Data Not Available, No. (%) (n = 839)	All Eligible SWOG 8516, No. (%) (n = 899)	Significant Difference
Age (>60 yr)	19 (32)	341 (41)	360 (40)	$P = .22$
Sex (male)	38 (63)	502 (60)	540 (60)	$P = .59$
Stage (III, IV)	49 (82)	708 (84)	757 (84)	$P = .58$
Performance status (not ambulatory)	4 (7)	48 (6)	52 (6)	$P = .76$
B symptoms	28 (47)	364 (43)	392 (44)	$P = .62$
LDH (>1 $\times$ normal)	36 (60)	470 (56)	506 (56)	$P = .55$
Extranodal sites (>1)	25 (42)	291 (35)	316 (35)	$P = .33$
Marrow involved	17 (28)	221 (26)	238 (26)	$P = .74$
Diffuse large cell type	41 (68)	560 (67)	601 (67)	$P = .80$
Treatment				
CHOP	13 (22)	212 (25)	225 (25)	
m-BACOD	13 (22)	210 (25)	223 (25)	$P = .27$
ProMACE-CytaBOM	22 (37)	211 (25)	233 (26)	
MACOP-B	12 (20)	206 (25)	218 (24)	

The clinical characteristics of patients having a Ki-67  $\geq 80\%$  were compared with the clinical features of patients having a Ki-67 less than 80% (Table 3). There were no significant differences between patient groups comparing the proportion of patients with older age, male sex, advanced stage, poor performance status, more than one extranodal site, high LDH, and bone marrow involvement. There was a nonsignificant trend for patients having a Ki-67  $\geq 80\%$  to present with systemic (B) symptoms of disease (74%) compared with patients having a Ki-67 less than 80% (41%,  $P = .09$ ). The newly proposed International Prognostic Index was also used to categorize patients into risk groups based on Ki-67 percentage (Table 4). There was no significant difference in distribution after comparing patients with low risk plus low-intermediate risk to patients with high-intermediate risk plus high risk groups according to Ki-67 percentage ( $P = .32$ ). It is noteworthy that 5 of the 11 patients having a Ki-67  $\geq 80\%$  were classified in the two lowest International Index risk groups. Of these 5 patients, 4 died within 1 year of study registration. Of 49 patients having a

Table 2. The Distribution of Patients in Risk Groups for 60 Patients Studied With Ki-67 MoAb Is Compared to All Other Eligible Patients Entered on SWOG 8516 (INT-0067) Using the Newly Proposed International Prognostic Index

International Index Risk Group	Ki-67 Data Available, No. (%) (n = 60)	Ki-67 Data Not Available, No. (%) (n = 839)	All Eligible SWOG 8516, No. (%) (n = 899)
Low	10 (17)	186 (22)	196 (22)
Low-intermediate	26 (43)	298 (36)	324 (36)
High-intermediate	18 (30)	253 (30)	271 (30)
High	6 (10)	102 (12)	108 (12)

There is no significant difference in risk group distribution between patients studied with Ki-67 and all other eligible patients ( $P = .58$ ).

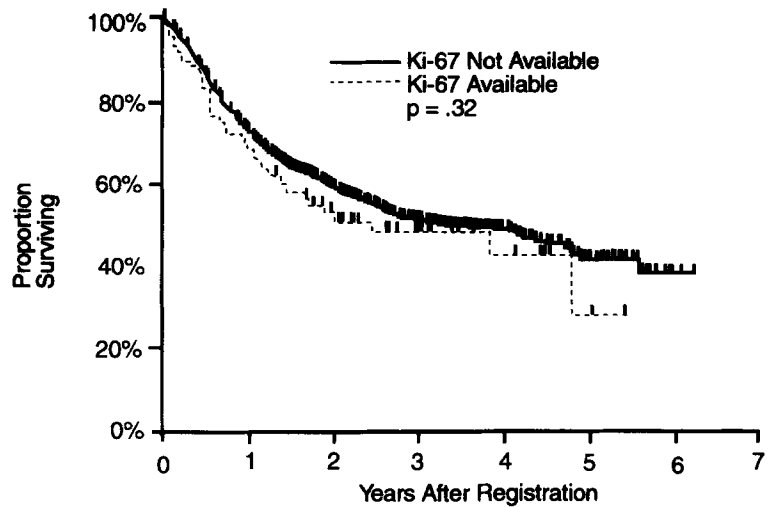


Fig 2. Outcome based on survival is compared for 60 patients in the study group (Ki-67 data available) and the remaining 839 patients on SWOG 8516. There is no difference in survival between the groups of patients ( $P = .32$ ).

Ki-67 less than 80%, 18 were included in the two highest International Index risk groups. Of these 18 patients, 12 have died, but only 4 deaths occurred within 1 year of study registration.

Further analysis of outcome was performed on 41 patients with DLCL (category G and H, Working Formulation), demonstrating a significant difference in overall survival based on Ki-67 ( $P = .02$ , Fig 4). For patients with Ki-67  $\geq 80\%$  (7 patients) the 1-year and 3-year survival estimates were 29%; whereas, the 1-year and 3-year estimates for patients with Ki-67 less than 80% (34 patients) were 85% and 59%, respectively.

A Cox regression model was used to assess survival adjusting for other known prognostic variables as used in the International Prognostic Index and as summarized in Table 5. Patients were stratified by randomized treatment arm. Covariates included in the model were pretreatment performance status (ambulatory  $v$  not ambulatory), age ( $<60 v \geq 60$ ), extranodal sites of disease ( $\leq 1$  site  $v > 1$  site), Ann

Arbor stage (I, II  $v$  III, IV), and serum LDH (normal  $v > 1 \times$  normal). The estimated relative risk of death for patients with Ki-67  $\geq 80\%$  was 5.9 (95% confidence interval [CI], 2.2, 16.1) compared with patients with Ki-67 less than 80%. The increased relative risk was larger than that seen for all other variables included in the model. Ki-67 was statistically significant in the multivariate survival model ( $P = .0005$ ).

SWOG 8516 compared regimens of variable planned dose intensity and schedules. Inasmuch as the third generation regimens were, in part, designed to improve efficacy against high proliferative tumors, outcome based on treatment regimen is of interest. Among 11 patients with Ki-67  $\geq 80\%$ , there have been 9 deaths. The 2 surviving patients were treated with m-BACOD (1 patient) and ProMACE-CytaBOM (1 patient).

DISCUSSION

The current study tests the utility of proliferative rate as measured by the Ki-67 MoAb to identify a group of patients

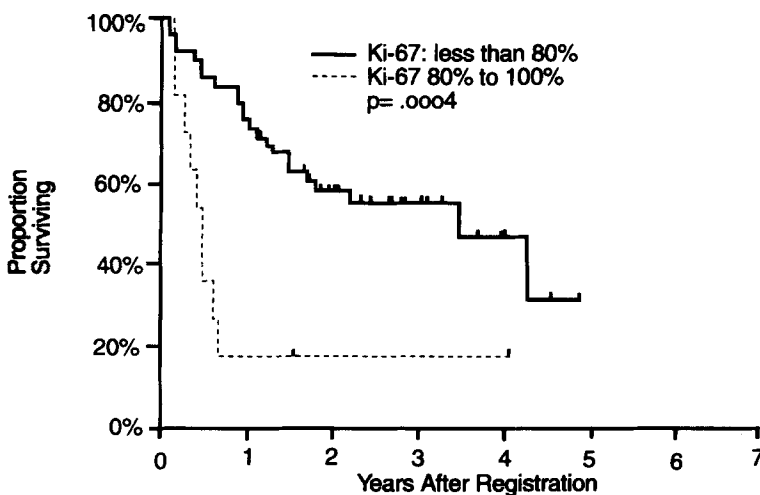


Fig 3. Outcome based on survival is compared for 11 patients having a proliferative index  $\geq 80\%$  to 49 patients having a proliferative index less than 80%. There is a significant difference in overall survival ( $P = .0004$ ).

**Table 3. The Clinical Features of 49 Patients Having a Ki-67 Less Than 80% Are Compared With 11 Patients Having a Ki-67  $\geq$ 80%**

Clinical Feature	Ki-67 <80%, No. (%) (n = 49)	Ki-67 $\geq$ 80%, No. (%) (n = 11)	Significant Difference
Age ( $\geq$ 60 yr)	16 (33)	3 (27)	$P = .90$
Sex (male)	31 (63)	7 (64)	$P = .98$
Stage (III, IV)	39 (80)	10 (91)	$P = .67$
Performance status (not ambulatory)	3 (6)	1 (9)	$P = .57$
B symptoms	20 (41)	8 (73)	$P = .09$
LDH ( $> 1 \times$ normal)	29 (59)	7 (64)	$P = .90$
Extranodal sites ( $> 1$ )	19 (39)	6 (54)	$P = .50$
Marrow involved	12 (24)	5 (45)	$P = .26$
Diffuse large cell type	34 (69)	7 (64)	$P = .73$
Treatment			
CHOP	10 (21)	3 (27)	
m-BACOD	11 (22)	2 (18)	$P = .81$
ProMACE- CytaBOM	19 (39)	3 (27)	
MACOP-B	9 (18)	3 (27)	

for whom currently available chemotherapy is clearly inadequate. A proliferative index of 80% or greater was shown to be an important pretreatment predictor of poor survival. Of 11 patients having a Ki-67 greater or equal to 80%, only 2 survived 1 year. In an effort to determine whether other clinical prognostic variables confounded our findings, a multivariate regression analysis was performed, confirming that the proliferative index was a significant determinate of survival and independent of well-established clinical markers of prognosis (relative risk estimate, 5.9; 95% CI, 2.2, 16.1). Further evidence of the utility of the Ki-67-determined proliferative index is the finding that 5 of 11 patients (45%) with high values were identified among the two lowest risk groups as defined by the new International Prognostic Index.<sup>18</sup>

Growth fraction, as determined by flow cytometric (S phase) DNA assessment or by gamma counter radioactive tritiated thymidine incorporation, is an established predictor of lymphoma outcome.<sup>6,7</sup> These methods have shown a high correlation between proliferation rates and histologic grade of lymphoma. Although generally very useful, these methods have limitations. In particular, flow cytometry uses a suspension of cells, which results in loss of microanatomic detail. Although radiolabeling may provide microanatomic features, it is nonetheless arduous and time-consuming, and raises laboratory safety issues requiring special facilities. A simple method to speedily overcome these constraints is to use tissue sections for immunohistochemical assessment of proliferation antigens.<sup>8,10</sup> The SWOG Lymphoma Repository and others studies have sought to determine the reliability of the MoAb Ki-67 in lymphoma diagnosis and in predicting lymphoma outcome. This MoAb applied to frozen sections, has proven comparable to the tritiated thymidine labeling index.<sup>12</sup> It detects a nuclear antigen found throughout the cell cycle ( $G_1$ ,  $G_2$ , S, M) and not in resting cells ( $G_0$ ), allowing delineation of the growth fraction of lymphoma cells.<sup>13</sup>

**Table 4. The Distribution of Patients in Risk Groups Having a Ki-67 Less Than 80% Is Compared With Patients Having a Ki-67  $\geq$ 80% Using the International Prognostic Index**

International Index Risk Group	All Patients, No. (%) (n = 60)	Ki-67 <80%, No. (%) (n = 49)	Ki-67 $\geq$ 80%, No. (%) (n = 11)
Low	10 (17)	9 (18)	1 (9)
Low-intermediate	26 (43)	22 (45)	4 (36)
High-intermediate	18 (30)	14 (29)	4 (36)
High	6 (10)	4 (8)	2 (18)

Comparing low risk plus low-intermediate risk groups to high-intermediate risk plus high risk groups using Fisher's exact test ( $P = .32$ ).

Among intermediate- and high-grade lymphomas, poor survival correlates with a Ki-67 exceeding 60% in two studies of large-cell lymphoma.<sup>8,9</sup> In one of these studies, median survival was 8 months for patients with Ki-67 less than 60% versus 39 months for patients with Ki-67 greater than 60%.<sup>8</sup> This was the first study of Ki-67 within a single histologic category of NHL with statistical adjustment for clinical prognostic factors. In contrast, other studies have shown the opposite effect with poor outcome related to low Ki-67.<sup>10,11</sup> However, the latter studies did not account for, or balance all known prognostic or treatment factors.

The utility of Ki-67 immunostaining has also been tested using retrospective designs among patients with lower grades of NHL.<sup>10,19</sup> The Stanford group found that Ki-67 values of greater than 20% identified a more aggressive clinical course among patients with diffuse small lymphocytic lymphomas.<sup>19</sup> We have previously shown that, within the single histologic group of diffuse small cleaved cell NHL, patients with a Ki-67 proliferative rate greater than 20% had a median survival of 20 months compared with 80 months for those with a lower index ( $P = .002$ ).<sup>20</sup> Thus, the Ki-67 proliferative index appears to have utility within several histologic subgroups of NHL and may prove valuable in selecting patients for more aggressive therapy among the low-grade histologic subtypes of NHL.

The current study addresses several concerns common to many of the previously published reports of the prognostic significance of growth rates on outcome, regardless of methods used. First, the current trial used a prospective design testing a pre-established endpoint. We chose 80% based on

**Table 5. Cox Regression Model for Survival Stratified by Treatment Regimen for 60 Patients With Ki-67 Data Available Using Variables Identified by the International Prognostic Index**

Prognostic Variable	Relative Risk Estimate	95% CI	Significance ( $P$ Value)
Ki-67 $\geq$ 80%	5.9	2.2, 16.1	.0005
Performance status (not ambulatory)	3.1	0.8, 11.8	.09
Age $\geq$ 60 yr	2.6	1.0, 6.8	.04
Extranodal disease ( $> 1$ site)	2.3	1.0, 5.4	.06
Stage III or IV	1.3	0.4, 4.2	.71
Serum LDH ( $> 1 \times$ normal)	0.8	0.3, 2.0	.56

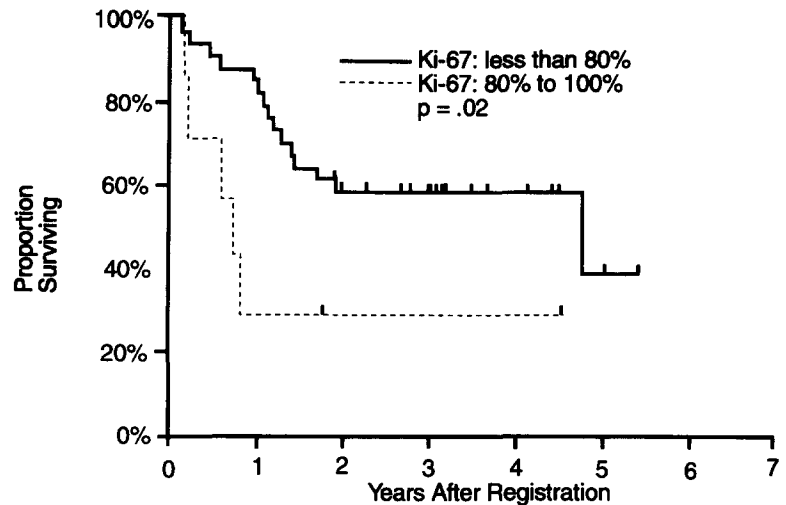


Fig 4. Outcome based on survival for patients with DLCL (categories G and H of Working Formulation) is compared for 7 patients having a proliferative index  $\geq 80\%$  to 34 patients having a proliferative index less than 80%. There is a significant difference in overall survival ( $P = .02$ ).

results of our previous retrospective study and after refinement of the methods used to quantitate Ki-67 positivity. We now include as positive all malignant cells with any degree of nuclear staining, including those cells with staining limited to the nucleolus. This change in technique increased the average proliferative index (median proliferative index in our prior study was 40% compared with 50% in the current study; thus, our cutpoint was adjusted). Second, only patients with snap-frozen tumor available for analysis were eligible for study eliminating loss of antigen through tissue fixation artifact. Third, all patients studied were simultaneously registered on a treatment-directed study using uniform staging and state-of-the-art doxorubicin-containing chemotherapy (the study group and the remaining patients appear to have similar clinical features and outcome, as summarized in Tables 1 and 2 and in Fig 2). Fourth, clinical outcome, proliferative rate, and statistical correlations were determined independently at separate sites. For these reasons, we believe the current trial is a definitive test of the prognostic value of the growth fraction on outcome.

In conclusion, the Ki-67-determined proliferative index identifies a group of patients (18%) with extremely poor outcome after treatment with currently available chemotherapy (18% 1-year survival). The utility of this test cuts across clinically defined risk groups, and is independent of established clinical prognostic factors. These findings provide some insight into the cause of treatment failure and lead to testable hypotheses for new treatment strategies. Within the SWOG, we plan to prospectively identify patients with very high proliferative rates and test the effect on outcome of high-dose intensity therapy with bone marrow rescue.

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