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# Assessment of Proliferative Activity in Ovarian Neoplasms by Flow and Static Cytometry

## Correlation with Prognostic Features

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***We undertook a prospective flow and static cytometric study of proliferative activity in 74 malignant, borderline and benign ovarian neoplasms. Proliferative activity as assessed by S phase fraction (SPF), and immunostaining of tissue sections with Ki-67 antibody was compared with prognostically important clinicopathologic features. Malignant neoplasms had higher median percentage Ki-67 staining (27.6%) than borderline (12.3%) and benign (2.7%) tumors ($P < 0.05$). Percentage Ki-67 staining correlated with SPF, DNA index, architectural grade, nucleolar grade, and mitotic count in malignant tumors; with nucleolar grade in benign tumors and with none of these variables in borderline tumors. Similarly, malignant neoplasms had a higher median SPF (11.5%) than borderline (3.4%) and benign (2.9%) neoplasms ($P < 0.05$). In malignant neoplasms, SPF correlated with percentage Ki-67 staining, DNA index, age, and stage, but with none of these in borderline or benign neoplasms. (Am J Pathol 1992, 141:699–706)***

Ovarian cancer is the fifth most common form of cancer in women. Because it usually becomes symptomatic at an advanced stage, this malignancy comprises a disproportionate number of cancer deaths, killing 12,000 women each year.[1] Factors shown to predict survival include FIGO (International Federation of Gynecology and Obstetrics) stage at diagnosis,[2–10] amount of residual tumor,[2,4,6–8,10] histologic subtype,[2,4,7] histologic grade,[3,4,6–8,10] and DNA ploidy.[5–10] Stage and amount of residual tumor reflect the extent and bulk of disease.[7] The histologic type and grade of tumor may give information on the malignant potential of tumor cells, but is of questionable utility because of lack of uniform criteria and marked interobserver and intraobserver variability.[11]

Quantitation of proliferative activity provides an estimation of the malignant potential of tumor cells. The percentage of cells in the S, or synthesis, phase of the cell cycle is a reflection of the proliferative fraction of a tumor. Analysis of flow cytometric DNA histograms provides a rapid and convenient method to determine the S phase fraction (SPF) of a tumor, and this method has been extensively employed to study a wide variety of neoplasms.[12–20] The role of SPF in predicting outcome in ovarian cancer, however, is still unclear.[7,10] Proliferative activity also can be assessed by immunostaining with Ki-67, a monoclonal antibody that reacts with nuclear antigens expressed in actively proliferating but not in resting (GO) cells.[21,22] Only scant data are known about Ki-67 staining in ovarian tumors.[19,23]

We have studied the proliferative activity of 74 malignant, borderline, and benign ovarian neoplasms. The proliferative activity was assessed by determination of SPF from DNA histograms and by quantitation of nuclear Ki-67 immunostaining of tissue sections using image analysis. These two methods of measuring proliferative activity were compared with each other and with the clinical and histologic factors shown to be of prognostic value in ovarian cancer.

## Materials and Methods

Between January 1987 and June 1989, 74 ovarian neoplasms with sufficient tissue for flow cytometric and im-

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munohistochemical studies were evaluated. A portion of each lesion was snap frozen in isopentane and stored at −80°C. The opposite face was processed for flow cytometry and was stored for less than 24 hours at 4°C in Hank's Balanced Salt Solution. The remaining tissue was routinely processed for pathologic diagnosis. One section per centimeter of maximum tumor diameter was examined in borderline and malignant tumors. There were 44 malignant, 12 borderline, and 18 benign neoplasms. All cases were classified based on World Health Organization criteria.[24] Malignant epithelial neoplasms were given an architectural grade of 1 (<5% solid), 2 (5% to 50% solid), or 3 (>50% solid). Nuclei in all lesions were graded from 1 to 3: grade 1 nuclei were uniform in size, shape, and chromatin distribution; grade 3 nuclei varied markedly in size and shape and had clumpy, condensed chromatin; and grade 2 nuclei had intermediate features. Nucleoli in all lesions were graded from 1 to 3: grade 1 had small, uniform nucleoli; grade 3 had large, irregular nucleoli; and grade 2 nucleoli had intermediate features. Mitotic activity was evaluated by determining the mean number of mitotic figures in 50 high-power (40×) fields. Age and new FIGO stage[1] were obtained from chart review.

Cryostat sections were reacted with Ki-67 antibody (DAKO-C, Dako Corporation, Santa Barbara, CA) using established immunoperoxidase techniques.[25] Briefly, sections were cut 3–5 μm, fixed for 6 minutes in 1% paraformaldehyde at 4°C, incubated with monoclonal Ki-67 antibody at a dilution of 1:15 for 1 hour, then rinsed in TRIS buffer. Peroxidase conjugated rabbit anti-mouse immunoglobulin (1:40 dilution) and swine anti-rabbit immunoglobulin (1:60 dilution) were used as secondary and tertiary staining reagents. A section from each case was incubated with buffer instead of primary antibody as a negative control. Diaminobenzidine (Aldrich Chemical Company, Milwaukee, WI) was used as a chromogen. Sections were counterstained with 2% methyl green after dehydration in alcohol and xylene. In each case a section was stained with hematoxylin and eosin (H&E) to confirm that representative tissue was present.

Quantitation of Ki-67 staining was performed using the CAS 100 system (Cell Analysis Systems, Lombard, IL).[16,26] The CAS 100 System is a microscope-based image analyzer with a solid-state video camera mounted on a light microscope that transmits image data to an IBM 386 computer equipped with a digital imaging board. The image data are stored in the form of pixel optical density values. Previous standardization and calibration of the instrument enables conversion of light intensity values to optical density values. Measurement of nuclear staining was performed as previously described.[22] Briefly, a representative field without positive staining was selected to determine and correct for the background level of staining. Then microscopic fields containing lesional tissue and excluding stroma to the greatest extent possible were selected randomly. Ten fields per case were analyzed, including fields with high percent reactivity and fields with low or absent reactivity with antibody. An image of the total nuclear area in a selected microscopic field was mapped using a red (650/10 nm bandpass) filter. Using a green (540/10 nm bandpass) filter on the same microscopic field, a map of positive nuclear staining was obtained. The percent positive staining was automatically calculated by the system software as percent nuclear area stained, and the mean percent staining was accumulated for sequential fields. In all cases, the variation in the cumulative percent positive area was less than 5% after analysis of 10 fields. All images were obtained using a 40× objective (numerical aperture 0.66) and a 10× eyepiece, yielding a final magnification of 400×.

Flow cytometric DNA analysis was performed on fresh tissue as described previously.[27] Detergent-isolated nuclei were stained with propidium iodide, and DNA histograms were generated using FACScan (Becton Dickinson, Mountain View, CA). Normal human lymphocytes served as an internal control for normal (diploid) DNA content in one duplicate sample. Histograms with a G0/G1 peak distinctly separate from diploid cells (confirmed by the addition of normal lymphocytes) were considered to have aneuploid DNA content. The DNA index (DI) was calculated as the ratio of the modal fluorescence channel numbers of the aneuploid and diploid (G0/G1) peaks according to convention.[28] The DNA ploidy data have been reported in part elsewhere.[29] The percentage of cells in the S phase of the cell cycle (SPF) was evaluable in 56 cases. This was done using the computer software program SOBR (Sum of Broadened Rectangles)[30] in 35 cases and the method of Baisch et al[31] in 16 cases. In five cases where the S phase was extremely low and these two methods were inappropriate, a crude estimate of percent S phase was estimated from the DNA histogram by determining the number of cells between G0/G1 and G2/M peaks as a percentage of the total number of cells analyzed.

All data analyses were performed with SAS (Statistical Analysis System, SAS Institute, Cary, NC) on a personal computer. Because neither SPF nor percent Ki-67 positive nuclear area were normally distributed, the medians and upper and lower quartile values are reported. One-way analysis of variance (ANOVA) and Duncan's Multiple Range test on the normalized ranks of SPF and percent Ki-67 were employed to look for differences between diploid and aneuploid tumors. We used Spearman correlation coefficients to examine associations between the clinical and histologic factors and SPF and percent Ki-67. The relationships between ranked percent Ki-67, and

ranked SPF for the three tumor types were examined by analysis of covariance.

## Results

Seventy-four ovarian neoplasms were analyzed. Specific diagnoses for these cases are detailed in Table 1. The mean patient ages for the malignant, borderline, and benign neoplasms were 58.1, 40.2, and 48.6 years, respectively.

Immunoperoxidase staining of cryostat sections with Ki-67 antibody produced clear nuclear staining in positive cells. Cytoplasmic staining was no greater than that of the background in the immunoperoxidase control. Positive nuclear staining for Ki-67 was present in varying proportions of tumor cells, ranging from cases with scattered positive nuclei (Figure 1) to cases with predominantly positive nuclei (Figure 2).

The median percent Ki-67 positive nuclear area for the three types of ovarian neoplasms are depicted in Figure 3. The median values for percent Ki-67 were: 2.7% in benign (range, 0.5 to 22.4); 12.3% in borderline (range, 2.5 to 74.7); and 27.6% in malignant tumors (range, 1.4 to 69.8). Duncan's multiple range test showed that the percent Ki-67 staining for malignant neoplasms was significantly higher than that for borderline ($P < 0.05$), which was, in turn, significantly higher than that for benign neoplasms ($P < 0.05$). Analysis of variance on the ranked percent Ki-67 for the various tumors showed that the benign, borderline, or malignant category status of the tumor explained 45% of the variability in percent Ki-67 staining ($P < 0.0001$).

**Table 1.** *Classification and Number of Cases of Malignant, Borderline, and Benign Ovarian Neoplasms*

| Diagnosis | Number |
|---|---|
| Malignant | 44 |
| Serous | 19 |
| Mucinous | 1 |
| Endometrioid | 6 |
| Clear cell | 3 |
| Surface serous | 2 |
| Mixed epithelial type | 2 |
| Adenocarcinoma, NOS | 1 |
| Undifferentiated carcinoma | 1 |
| Mixed mesodermal | 3 |
| Dysgerminoma | 1 |
| Malignant Brenner | 2 |
| Granulosa cell | 3 |
| Borderline neoplasms | 12 |
| Serous | 7 |
| Mucinous | 4 |
| Mixed epithelial type | 1 |
| Benign neoplasms | 18 |
| Serous cystadenoma | 3 |
| Serous cystadenofibroma | 5 |
| Lipid cell | 1 |
| Mature cystic teratoma | 1 |
| Fibroma-thecoma | 8 |

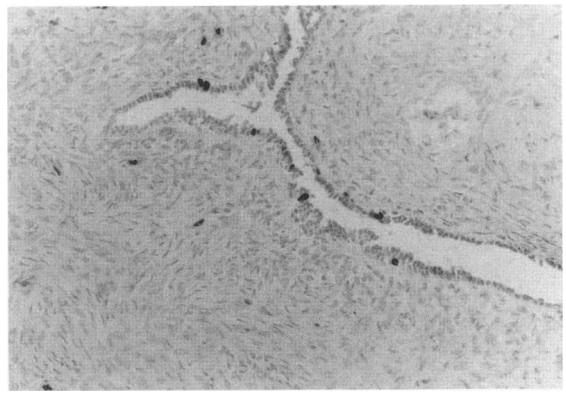

**Figure 1.** *Nuclear staining by Ki-67 antibody in a serous cystadenofibroma of the ovary. Methyl green counterstain (×200)*

Diagnostic DNA histograms were obtained in 70 of these tumor samples: 42 malignant, 11 borderline, and 17 benign neoplasms. Of the malignant neoplasms, 24 (57.1%) were DNA aneuploid and 18 (42.9%) were diploid. All 11 borderline tumors were diploid. Three (16.7%) of the benign neoplasms exhibited DNA aneuploidy (a cystadenofibroma, a fibrothecoma, and a lipid cell tumor).[29,32]

The median SPF for the three types of ovarian neoplasms were calculated and are shown in Figure 3. The median SPF of the tumors were as follows: 2.9% in benign (range, 1.0 to 14.0); 3.4% in borderline (range, 1.5 to 7.0); and 11.5% in malignant tumors (range, 1.0 to 33.0). Duncan's multiple range test showed that the median SPF value for malignant neoplasms was significantly higher than that of borderline and benign neoplasms ($P < 0.05$) and that the values for borderline and benign neoplasms were not different from each other. Analysis of variance on the ranked SPF showed that the category of neoplasm (benign, borderline, or malignant) explained only 20% of the variability in the SPF ($P = 0.003$).

We also were interested in evaluating the relationship between DNA ploidy and SPF or percent Ki-67 positive nuclear area. The 70 ovarian tumors for which evaluable DNA histograms were obtained were divided into diploid (42 cases) and aneuploid (28 cases) categories. The median percent Ki-67 staining was 8.1% (range, 0.5 to 74.7) for diploid tumors *versus* 29.5% (range, 1.7 to 61.0) for aneuploid tumors (Figure 4). A lower median SPF of 3.0% (range, 1.0 to 14.0) was found in the diploid tumors compared with 14.3% (range, 2.2 to 33.0) in the aneuploid tumors (Figure 4). Both the median percent Ki-67 positive nuclear area and the SPF were significantly higher in aneuploid *versus* diploid tumors ($P < 0.0001$). The ANOVA on the ranked values showed that ploidy status explained 29% of the variability observed in percent Ki-67 staining and 43% of the variability in SPF.

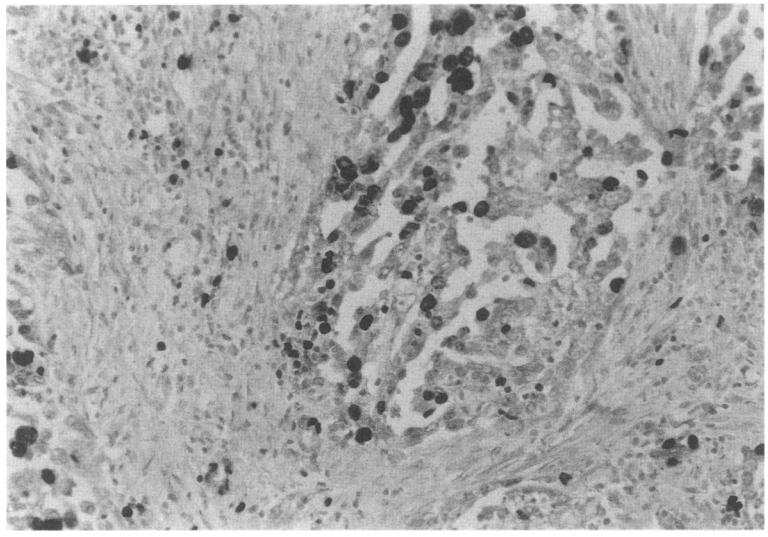

**Figure 2.** *Nuclear staining by Ki-67 antibody in a serous adenocarcinoma of the ovary. Methyl green counterstain (×200).*

We then examined the effects of DNA aneuploid *versus* diploid status on SPF and percent Ki-67 staining in each of the three tumor categories. There were 35 diploid tumors in which SPF was evaluated: 12 benign, 10 borderline, and 13 malignant. The median SPF was 2.9% in benign, 3.4% in borderline, and 4.0 in malignant diploid tumors. There were 21 aneuploid tumors in which SPF was evaluated: two benign and 19 malignant. The median SPF was 5.2% in the benign and 15.4% in the malignant aneuploid tumors. The malignant aneuploid tumors had significantly higher SPF than all other combinations ($P < 0.002$). There were 42 diploid tumors in which Ki-67 staining was evaluated: 13 benign, 11 borderline, and 18 malignant. The median percent Ki-67 staining was 2.2% in benign, 13.3% in borderline, and 15.4% in malignant diploid tumors. There were 28 aneuploid tumors in which Ki-67 staining was evaluated: four benign and 24 malignant. The median percent Ki-67 values were higher in both benign and malignant aneuploid tumors: 7.8% in benign and 31.7% in malignant aneuploid tumors ($P < 0.003$).

Univariate analysis was performed to see how histologic and clinical factors related to percent Ki-67 positive nuclear area and SPF. The results are shown in Table 2. In the malignant neoplasms, percent Ki-67 was significantly and positively associated with SPF, DNA index, architectural grade, nucleolar grade, and mitotic count. There was no relationship with nuclear grade, age, or clinical stage. There were no significant correlations between any of the clinical or histologic factors evaluated and SPF or percent Ki-67 staining in the borderline group (Table 2). In the benign group, only nucleolar grade was significantly correlated with percent Ki-67 staining.

In contrast, SPF was significantly and positively correlated with all parameters evaluated in the malignant tumors: percent Ki-67, DNA index, architectural grade,

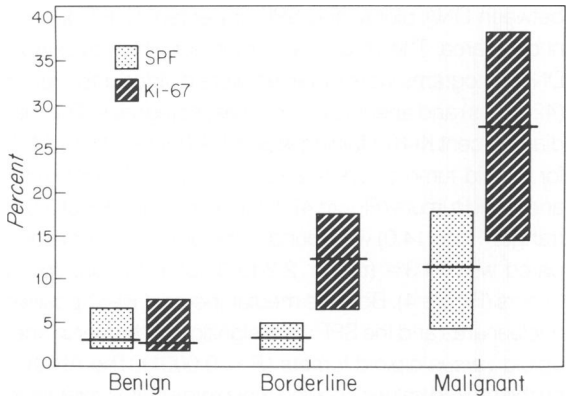


**Figure 3.** *Median % Ki-67 positive nuclear area and S phase fraction (+/− lowest and highest quartiles) for benign, borderline, and malignant ovarian neoplasms.*

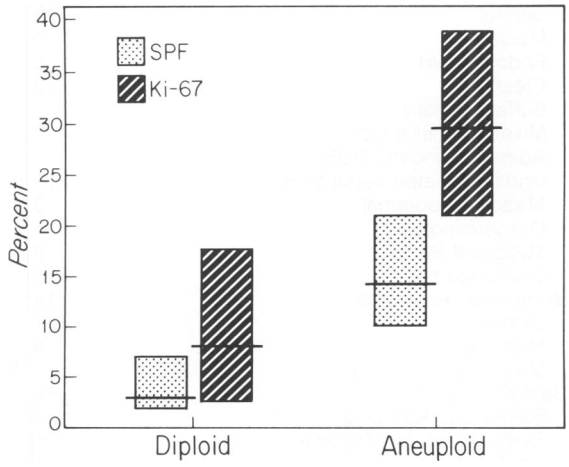


**Figure 4.** *Median % Ki-67 positive nuclear area and S phase fraction (+/− lowest and highest quartiles) for diploid and aneuploid neoplasms.*

Table 2. *Spearman Correlation Coefficients for Univariate Analysis of % Ki-67–positive Nuclear Area and SPF and Clinical and Histologic Prognostic Parameters for Malignant, Borderline, and Benign Ovarian Neoplasms*

| | Malignant | | Borderline | | Benign | |
|---|---|---|---|---|---|---|
| | % Ki-67 (N = 44) | SPF (N = 32) | % Ki-67 (N = 12) | SPF (N = 10) | % Ki-67 (N = 18) | SPF (N = 14) |
| S Phase fraction | **.59** | | −.01 | | .20 | |
| DNA index | **.59** | **.56** | * | * | .46 | .13 |
| Architect. grade | **.53** | **.52** | ** | ** | ** | ** |
| Nuclear grade | .26 | **.64** | −.02 | −.02 | .40 | −.10 |
| Nucleolar grade | **.45** | **.59** | .02 | .14 | **.50** | −.10 |
| Mitotic index | **.54** | **.52** | .27 | .07 | *** | *** |
| Stage | .28 | **.48** | .34 | −.28 | *** | *** |

* Architectural grading was not performed on borderline and benign neoplasms.
** Analysis was not possible because all borderline tumors were diploid and all benign tumors had mean mitotic counts of zero and are not staged (Stage 0).
Boldface coefficients show significant associations ($P < 0.05$).

nuclear grade, nucleolar grade, mitotic index, age, and clinical stage. No associations between any of these parameters and percent Ki-67 or SPF were found in borderline and benign neoplasms.

Ranked percent Ki-67 positive nuclear area *versus* ranked SPF for the three types of ovarian neoplasms are shown in Figure 5. These two measures of proliferative activity are positively correlated ($r = 0.59$) for malignant neoplasms but not for borderline or benign neoplasms.

## Discussion

We assessed the proliferative activity of 74 ovarian tumors by two different methods, SPF estimated from flow cytometric DNA histograms and Ki-67 immunostaining of tissue sections quantitated by image analysis, and compared this activity with clinical and histologic parameters useful in predicting prognosis. The median SPF was significantly higher in malignant neoplasms than in borderline neoplasms or benign neoplasms. By univariate analysis, SPF was positively correlated with percent Ki-67 positive nuclear area, DNA index, architectural grade, nuclear grade, nucleolar grade, mitotic count, age, and stage. None of these features positively correlated with SPF in borderline or benign neoplasms.

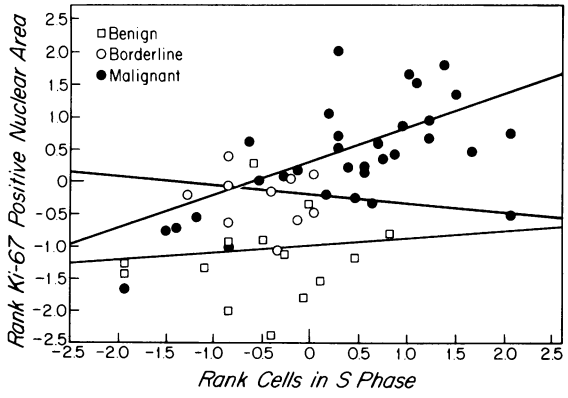


Figure 5. *Ranked Ki-67 positive nuclear area and S phase fraction for malignant, borderline, and benign ovarian neoplasms.*

The number of actively proliferating cells (those in G1, S, and G2/M) was determined by staining tissue sections with the monoclonal antibody Ki-67. As expected, median percent Ki-67 positive nuclear area was significantly higher in malignant neoplasms than in borderline or benign neoplasms. By univariate analysis, percent Ki-67 staining correlated positively with SPF, DNA index, architectural grade, nucleolar grade, and mitotic count for malignant neoplasms; only with nucleolar grade in benign neoplasms; and not with any of these parameters in borderline neoplasms.

Many clinical and histologic factors including FIGO stage,[2–10] amount of residual tumor,[2,4,6–8,10] histologic subtype,[2,4,7] histologic grade,[3,4,6–8,10] and DNA ploidy,[5–10] have been correlated with survival in ovarian cancer. Many histologically assessed parameters lack uniform criteria and suffer from marked interobserver and intraobserver variability.[11] Generation of a flow cytometric DNA histogram, thus allowing for analysis of DNA ploidy and cell cycle kinetics, including S phase, has the advantages of objectivity, speed, and analysis of large numbers of cells, generally 5000 to 10,000 per case. Normal stromal and inflammatory cells present in the sample, however, cannot be separated from tumor cells in routine flow cytometry.

The measurement of proliferative activity provides an objective means of determining the malignant potential of a tumor. Estimation of SPF from DNA histograms compares favorably with thymidine labeling in a variety of human tumors, a more rigorous technique for determining the proportion of actively proliferating cells.[15] The prognostic value of SPF has been demonstrated for certain groups of patients with breast cancer, non–small cell lung cancer, and colon cancer as well as ovarian cancer.[19,20] In addition to survival, SPF has been correlated with known prognostic factors in ovarian cancer such as

stage, nuclear grade, mitotic count, and DNA ploidy.[7,13,14,19] Our findings are in agreement with the results of these studies.

Ki-67 staining has been examined in ovarian tumors in two studies.[19,23] Wong and Tattersall[23] found a positive correlation between Ki-67 value and histologic grade (architectural pattern) and stage. Ki-67 values correlated highly with SPF and survival, but not with clinical stage of disease in the study by Isola et al.[19] Our results confirm the correlation between percent Ki-67 with SPF, and further extend this association to encompass other prognostic factors in ovarian tumors: architectural grade, nucleolar grade, mitotic index, and DNA index.

Ki-67 staining has been found to positively correlate with poor prognosis or features associated with poor prognosis in soft tissue tumors,[33,34] non-Hodgkin's lymphoma,[16,35] breast carcinoma,[18,36,37] and gliomas.[38] Studies failing to find such associations, however, include reports of breast,[39] colonic,[40] and cervical carcinomas,[41] as well as gliomas.[42]

Although we found that measurements of proliferative activity by SPF from DNA histogram analysis and by Ki-67 immunostaining correlated with known prognostic factors in ovarian cancer and had the advantage of objectivity, each method had inherent disadvantages. Estimating SPF from DNA histograms is not possible in many cases because of merging of diploid and aneuploid G0/G1 peaks. Of the 70 cases with diagnostic DNA histograms in this study, S phase could be estimated in only 56 cases. Friedlander et al[12] found that overlapping peaks precluded S phase analysis in as many as 50% of aneuploid tumors. In diploid tumors, the S phase of tumor cells cannot be distinguished from the S phase of normal stromal or inflammatory cells present in the sample. This may artificially and unpredictably lower the estimate of SPF in diploid tumors, depending on the proportion of stromal cells to tumor cells.[17]

Ki-67 staining is distinct and relatively easy to interpret. Image analysis provides objective quantitation of immunohistochemical staining. Furthermore, the operator chooses the areas to be studied, ensuring that only tumor cells are analyzed. Specific evaluation of tumor cells is not possible in routine flow cytometry, even with the use of additional cell markers. Therefore, *in situ* methods of assessing proliferative activity may eventually prove more practical and accurate than flow cytometry.

Heterogeneity in Ki-67 staining in different areas of the same tumor occurs frequently.[43] We choose to use mean percent positive Ki-67 staining in the statistical analysis. Prior work from our group has shown that mean Ki-67 staining rather than the highest Ki-67 value correlates best with grade in a series of non-Hodgkin's lymphomas.[16] It may be that the peak Ki-67 value or the coefficient of variance for Ki-67 correlates better with various prognostic factors in ovarian tumors. This would be an interesting area for future study.

Occasional stromal cell nuclei demonstrated positive immunostaining with Ki-67. Analysis of stromal proliferative activity using Ki-67 was not undertaken in this study and has not been reported in the literature. As Dvorak[44] has pointed out in a review of aspects of tumor stroma generation, the stroma provides essential support for the survival and growth of tumor. Several cell types within tumor stroma, including fibroblasts and endothelial cells, replicate. Perhaps the proliferative activity of the stromal compartment specifically, as measured by Ki-67 immunostaining, will correlate with prognostic factors in ovarian and other tumors and prove useful clinically.

Analysis of the DNA ploidy of a large series of ovarian tumors including most of those in this study have been reported elsewhere.[29] The current study was designed to additionally examine SPF and percent Ki-67 staining in ovarian tumors. In this subset of the study, 57.1% of the malignant neoplasms were aneuploid and 42.9% were diploid. This agrees well with other series of ovarian tumors.[7,9] All of our borderline tumors were diploid. Other series have found that most borderline tumors are diploid.[8,45] When we compared the SPF and percent Ki-67 staining of diploid *versus* aneuploid tumors, we found that both were higher in aneuploid tumors. The highest values of both were obtained in the malignant aneuploid category and, overall, aneuploid tumors had significantly higher percent Ki-67 staining and SPF than diploid tumors.

Few studies have examined proliferative activity in ovarian cancers by staining with Ki-67. We and others[19,23] have shown that the number of Ki-67–positive nuclei (or nuclear area) correlates with various prognostic factors for ovarian cancer. Further studies are needed to confirm these findings and should directly address the relationship of Ki-67 staining with survival.

In summary, we studied the proliferative activity of 74 ovarian tumors by measuring the SPF from DNA histograms and by staining tissue sections with Ki-67 antibody. We found that percent Ki-67 positive nuclear area correlated with SPF, DNA index, architectural grade, nucleolar grade, and mitotic index in malignant tumors. In contrast, SPF correlated with percent Ki-67, DNA index, architectural grade, nuclear grade, nucleolar grade, mitotic index, stage, and age. Further clinical studies are needed to assess the prognostic significance of SPF and percent Ki-67 staining in ovarian neoplasms.

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