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Quantitative Evaluation of Malignant Potential of Early Breast Cancer Using High Resolution Image Cytometry

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Abstract We have been testing two independent hypotheses which postulate that the malignant potential of early breast cancer can be objectively assessed by measuring nuclear features of the diagnostic-malignant cells (hypothesis I) and/or normal-appearing epithelial cells found in the vicinity of the carcinoma (hypothesis II). In preliminary experiments, we tested some of these hypotheses using historical samples and a high resolution image cytometry apparatus. Tissue sections were stained with our stoichiometric stain and over 60 nuclear features, primarily texture features describing the DNA distribution in the nuclei, were employed in the multivariate analyses. Data derived from measurements of ductal carcinoma *in situ* (DCIS) with and without the invasive component indicated that the malignant potential of these lesions can be estimated with a sensitivity and specificity of at least 80%. The analysis of the tissue surrounding an invasive breast carcinoma showed that the existing malignancy can be predicted solely from the measurements of normal nuclei (normal-appearing breast lobules) in more than 85% of patients. This result indicates that the analysis of benign tissues also could give prognostically valid information.

These results can be greatly improved using larger sample sizes and other improvements, including technical improvements of the cytometry device. We believe that this approach can be developed into a practical diagnostic and prognostic tool for better management of early breast cancer.

Key words: Early breast cancer, image cytometry, malignant potential, nuclear features

One of the best means to reduce mortality of malignant cancer is to detect the disease in its early, non-invasive stage. For instance, the mortality rate from cervical cancer in some countries has dropped several-fold due to regular screening [1], which discovers cervical precancerous and/or cancerous lesions at such an early stage that nearly 100% can be cured.

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Recent developments in breast examination, particularly including x-ray mammography, may also lead to better management of breast cancer. In many countries, women over age 50 have annual mammography examinations. This process has already significantly increased the rate of detection of many precancerous lesions, as well as ductal carcinomas *in situ* (DCIS); the proportion of carcinoma *in situ* found in malignant breast biopsies increased nearly *five-fold* [2].

At this stage, it is virtually impossible to predict which of these early lesions will rapidly

progress to malignancy and which will remain benign or even regress without further intervention. This presents a dilemma: How do we treat such early lesions? DCIS tumors have been successfully treated by mastectomy, but in many cases, a significant proportion could be successfully treated with more conservative therapy. Ideally, one could predict which DCIS lesions could be eradicated by minimal intervention and which must be treated more aggressively. If we could accurately predict the recurrence or progression of DCIS, differential treatment protocols could be established, leading to a much better quality of life for most DCIS patients, while saving the lives of those for whom present treatment protocols are inadequate. Currently, there are no known means to estimate the malignant potential of either DCIS or such premalignant lesions of the breast as atypical hyperplasia.

We postulate that the malignant potential of early breast lesions of both precancerous and cancerous types can be evaluated by multivariate analysis of nuclear features of diagnostic cells and/or normal epithelial cells surrounding the diseased site. It has long been recognized that some morphometric features of diagnostic cells such as nuclear DNA amount, chromatin pattern, nuclear size and shape, *etc.*, carry some information of the malignant potential of the cancerous diseases. The effect of a malignant growth on surrounding normal tissue cells has also been well-documented in several tissues through a phenomenon known as malignancy-associated changes (MACs) [3–12]. We believe that both of the above effects could be used independently or combined for an objective evaluation of malignant potential of early breast lesions.

A prerequisite for this approach is an accurate measurement of a variety of nuclear features extracted from digitized images of cell nuclei stained with a stoichiometric DNA stain using high precision image cytometry. This paper describes some preliminary results using a high resolution image cytometry system specially developed for quantitative pathology and cytology. The results are consistent with the postulates of the two hypotheses. We plan to launch a more detailed study to prove/disprove these postulates. If they prove to be correct, a simple and inexpensive method could be established for routine clinical work to evaluate malignant potential of early breast lesions.

MATERIALS AND METHODS

Tissue Sections

For most of the studies described in this work, 4 μm thick tissue sections were obtained from paraffin-embedded tissues which originated from fine wire biopsies taken as a result of positive mammography screening or from mastectomy material. In total, 144 diseased cases were studied (8 mild hyperplasia, 18 moderate and severe hyperplasia and papilloma, 60 non-comedo DCIS, 21 comedo DCIS, and 37 invasive ductal carcinoma) with 53 control (normal tissue) cases. Tissue sections were first deparaffinized and rehydrated, and then stained with the Thionin-SO_2 stain (Xillix Technologies Corp.) using Feulgen procedures. This stain has consistently shown the best DNA stoichiometric properties using absorbance [13] and is believed to be a prerequisite for the work described below.

Image Cytometry

High resolution image cytometry was used to capture digitized images of cell nuclei at the diffraction limit of spatial resolution (≤0.3 μm). A specially designed image cytometry system was first employed [14] and has since been replaced by a superior high resolution image cytometer (Cyto-Savant™), developed by us in collaboration with Xillix Technologies Corp., Vancouver, BC, Canada [15,16]. The latter employs a light transducer in the form of scientific charge-coupled device (CCD) with a host of properties, ideally suited for precision work in quantitative pathology and cytology. The system features small sensors (6.8 μm × 6.8 μm) which sense in their entire surface (100% fill factor). The transducer is positioned in the primary image plane of the aberration-free objective (PlanApo 20×/0.75) which results in a pixel size of 0.34 μm × 0.34 μm (0.1 μm^2) at 20× magnification with a signal-to-noise ratio of better than 500:1.

For each measurement of atypical cells or normal epithelial cells in the tissue section, 100–200 non-overlapping cell nuclei were selected. These were placed in exact focus and were segmented semi-automatically. Digitized images were captured in the computer memory for further processing. Nuclear masks were examined and, if necessary, were corrected manually to

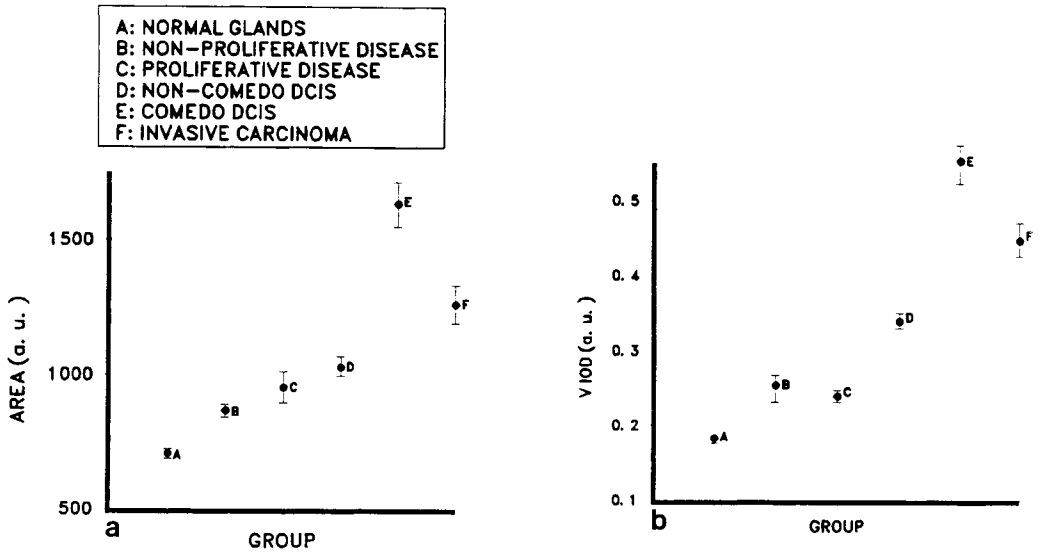

Fig. 1. Nuclear features in different groups of breast diseases. The mean of the nuclear area **(a)** and variance of integrated optical density (V IOD) **(b)** are plotted with the standard error of the mean. V IOD corresponds to the variation of the cellular DNA content on individual slides.

define the nuclear boundary pixels. From each nuclear image, over 60 nuclear features were extracted. They ranged from single bulk features (size, shape, DNA amount) to more sophisticated texture features (both discrete and continuous) describing the chromatin distribution in the nuclei. Lymphocytes were used as internal diploid DNA controls to normalize each slide to correct staining variations. Statistical analyses, including discriminant function analysis, were then performed on the data [17,18].

RESULTS

Correlation of Nuclear Features With Disease Classification

One of the first tests of the usefulness of the nuclear features was to examine their discriminant power between different types of breast diseases. A large number of features have significantly different mean values as well as variances when compared among the representative groups [normal; non-proliferative (mild ductal hyperplasia); proliferative (moderate and severe ductal hyperplasia, papilloma, and sclerosing adenosis); non-comedo DCIS; comedo DCIS; and invasive carcinoma]. These feature values generally progressively increase/decrease according to the disease severity. Nuclear features of all types (DNA content, size, shape, and texture) could be found with some discriminant power between the groups. Figures 1a and 1b show how a single feature changes from group to group. Using only two independent features (several such couples could be used), a high correlation can be shown between feature values and disease type. Using a combination of features, discriminant function analysis showed distinctive groups with a significant overlap. The scatter plot of two canonical variables acquired with discriminant function analyses is shown in Figure 2. These preliminary results indicate that nuclear features could be used as a tool for more objective classification of disease types, especially important in premalignant breast disease and carcinoma *in situ*. Other feature combinations, much greater sample size, more precisely specified diagnostic categories, and control of interobserver disagreements in the histopathological diagnosis should be used for superior results.

Nuclear Features of Different Histological Types of DCIS

A study was performed to test whether or not it is possible to demonstrate differences in nuclear features between different subtypes of non-

TABLE I. Jackknifed Classification of Non-Comedo and Comedo DCIS Nuclei

	Classified Groups		
Actual Groups	Non-Comedo	Comedo	Total
Non-Comedo	8185 (86%)	1356 (14%)	9541
Comedo	736 (26%)	2090 (74%)	2826

Overall classification is correct in 83% (10275/12367) of nuclei. Using this approach, sample-by-sample classification is expected to yield even better results, possibly over 90%.

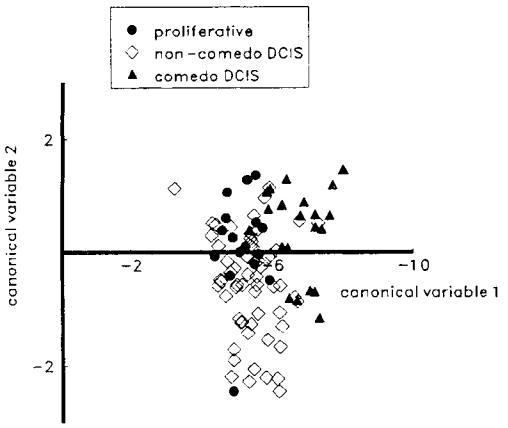

Fig. 2. Separation of different groups of breast diseases with discriminant function analyses. Two canonical variables were calculated from the discriminant function analyses used to discriminate six groups of breast diseases. Only cases of proliferative disease, non-comedo DCIS, and comedo DCIS are plotted.

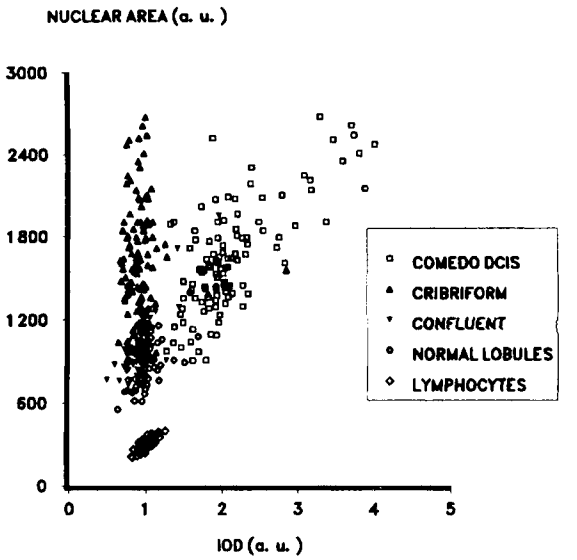

Fig. 3. Area versus IOD scatter plot of various DCIS types present in the same patient. Comedo, cribriform, and confluent DCIS were present in the same tissue section. DCIS cells of different histological types differ in DNA content as well as in nuclear size.

comedo DCIS. Significant discrimination between comedo and non-comedo DCIS can be readily achieved by nuclear features (Table I). Heterogeneity of DCIS was demonstrated in a few patients when different non-comedo types were present in the same tissue section (Fig. 3). The analysis of various non-comedo types showed that several features (size, shape, and texture) assumed different average values between these subtypes (cribriform, papillary, confluent/acinar, mixed, and non-specific). However, these differences were not as significant as those found between different disease types (Figs. 4a, 4b, and 4c). Nevertheless, we believe that by using a combination of these features, as well as feature variances, a multivariate analysis could place these subtypes on a quantitative scale for more objective classification of non-comedo DCIS.

Use of Nuclear Features to Distinguish DCIS With or Without Invasive Component

In the sample of 60 non-comedo DCIS cases, no invasive cancer was found elsewhere in the mastectomy tissue in 31 cases, while 29 cases of non-comedo DCIS did have an invasive component elsewhere in the mastectomy. Similarly, of the 21 comedo types, there were 6 without and 15 with an invasive component. A discriminant

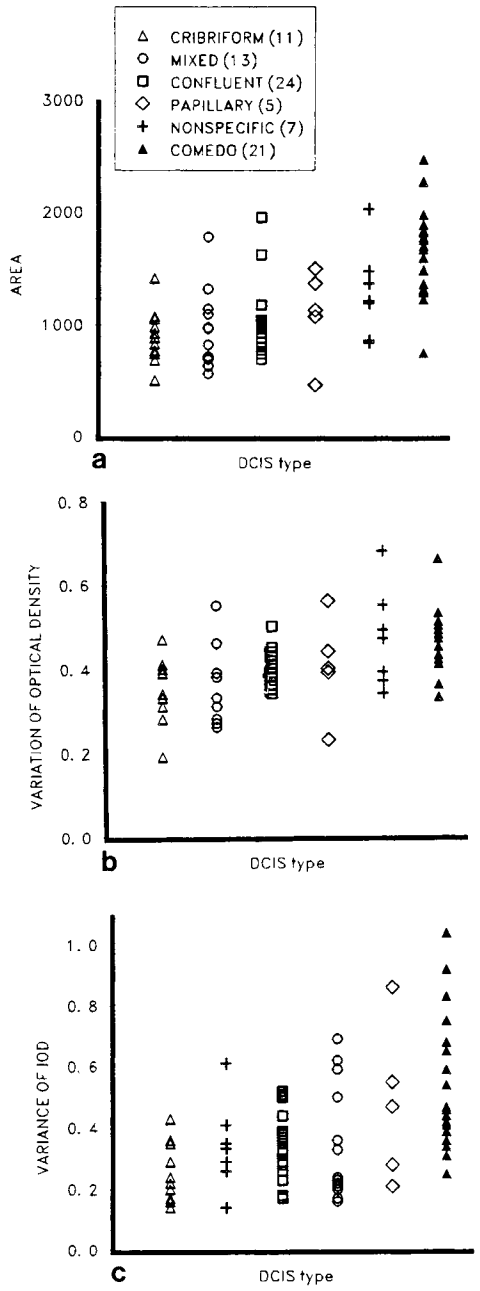

Fig. 4. Nuclear features in different DCIS types. Cribriform and mixed type have smaller nuclear area than other histological types of DCIS, and comedo type has the largest nuclear area (**a**). Variation of optical density represents the variation in the distribution of optical density values over the pixels of a nucleus. Cribriform and mixed types have more homogeneously stained chromatin than other types (**b**). The variation in the DNA content between nuclei is represented by the variance of integrated optical density (V IOD); the variation on individual slides is the lowest for the cribriform DCIS, while the comedo type has the highest intra-slide variation of the DNA content (**c**).

function analysis was performed to examine if DCIS with or without an invasive component could be separated (comedo and non-comedo cases were treated separately). The results [19] showed that cell-by-cell classification of DCIS with and without invasive components can be correctly achieved in approximately 70% and 80% of cases for non-comedo and comedo type, respectively. Due to the small sample size, the number of features in the multivariate analysis was very limited. However, a correct sample classification of DCIS with and DCIS without invasive component was correctly achieved in 80% of non-comedo and 100% of comedo DCIS.

MACs and Their Use in Discriminating Between Benign Breast Disease and Invasive Carcinoma Patients

With the analysis of nuclear features of normal epithelial cells selected from normal-appearing lobules, we tested the hypothesis that patients with benign disease (normal and non-proliferative cases were combined in one class) can be distinguished from patients with malignant disease (invasive carcinoma). In all cases, only nuclei from normal-appearing glands were analyzed. Overall classification of cell nuclei was correct in 76% of all cells. Using a single sample feature, samples were correctly classified in 86% of the cases [20]. If a sample contained over one-third of all measured lobular cells exhibiting MACs, then the sample was designated as containing invasive cancer; otherwise, it was designated as benign (Fig. 5). Despite the small sample size and use of a single sample feature, results are very good and provide impetus to pursue further studies. We believe that these results can be greatly improved with a larger number of features, which will require analyzing a much larger sample size.

DISCUSSION

Our work with high resolution image cytometry shows that this tool can be employed not only as an aid for more objective and reproducible classification of breast diseases, but that it could also be important in the prognostic evaluation of these patients. There have been many attempts to find a marker to predict the malignant potential of early cancers in breast as well

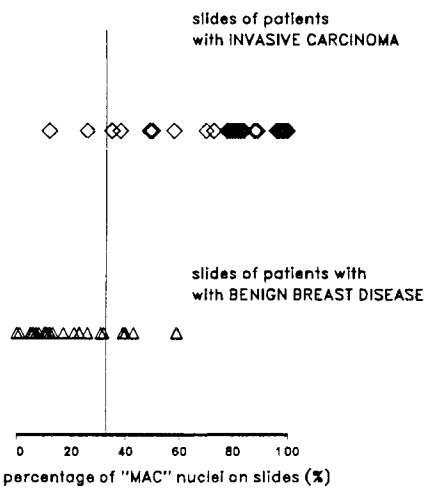

Fig. 5. Classification of patients with benign breast disease and patients with invasive carcinoma using malignancy-associated changes (MACs). The classification of cases was based on the proportion of MAC nuclei on individual slides. A threshold of 34% was found to best separate the two groups of patients.

as other tissues. To date, no such markers have been found for DCIS and/or other precancerous breast lesions. DNA aneuploidy has been suggested as a prognostic indicator for patients with invasive breast carcinoma [21]. However, this single nuclear morphometric feature alone may not be very useful in predicting the malignant potential of breast lesions.

We postulate that a multivariate analysis of nuclear features of diagnostic cells, or of normal cells near a lesion, could be an indicator of prognosis. This postulate is based on data from other tissue sites. For example, progression and regression of cervical precancerous lesions (moderate and severe dysplasia) can be predicted with a high degree of probability by either nuclear features of atypical cells or by the surrounding, normal-appearing intermediate cells [22]. Similarly, a study involving early lung cancer found both diagnostic cells and/or the surrounding normal epithelial cells have been used for objective classification of early lung cancer, with indications that these measurements could be used to predict the malignancy of these lesions [17].

Our own preliminary data on breast diseases presented in this paper are consistent with the above hypotheses. The differences in nuclear morphology between DCIS without an invasive component and DCIS with an associated invasive carcinoma may be related to the subsequent behavior of DCIS and could predict the invasive or recurrence potential after local treatment. In addition to diagnostic implications, the changes in nuclear morphology of normal breast tissue adjacent to a malignancy (MAC) could be very important in the prognostic evaluation of patients with breast diseases. In our opinion, these nuclear changes are not limited to tissues adjacent to invasive carcinoma; it should be possible to detect them in tissues adjacent to premalignant lesions as well.

These findings are highly relevant in clinical evaluation of patients with breast diseases. Therefore, it is very important to establish and expand these results in much larger samples using retrospective as well as prospective approaches. Further improvements are anticipated with the use of better image cytometry technology, including better algorithms for focusing, segmentation, and feature calculations. This method can be readily developed into a fast, inexpensive, and objective means for routine clinical practice.

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REFERENCES

1. Anderson GH, Boyes DA, Benedet JL, LeRiche JC, Matisic JP, Suen KC, Worth AJ, Millner A, Bennett OM. (1988) The organization and results of the cervical cytology screening program in British Columbia from 1955 to 1985. Br Med J 295:975–978.
2. Stacey-Clear A, McCarthy KA, Hall DA, Pile-Spellman E, White G, Hulka C, Whitman GJ, Mahoney E, Kopans DB. (1992) Breast cancer survival among women under age 50: Is mammography detrimental? Lancet 340:991–994.
3. Wied GL, Bibbo M, Pishotta FT, Bartels PH. (1984) Intermediate cell markers for malignancy. Consistency of expression. Anal Quant Cytol 6:243–246.
4. Montag AG, Bartels PH, Lerma-Puertas E, Dytch HE, Leelakusolvong S, Bibbo M. (1989) Karyometric marker features in tissue adjacent to *in situ* cervical carcinomas. Anal Quant Cytol Histol 11:275–280.

5. Haroske G, Bergander S, König R, Meyer W. (1990) Application of malignancy-associated changes of the cervical epithelium in a hierarchic classification concept. Anal Cell Pathol 2:189–198.
6. Bibbo M, Bartels PH, Galera-Davidson H, Dytch HE, Wied GL. (1986) Markers for malignancy in the nuclear texture of histologically normal tissues from patients with thyroid tumors. Anal Quant Cytol Histol 8:167–175.
7. Bibbo M, Montag AG, Lerma-Puertas E, Dytch HE, Leelakusolvong S, Bartels PH. (1989) Karyometric marker features in tissue adjacent to invasive cervical carcinomas. Anal Quant Cytol Histol 11:281–285.
8. Bibbo M, Michelassi F, Bartels PH, Bania C, Lerma E, Montag AG. (1990) Karyometric marker features in normal-appearing glands adjacent to human colonic adenocarcinoma. Cancer Res 50:147–151.
9. Zahniser DJ, Wong KL, Brenner JF, Ball HG, Garcia GL, Hutchinson ML. (1991) Contextual analysis and intermediate cell markers enhance high-resolution cell image analysis for automated cervical smear diagnosis. Cytometry 12:10–14.
10. Hutchinson ML, Isenstein LM, Martin JJ, Zahniser DJ. (1992) Measurement of subvisual changes in cervical squamous metaplastic cells for detecting abnormality. Anal Quant Cytol Histol 14:330–334.
11. Montag AG, Bartels PH, Dytch HE, Lerma-Puertas E, Michelassi F, Bibbo M. (1991) Karyometric features in nuclei near colonic adenocarcinoma. Statistical analysis. Anal Quant Cytol Histol 13:159–167.
12. Swank PR, Greenberg DS, Hunter NR, Spjut HJ, Estrada R, Trahan EB, Montalvo J. (1989) Identification of features of metaplastic cells in sputum for the detection of squamous-cell carcinoma of the lung. Diagn Cytopathol 5:98–103.
13. Tezcan A, Garner D, Ferguson G, Palcic B. (1993) Quantitative analysis of thionin, gallocyanin and hematoxylin for automated image cytometry. 8th Ann Mtg Clinical Appl of Cytometry, Charleston, SC, Sept 15–18, Abstract #65.
14. Jaggi B, Poon SSS, MacAulay C, Palcic B. (1988) Imaging system for morphometric assessment of conventionally and fluorescently stained cells. Cytometry 9:566–572.
15. Jaggi B, Poon SSS, Pontifex B, Fengler J, Marquis J, Palcic B. (1991) A quantitative microscope for image cytometry. Proc SPIE 1448:89–97.
16. Garner D, Ferguson G, Palcic B. (1993) The Cyto-Savant system. For publication in Grohs HK, Hüsoin OAN (eds): "The Automation of Cervical Cancer Screening." (accepted).
17. Palcic B, MacAulay C, Shlien S, Treurniet W, Tezcan H, Anderson G. (1992) Comparison of three different methods for automated classification of cervical cells. Anal Cell Pathol 4:429–441.
18. MacAulay C, Lam S, Payne P, Palcic B. (1993) Malignancy associated changes in bronchial epithelial cells in biopsies. XVI Congress Intl Soc Analytical Cytology, Colorado Springs, CO, March 21–26. Cytometry Suppl 6:84, Abstract #443.
19. Susnik B, Worth AJ, LeRiche JC, Palcic B. (1993) The value of nuclear features in distinguishing ductal carcinomas *in situ* with and without an invasive component. Pathol Res Pract 189:821.
20. Susnik B. Quantitative nuclear features analysis in the prognosis of benign breast disease and ductal carcinoma *in situ*. PhD thesis, University of British Columbia, defended, November 1993.
21. Auer G, Caspersson T, Wallgren A. (1980) DNA content and survival in mammary carcinoma. Anal Quant Cytol 3:75–79.
22. Hanselaar A, MacAulay C, Palcic B, Garner D, LeRiche J. (1992) Discrimination between progressive and regressive cervical intraepithelial neoplasia (CIN) by DNA cytometry. Anal Cell Pathol 4(3):165 (abstract).