# Quantitative Nuclear Morphometry by Image Analysis for Prediction of Recurrence of Ductal Carcinoma *in Situ* of the Breast[1]

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## Abstract

**Clinical management of ductal carcinoma *in situ* (DCIS) remains a challenge because significant proportions of patients experience recurrence after conservative surgical treatment. Unfortunately, it is difficult to prospectively identify, using objective criteria, patients who are at high risk of recurrence and might benefit from additional treatment. We conducted a multi-institutional, collaborative case-control study to identify nuclear morphometric features that would be useful for identifying women with DCIS at the highest risk of recurrence. Tissue sections of archival breast tissue of 29 women with recurrent and 73 matched women with nonrecurrent DCIS were stained for DNA, and nuclei in the DCIS lesions were evaluated by image analysis. A clear correlation between mean fractal2_area (FA2) and nuclear grade was observed ($P < 0.001$), allowing an objective determination of nuclear grade. Several nuclear morphometric features, including mean and variance of variation of radius, mean area, mean and variance of frequency of high boundary harmonics (FQH), and variance in sphericity, were found to be useful in discriminating recurrent from nonrecurrent DCIS subjects. However, the nuclear features associated with recurrence differed between high- and low-grade lesions. For lesions with high FA2 (nuclear grade 3), mean variation of radius, mean FQH, and mean area alone yielded recurrence odds ratios of 4.55 [95% confidence interval (CI) 0.45–45.96], 3.86 (95% CI, 0.88–16.98), 2.90 (95% CI, 0.31–27.2), respectively. Using a summed feature model, high-FA2 lesions showing three poor prognostic features had an odds ratio of 15.63 (95% CI, 1.22–200), compared with those with zero or one poor prognostic feature. Lesions with low mean FA2 (nuclear grade 1 or 2) showing high variances in sphericity and FQH had an odds ratio of 7.71 (95% CI, 1.77–33.60). Addition of other features did not enhance the odds ratio or its significance. These results suggest that nuclear image analysis of DCIS lesions may provide an adjunctive tool to conventional pathological analysis, both for the objective assessment of nuclear grade and for the identification of features that predict patient outcome.**

## Introduction

The clinical management of breast DCIS[3] remains a critical dilemma for the oncologist and the patient because of the tumor's diverse biological behavior (1). In most cases, conservative surgery is sufficient for removing all clinically detectable disease. Nevertheless, after conservative surgery, the risk of local recurrence of DCIS or subsequent invasive disease is estimated to be 10–25% (2–9). Total mastectomy has been shown to reduce the risk of recurrence to less than 1% after DCIS, and radiation therapy after conservative surgery has been shown to reduce the risk of recurrence to 8–16% at 8 years of follow-up (2, 10, 11). Although such treatments are likely to benefit patients destined for recurrence, it could also be said that the remaining 75% of patients may suffer the morbidity of additional definitive treatment without additional benefit. In addition, tamoxifen treatment for 5 years has been shown to reduce the risk of recurrence even further (12). However, tamoxifen treatment is also associated with significant side effects, some of which can be potentially life-threatening, such as the risk of thromboembolism and endometrial cancer (13). For this reason, there is a need to identify that subgroup of patients with DCIS who are at a low risk of recurrence after conservative surgery so that these patients can be spared the added morbidity of irradiation and/or tamoxifen.

A number of approaches have been developed recently to identify the patients at highest risk of recurrence. Some of these methods focus on examining the pathological specimens for features that predict recurrence. Several studies have shown that the presence of tumor at the surgical margin predicts tumor recurrence (14, 15). For example, a recent report indicated that

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[3] The abbreviations used are: DCIS, ductal carcinoma *in situ*; OR, odds ratio; CI, confidence interval; FA2, fractal2_area; var_radi, variation of radius; FQH or freq_high_fft, frequency of high boundary harmonics; FQL or freq_low_fft, frequency of low boundary harmonics; MAC, malignancy-associated change.

margin width is a strong independent predictor of local recurrence, which ranges from an estimated rate of 5% for lesions with a margin of more than 10 mm to a rate of 63% for a margin smaller than 1 mm (2). However, the definition of a negative margin varied among reported studies, making it difficult to compare results from different sources (14, 16). Other studies have looked at conventional histological features to identify a subset of DCIS that behaves more aggressively than others (17–21). For example, nuclear grade and comedo necrosis have been reported to be valuable predictors of recurrence of both *in situ* and invasive carcinoma (17, 18, 21, 22). However, the value of nuclear grading in predicting recurrence remains an active focus of discussion, perhaps because the accuracy of subjective assessments is limited by a considerable degree of intra- and interobserver variability (23–26).

One potential way to overcome the problems associated with subjective histological assessments is to develop objective and reproducible analytical techniques to quantitate the features that pathologists use to describe the histopathological character of the breast lesion. For nuclear grading of DCIS lesions, pathologists usually evaluate such features as cell pleomorphism, pattern of irregularity in spacing among cells, variation in nuclear size, irregularity in nuclear contours, chromatin texture, nuclear:cytoplasmic ratio, position of nucleus inside the cell, number of nucleoli, presence or absence of mitosis, necrosis, and polarization of cells. Many of these features are amenable to quantitative evaluation by computer-assisted image analysis. For example, several investigators have reported high reproducibility in the assessment of morphometric features such as nuclear area, contour length, DNA content, and nuclear roundness or ellipticity using computer-assisted image analysis techniques and have related these features to prognosis in a variety of diseases (27–35). In the setting of breast cancer, for example, some investigators have reported a significant correlation between anaplastic nuclear morphometry and recurrence in stage I estrogen receptor-negative breast cancer (36, 37).

The goals of the present study were 2-fold. First, we wanted to determine whether image analysis techniques could provide an objective assessment of nuclear morphology that could complement conventional histopathological analysis. Second, we wanted to determine whether specific nuclear morphological features could identify those patients with DCIS who are at the highest risk of treatment failure. If specific nuclear morphological features of prognostic value could be identified, they might provide insight into the biological processes that underlie recurrence of DCIS, and this could lead to directed interventions to decrease the risk of recurrence. To approach these goals, a multi-institutional retrospective study was carried out using a case-control design in which DCIS lumpectomy specimens from patients who subsequently experienced recurrence were compared with specimens from matched patients whose disease did not recur. The results reported here suggest that image analysis techniques allow the identification of objective nuclear features useful for predicting local recurrence in DCIS.

## Materials and Methods

**Identification of Patient Material.** Tissue sections from lumpectomy/local excision specimens of subjects with subsequent recurrent DCIS with or without invasive cancer and subjects without recurrence were obtained through a multi-institutional collaboration that included The University of Texas M. D. Anderson Cancer Center (Houston, Texas), The Jefferson University Hospital (Philadelphia, PA), and the Van Nuys Breast Center (Van Nuys, California). These institutions were selected because they had access to large numbers of patients with DCIS who have been treated with lumpectomy (with or without radiation therapy), and they had follow-up records available. Potentially eligible subjects included women who underwent lumpectomy/local excision for DCIS, did or did not receive postsurgical radiation therapy, and either experienced recurrent disease or had no recurrence. Individuals with recurrence were first identified from the medical records, and investigators at the respective institutions reviewed the pathology reports of each patient. Two matching nonrecurrent subjects were identified for each subject with recurrent disease on the basis of age ($\pm$5 years), follow-up interval ($\pm$1 year), and radiation therapy (yes/no). On completion of the initial medical record reviews, a large number of potential subjects were deemed ineligible for various reasons, including: unavailability of the medical records, pathology slides, or tissue blocks; lack of follow-up information; or diagnosis other than DCIS (thus excluding invasive cancer, atypical ductal hyperplasia, lobular carcinoma *in situ*, or benign lesion). In addition, patients treated with total mastectomy, hormone therapy, or chemotherapy were excluded.

A total of 217 H&E-stained tissue section slides from potential subjects were obtained from the collaborating institutions, including 82 sample slides from M. D. Anderson, 50 from the Van Nuys Breast Center, and 85 from Jefferson University Hospital. These slides were reviewed by M. D. Anderson pathologists to verify the diagnosis of DCIS before review by a consensus pathology panel. Of the 217 H&E slides submitted for evaluation, 67 slides (31%) had no or insufficient tissue blocks available. An additional 9 slides (4%) were found to have a diagnosis other than DCIS. Of the remaining 141 slides with confirmed diagnoses of DCIS, 43 and 98 were associated with recurrent and nonrecurrent subjects, respectively. One slide (recurrent disease) was excluded because of a lack of clinical information, and 14 slides (nonrecurrent disease) were excluded because of lack of matching.

A consensus pathology panel consisting of six breast pathologists from the three institutions was then assembled to review the remaining 126 slides (42 from recurrent and 84 from nonrecurrent subjects) during the final selection process. A nuclear grading score was assigned to each patient sample according to the Lagios grading criteria (18). Each pathologist independently recorded the nuclear grade for each slide from recurrent and nonrecurrent subjects. The pathologists were blinded to the recurrence or nonrecurrence status of the study subjects. Slides for which agreement on grade was not unanimous were reviewed at a multiheaded microscope, and a consensus was achieved and recorded. The consensual nuclear grade was used for all analyses. Interobserver variability among the pathologists has been described elsewhere (23).

On completion of the consensus pathology panel review, 105 subjects were deemed eligible for this analysis: 39 patients (10 recurrent and 29 nonrecurrent) from M. D. Anderson, 16 patients (4 recurrent and 12 nonrecurrent) from the Van Nuys Breast Center, and 50 patients (18 recurrent and 32 nonrecurrent) from Jefferson University Hospital. The primary cause for excluding 21 potentially eligible subjects after the consensus panel review was that the consensus slide was subsequently found not to represent the primary lesion. Thus, slides from 32 recurrent and 73 nonrecurrent subjects, were deemed eligible for image analysis.

**Nuclear Morphometric Analysis.** Paraffin-embedded breast tissue blocks were cut in serial 4-$\mu$m-thick slices, dewaxed in

xylene, and rehydrated through an ethanol series. One set of slides was stained with H&E for light-microscopic evaluation. A second set of slides was stained for DNA content using the protocol of Xillix Technology Co. (Vancouver, British Columbia, Canada), including postfixation in Boehm-Sprenger fixative for 45 min, 45 min of acid hydrolysis in 5 N HCl at room temperature, staining in 0.5 mg/ml of thionin-sulfite DNA stoichiometric stain, and stopping in freshly prepared bisulfite solution. The slides from 3 recurrent DCIS subjects were found to be inevaluable, leaving 29 recurrent and 73 nonrecurrent subjects suitable for evaluation.

Nuclear morphometric analysis of the patient sample material was carried out using the Cyto-Savant computer-assisted image analysis system (Oncometrics Imaging Co., Vancouver, British Columbia, Canada). Prior to acquisition of nuclear images, the pathologist (N. S.) examined the H&E-stained specimen to locate the relevant DCIS region and then marked the DCIS region of interest on the thionin-stained slide. Each nuclear image was captured using a 20× objective with a numerical aperture of 0.75 using a high resolution 12-bit digital camera (1280 × 1024 pixels), for which each pixel had a 100% fill factor. Each pixel was 6.8 $\mu$m × 6.8 $\mu$m in size. The pixel sampling spacing within the image plane was 0.34 $\mu$m with a 0.116 $\mu m^2$ effective pixel spacing area. An average of 200–300 relevant DCIS epithelial cell nuclei and 100 lymphocyte nuclei were then selected from the marked regions by a pathologist (I. V. B.) in an interactive mode, and their nuclear images were digitally captured and stored in computer memory. Only nonoverlapping nuclei with easily detected boundaries and without "capping" were chosen. Lymphocytes were used as internal normalization controls to correct for any staining variation among slides. The ability to display the stored nuclear images at higher magnification allowed the pathologist to review all of the captured nuclei and to eliminate fragmented cells from the final analysis.

The Cyto-Savant imaging system measures 116 features based on the digital nuclear images using computational algorithms described previously (38). Features measured by the Cyto-Savant imaging system can be grouped into three general categories: nuclear morphological features, chromatin texture, and DNA content. Morphological features include nuclear area and shape parameters that reflect variations from nuclear roundness, angular variation in nuclear contour, and raggedness of the nuclear boundary. Textural features describe variations in absorbance over the nuclear area, and DNA content reflects the integrated absorbance over the nuclear area of the cell relative to that of internal control lymphocytes.

**Selection of Independent Nuclear Features.** The mean values of each of 116 nuclear features by the Cyto-Savant System were recorded as a continuous variable. Many of the nuclear features measure similar aspects of cell structure; thus, a high degree of collinearity was observed in this data set. Because the presence of collinear features can degrade the performance of an algorithm as well as increase type I error, the number of morphometric variables was reduced to a manageable level using a diagnostic of collinearity devised by Belsley (39). Briefly, a set of variables was considered collinear if a high-scaled condition index was associated with high-scaled variance-decomposition proportions for two or more estimated regression coefficient variances. Condition indices were deemed high when they were >30, and variance proportions were deemed high when they were >0.5. This variable selection procedure was essentially a forward selection procedure. Given a set of (scaled) variables already selected for inclusion in the model, each remaining variable was examined in turn to determine whether its inclusion would result in unacceptable collinearity. Then, from the set of variables that would not give rise to collinearity, variables were selected if, when added to the variables already included, they produced a data set with the smallest overall condition number. The condition number is a measure of the overall level of collinearity present in a data set. This process was repeated until no candidate variables were able to pass the entrance screening procedure. The set of variables selected in this fashion does not necessarily represent the minimum size nor does it possess the lowest degree of collinearity of all subsets of equal size. Rather, it is a subset of variables whose acceptable level of collinearity is such that no variable can be added without raising collinearity to an unacceptable level. To address the absence of uniqueness, the procedure was repeated using each variable in turn as the sole initial candidate.

We also ran backward stepwise logistic regression analyses using varying stringencies as an initial variable screen using both means and variances of nuclear features. Backward selections with stringencies of 0.01, 0.02, 0.05, and 0.10 were performed.

**Statistical Analysis.** The Mann-Whitney *U* test was used to compare values of each morphological feature between recurrent and nonrecurrent study subjects. Each nuclear feature was compared between these two groups, stratified by the consensual nuclear grade of each sample using a box-plot analysis. The mean nuclear feature values were dichotomized into low and high values using the median value of the whole study population as cutoff point (excluding nuclear grade 1 data).

One objective of this study was to estimate the risk of recurrence among patients who were diagnosed with DCIS after lumpectomy/local excision. ORs and 95% CIs for each feature were computed by a logistic regression model based on a maximum likelihood method using the Statistical Package for the Social Sciences (SPSS Inc., Chicago, Illinois) and STATA computer software programs (Stata Corporation, College Station, Texas). Individual matching on age and follow-up interval was suboptimal in some instances; therefore, these variables were adjusted during unconditional multivariate modeling. A *P* below 0.05 was considered statistically significant.

## Results

A total of 432 candidate patients with a diagnosis of DCIS during the period 1973–1995 were initially identified by the three participating institutions, including 83 patients (17 recurrent and 66 nonrecurrent subjects) from M. D. Anderson Cancer Center, 163 patients (20 recurrent and 143 nonrecurrent subjects) from the Van Nuys Breast Center, and 186 patients (34 recurrent and 152 nonrecurrent subjects) from Jefferson University Hospital. As described above, 59% of the potentially eligible recurrent DCIS subjects and 80% of potentially eligible nonrecurrent DCIS subjects did not meet the study's eligibility requirements by the end of the consensus review or did not have evaluable DCIS specimens, leaving a total of 102 evaluable patients (29 recurrent and 73 nonrecurrent).

As shown in Table 1, the median age was similar among subjects with or without recurrence. The median length of follow-up appeared to be longer for nonrecurrent than for recurrent subjects, partly because disease recurrence interrupted follow-up. During selection of nonrecurrent DCIS subjects, an attempt was made to find patients whose surgery was performed in close proximity to the time of surgery of the relevant recurrent subjects (±5 years) to ensure that changes in patterns

*Table 1* Patient characteristics

| Variable | No. of subjects | |
|---|---|---|
| | Recurrent DCIS | Nonrecurrent DCIS |
| Total | 29 | 73 |
| Median age, yr | 49 | 53 |
| (range) | (30–87) | (35–93) |
| Median length of follow-up, mo | 25 | 55 |
| (range) | (11–184) | (7–269) |
| Lagios nuclear grade (%) | | |
| 1 | 5 (17.2) | 4 (5.5) |
| 2 | 14 (48.3) | 40 (54.8) |
| 3 | 10 (34.5) | 29 (39.7) |
| Necrosis[a] (%) | | |
| Absent | 6 (21.4) | 18 (24.7) |
| Punctate | 7 (25.0) | 19 (26.0) |
| Extensive | 15 (53.6) | 36 (49.3) |
| Received radiotherapy (%) | | |
| Yes | 8 (27.6) | 28 (38.4) |
| No | 21 (72.4) | 45 (61.6) |

[a] One case was excluded from the necrosis evaluation.

of care over time did not bias the analysis. When this was not possible, nonrecurrent subjects from earlier time periods were chosen. Fifty-four % of recurrent and 49% of nonrecurrent subjects had extensive necrosis, whereas absence of necrosis was more common among the nonrecurrent group (25%) than the recurrent group (21%). These differences were not statistically significant. Whereas the percentage of grade 3 lesions was similar among the two groups, grade 2 lesions were slightly more numerous among nonrecurrent subjects. ($P$ was not significant). No significant difference was seen between recurrent and nonrecurrent subjects with regard to radiation treatment; however, the participating institutions differed in patient management. The majority of patients (86%) treated at the M. D. Anderson Cancer Center received radiation therapy, whereas none received radiation at Jefferson University Hospital, and only 31% treated at the Van Nuys Breast Center did so.

Tissue sections from patients' resected tissue or biopsies were subjected to a DNA-staining protocol, and the nuclei in the DCIS lesions were analyzed for DNA content, nuclear morphometry, and nuclear texture by image analysis techniques. To limit the focus of statistical analyses to independent and relevant features, three strategies were used. First, the level of collinearity among nuclear features was assessed, and independent features were chosen. Second, backward step-wise logistic regression analyses were performed to identify potentially prognostic features. And third, nuclear features were examined that were believed to reflect histological characteristics often used by pathologists to grade DCIS. For the sake of the present analysis, the population means and variances for both independent and relevant features were used for statistical analyses.

Because many of the quantified features provided by the Cyto-Savant image analysis system reflect similar aspects of nuclear structure, a high degree of collinearity between several variables was observed. After a procedure described by Belsley (39) was applied to identify collinearity, the means of eight features turned out to be independent and informative, including the following: (*a*) low_den_obj (a measure of the number of grouped pixels with low absorbance); (*b*) lowDNAarea (a measure of the fraction of the nucleus occupied by low-density pixels); (*c*) var_radi (a measure of the variance of length of the object's radius vectors); (*d*) FA2 (FA2, a measure of nuclear texture that reflects the frequency of changes from low-absorbance regions to high-absorbance regions); (*e*) harmon3 (a nuclear morphometric measure that estimates low harmonic changes in the nuclear boundary); (*f*) range_extreme (a measure of nuclear texture that describes intensity differences between the largest local maximum and the smallest local minimum, normalized against the slide DNA content); (*g*) hiDNAcomp (a nuclear texture feature that represents the degree of compactness of the medium- and high-density regions, treated as a single object); and (*h*) high_cnt_mass (a measure of the separation between the geometric center of the high-absorbance clusters and geometric center of the whole object, normalized by its mean_radius). No statistically significant differences were observed in the median values of recurrent and nonrecurrent subjects for these features.

We also ran backward step-wise logistic regression analyses using varying stringencies for selection of nuclear features using both mean and variance data. Although several new features were identified by backward step-wise logistic regression analyses, none proved to be stronger discriminators than features chosen by Belsley's method (39). However, variances in nuclear sphericity and FQH showed stronger discriminatory power to separate recurrent DCIS from nonrecurrent DCIS with low FA2.

One of the goals of this study was to use image analysis techniques to quantify nuclear features (*e.g.*, size, pleomorphism) commonly used by pathologists to describe and grade DCIS. We, therefore, examined the usefulness of means of the following eight features provided by the Cyto-Savant instrument: (*a*) nuclear area (total number of pixels belonging to the object); (*b*) elongation (the extent of the object along the major axis *versus* the perpendicular direction); (*c*) eccentricity (the ratio of the major axis to minor axis of the best-fit ellipse); (*d*) sphericity (minimum-to-maximum radius ratios, measured from the centroid of the nucleus); (*e*) compactness (a measure of the overall absorbance of the nucleus); (*f*) var_radi (a measure of the variation of the radius of the nucleus); (*g*) freq_low_fft (FQL, a measure of the low harmonic variations in the boundary of the nucleus); and (*h*) freq_high_fft (FQH, a measure of the high harmonic variations in the boundary of the nucleus). The mean values of recurrent and nonrecurrent subjects were dichotomized into high- and low-value groups for each feature based on the median for the total population. As shown in Table 2, trends for differences between recurrent and nonrecurrent subjects were observed for several of these nuclear features. For example, the median value of elongation was found to be significantly higher among recurrent compared with nonrecurrent subjects ($P = 0.04$). Likewise, the median value of eccentricity was also found to be significantly higher among recurrent than nonrecurrent subjects ($P = 0.05$).

Because nuclear grade has been suggested to predict likelihood of recurrence, we compared features among recurrent and nonrecurrent subjects stratified by nuclear grade. Interestingly, differences in the median values of some features between recurrent and nonrecurrent subjects were found for nuclear grade 2 but not for nuclear grade 3, and *vice versa*. For example, as shown in Fig. 1, high *versus* low median nuclear area distinguished recurrent and nonrecurrent subjects with grade 3 lesions, but not those with grade 1 and 2 lesions. In contrast, nuclear elongation was a better discriminator between recurrent and nonrecurrent subjects with grade 1 and 2 lesions (Fig. 2). Other features statistically associated with outcome of grade 2 lesions were eccentricity, sphericity, and compactness.

Although several investigators have suggested an association between higher nuclear grade and higher risk of local

*Table 2* Comparison of mean nuclear features that reflect histological characteristics

| Nuclear morphological features | Recurrent DCIS ($n$ = 29), median (range) | Nonrecurrent DCIS ($n$ = 73), median (range) | $P$ |
|---|---|---|---|
| Area | 438.07 (201.92–688.29) | 456.75 (249.60–822.02) | 0.43 |
| Eccentricity | 1.44 (1.19–1.62) | 1.41 (1.22–1.59) | 0.05 |
| Elongation | 1.49 (1.20–1.68) | 1.45 (1.24–1.64) | 0.04 |
| Sphericity | 0.61 (0.53–0.75) | 0.62 (0.54–0.74) | 0.08 |
| Compactness | 1.19 (1.12–1.29) | 1.19 (1.11–1.27) | 0.13 |
| Freq_low_fft | 28.87 (8.80–62.65) | 25.29 (7.25–49.57) | 0.13 |
| Freq_high_fft | 2.20 (1.62–3.29) | 2.08 (1.40–2.89) | 0.06 |

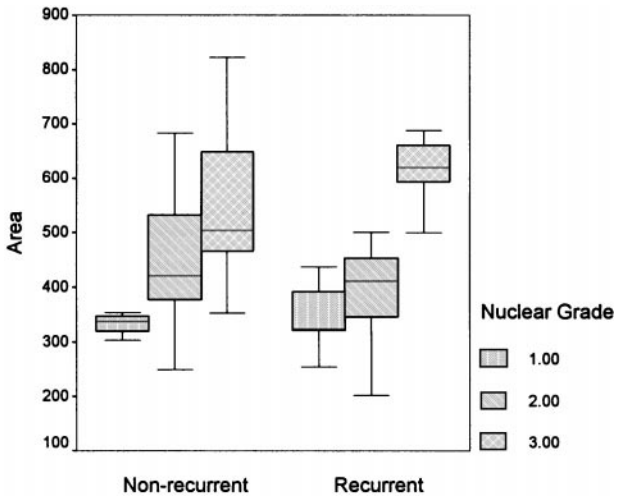


*Fig. 1.* Relationship between mean nuclear area and nuclear grade for recurrent and nonrecurrent DCIS subjects.

recurrence, this claim remains controversial, perhaps because of the variability among pathologists in grading DCIS lesions. For example, in our own study in which six highly experienced breast pathologists formed a consensus panel, interobserver reproducibility in grading DCIS lesions was much higher at nuclear grades 3 *versus* 2 than at grades 1 *versus* 2 (27). We, therefore, sought nuclear features that could be measured objectively by image analysis techniques and would be highly correlated with nuclear grade. As shown in Fig. 3, a clear correlation between FA2 and nuclear grade was observed ($P <$ 0.001). Representative nuclear images of low and high FA2 are shown in Fig. 4, *a* and *b*.

Because mean FA2 correlated with nuclear grade, and because different nuclear features were found useful for discriminating between nuclear grades of recurrent and nonrecurrent subjects, we chose to dichotomize subsequent analyses into high and low FA2 groups using the median FA2 value (*i.e.*, 968.9) of the total study population as a cutoff point. We then reexamined the nuclear features that showed promise for distinguishing recurrent and nonrecurrent subjects in the whole population and applied these separately to the high and low FA2 groups. For each nuclear feature, the high- *versus* low-value cutoff was determined based on the median value for the grade 2 and 3 lesions, and ORs were determined. Grade 1 lesions were not included in the cutoff determination because

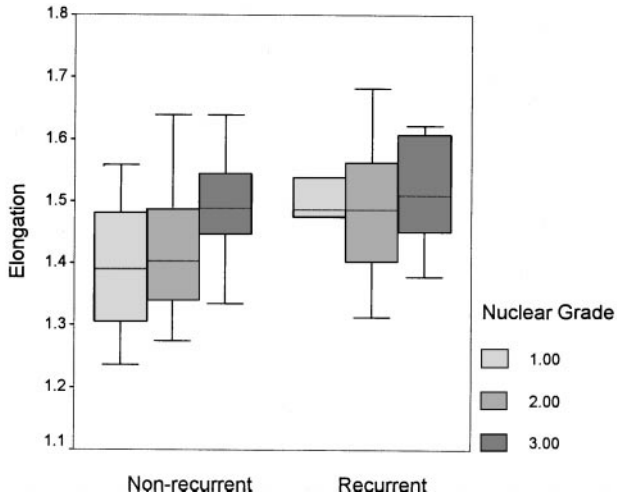


*Fig. 2.* Relationship between mean nuclear elongation and nuclear grade for recurrent and nonrecurrent DCIS subjects.

they were few in number and considered highly selected. As shown in Table 3A, the means of different nuclear features were found to be useful for discriminating recurrent and nonrecurrent subjects in the high and low FA2 populations. Features showing highest ORs for the high FA2 group included var_radi (OR, 4.55; $P$ = 0.20), nuclear area (OR, 2.90; $P$ = 0.35), and FQH (OR, 3.86; $P$ = 0.07). In contrast, nuclear features yielding the highest ORs for subjects with low FA2 values included var_radi (OR, 4.93; $P$ = 0.07), elongation (OR, 4.02; $P$ = 0.04), and FQL (OR, 3.15; $P$ = 0.09).

A variety of algorithms have been used for the identification of predictive nuclear features (40–42). Some authors have found it useful to examine variances of nuclear features rather than means. We, therefore, reexamined selected features using variance data. High and low FA2 groups were determined using the same median FA2 mean value of the total study population as a cutoff point. However, for variance analysis, the high- *versus* low-value cutoff for each nuclear feature was determined based on the median value for the grade 2 and 3 lesions, and odd ratios were determined. Again, different nuclear features were found to be useful for separating recurrent and nonrecurrent individuals in the high and low FA2 populations. As shown in Table 3B, features showing highest ORs for the high FA2 group included var_radi (OR, 4.24; $P$ = 0.26), nuclear sphericity (OR, 3.00; $P$ = 0.21), and nuclear eccentricity (OR, 2.17; $P$ = 0.36). In contrast, nuclear features yielding the highest ORs for subjects with low FA2 values were sphericity (5.76; $P$ = 0.02), FQH (4.80; $P$ = 0.02), var_radi (OR, 4.93; $P$ = 0.07), eccentricity (OR, 4.73; $P$ = 0.02), and elongation (OR, 4.21; $P$ = 0.03). Representative nuclear images of cells with high and low sphericity, area, and variation in radius are shown in Fig. 4, *c*–*h*.

To determine whether a combination of nuclear features might provide better discrimination between recurrent and nonrecurrent subjects, multiple feature sum-score models were developed in which a value of one was assigned to a high feature value associated with recurrence, a value of zero was assigned to a low feature value not associated with recurrence, and then a sum was determined for each subject. These analyses were performed separately for the high and low FA2 groups. To determine the significance of an OR based on the resulting

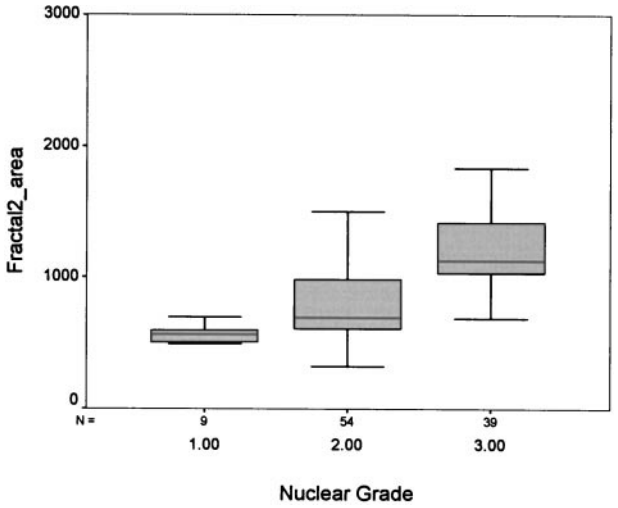


*Fig. 3.* Relationship between mean FA2 and nuclear grade.

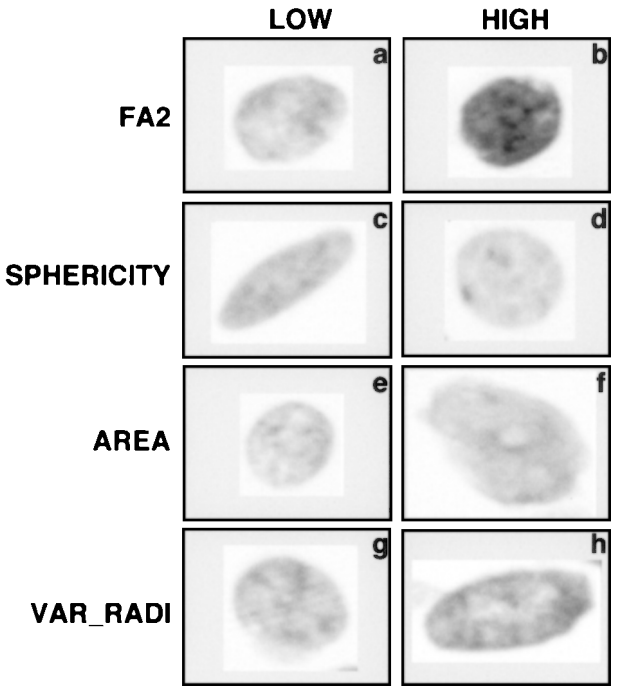


*Fig. 4.* Nuclear images of cells with high and low feature values. Examples of nuclei with low (*a*) or high (*b*) FA2; sphericity, low (*c*) or high (*d*); area, low (*e*) or high (*f*); and var_radi, low (*g*) or high (*h*).

sums of feature values, the summed scores were dichotomized into low and high categories depending on the proportion of recurrent and nonrecurrent subjects in each category. For example, as shown in Table 4, subjects with a high mean FA2 who also exhibited both high mean nuclear area and high mean var_radi had a 7-fold higher likelihood of experiencing a recurrence than subjects not showing both high feature values ($P = 0.09$). Moreover, subjects with evidence of three poor morphometric prognostic features (*i.e.*, high mean nuclear area, high mean variation in radius, and high mean FQH had only a slightly higher OR (8.74); however, the result was highly significant ($P = 0.01$).

A similar type of summed analysis was carried out for the subjects with low FA2. Because it was found that the variance of nuclear features provided stronger discriminating power between recurrent and nonrecurrent subjects in this setting, we used the variances of nuclear features for subsequent analyses of subjects with a low mean FA2. As was shown in a univariate analysis (Table 3B), subjects whose nuclei showed high variance in sphericity had an OR of 5.76 for recurrence ($P = 0.02$). The addition of variance in FQH increased this OR to 7.71 for recurrence ($P = 0.007$; Table 5). However, the addition of variance in var_radi did not further enhance this OR or its significance (Table 5). Therefore, we took variances in sphericity and FQH as the most discriminating features for recurrent subjects with low mean FA2.

A summary of these findings is presented as a decision tree in Fig. 5. The patients were first dichotomized according to their specimen's mean FA2 value. For this group, for which recurrent and nonrecurrent subjects were selected independent of nuclear grade, 38% of recurrent and 49% of nonrecurrent subjects had high mean FA2 (≥968.9). However, 6 (55%) of 11 of the recurrent subjects with high mean FA2 and three poor nuclear features showed recurrence, compared with 5 (13.9%) of 36 of the high mean FA2 cases without all three poor features. Similarly, for the subjects with low mean FA2 values, 12 (55%) of 22 of the recurrent subjects with high variances in sphericity and FQH showed recurrence compared with 6 (18%) of 33 of the subjects with low variance values.

The results of these summary scores were dichotomized into high- and low-risk categories for the sake of statistical analysis. However, this approach does not permit distinction of intermediate and very high levels of risk. To determine whether the nuclear features and the level of risk have a dose-response relationship, the ORs were plotted as a function of summary score for the subjects with high mean FA2 values. As shown in Fig. 6, a dose-response relationship was observed between the risk of recurrence and increasing summary scores: subjects with two poor morphometric prognostic features had an OR for recurrence of 2.25 (95% CI, 0.19–27.18; $P = 0.52$), whereas subjects with three poor morphometric prognostic features had an OR for recurrence of 15.63 (95% CI, 1.22–200.19; $P = 0.04$).

## Discussion

The long-term goal of these studies is to identify individuals with DCIS of the breast who are at greatest risk of recurrence and would most benefit from definitive treatment such as adjuvant radiotherapy. The approach taken in this study was to carry out a case-control type analysis using nuclear morphometric image analysis techniques to objectively identify the characteristics of lesions that discriminate high-risk DCIS patients. Several nuclear morphometric features (*i.e.*, mean FA2, mean and variance in var_radi, mean area, mean and variance FQH, and variance in sphericity) were found most useful. Interestingly, however, the nuclear features associated with increased likelihood of recurrence were different for subjects with nuclear grade 2 lesions (low FA2) than for subjects with nuclear grade 3 lesions (high FA2). For example, area, var_radi, and the frequency of high harmonics in the nuclear perimeter (FQH) were found to be important in discriminating recurrent from nonrecurrent subjects with high FA2. In contrast, variances in sphericity, FQH, and var_radi were found to be important in separating recurrent from nonrecurrent subjects with low FA2. In addition, we found a dose-response relationship

*Table 3* ORs for recurrence of DCIS

| Nuclear morphometric features[b] | High fractal2_area[c] | | Low fractal2_area[d] | |
|---|---|---|---|---|
| | OR (95% CI) | *P* | OR (95% CI) | *P* |
| A. ORs[a] for recurrence based on means of individual morphometric features | | | | |
| Low area | 1.0 | | 1.0 | |
| High area | 2.90 (0.31–27.2) | 0.35 | 0.61 (0.09–4.21) | 0.61 |
| Low eccentricity | 1.0 | | 1.0 | |
| High eccentricity | 1.15 (0.24–5.5) | 0.87 | 2.9 (0.77–11.56) | 0.11 |
| Low elongation | 1.0 | | 1.0 | |
| High elongation | 1.33 (0.28–6.3) | 0.72 | 4.02 (1.04–15.63) | 0.04 |
| Low sphericity | 1.0 | | 1.0 | |
| High sphericity | 1.0 (0.20–4.89) | 0.99 | 0.36 (0.10–1.36) | 0.13 |
| Low compactness | 1.0 | | 1.0 | |
| High compactness | 1.17 (0.24–5.77) | 0.85 | 3.0 (0.70–12.98) | 0.14 |
| Low var_radi | 1.0 | | 1.0 | |
| High var_radi | 4.55 (0.45–45.96) | 0.20 | 4.93 (0.86–28.25) | 0.07 |
| Low freq_high_fft | 1.0 | | 1.0 | |
| High freq_high_fft | 3.86 (0.88–16.98) | 0.07 | 1.17 (0.31–4.47) | 0.82 |
| Low freq_low_fft | 1.0 | | 1 | |
| High freq_low_fft | 2.55 (0.43–14.98) | 0.30 | 3.15 (0.83–11.88) | 0.09 |
| B. ORs[a] for recurrence based on variances of individual morphometric features | | | | |
| Low area | 1.0 | | 1.0 | |
| High area | 0.96 (0.14–6.80) | 0.97 | 0.24 (0.02–2.78) | 0.25 |
| Low eccentricity | 1.0 | | 1.0 | |
| High eccentricity | 2.17 (0.41–11.51) | 0.36 | 4.73 (1.24–18.05) | 0.02 |
| Low elongation | 1.0 | | 1.0 | |
| High elongation | 1.84 (0.35–9.63) | 0.47 | 4.21 (1.13–15.78) | 0.03 |
| Low sphericity | 1.0 | | 1.0 | |
| High sphericity | 3.00 (0.55–16.40) | 0.21 | 5.76 (1.38–23.94) | 0.02 |
| Low compactness | 1.0 | | 1.0 | |
| High compactness | 1.70 (0.32–8.94) | 0.53 | 2.67 (0.70–10.16) | 0.15 |
| Low var_radi | 1.0 | | 1.0 | |
| High var_radi | 4.24 (0.35–51.20) | 0.26 | 4.62 (0.77–27.55) | 0.09 |
| Low freq_high_fft | 1.0 | | 1.0 | |
| High freq_high_fft | 1.17 (0.27–5.14) | 0.84 | 4.80 (1.24–18.67) | 0.02 |
| Low freq_low_fft | 1.0 | | 1.0 | |
| High freq_low_fft | 2.04 (0.41–10.34) | 0.39 | 1.66 (0.44–6.25) | 0.45 |

[a] Odds ratios are adjusted for age at diagnosis, follow-up intervals, radiotherapy, and nuclear grade.
[b] High value is defined by a value above the median of the whole group (excluding grade 1) subjects.
[c] Represents 11 recurrent and 36 non-recurrent subjects.
[d] Represents 18 recurrent and 37 non-recurrent subjects.

between risk of recurrence and increasing summed scores computed by combining "bad" features (mean area, var_radi, and FQH) in subjects with high mean FA2.

On the basis of the previous literature, we expected nuclear grade to be predictive of recurrence. However, despite the fact that the recurrent and nonrecurrent subjects were not matched histologically, nuclear grade did not predict recurrence in this population. The reason for this finding is not well understood. One possible explanation may be the problem of interobserver variability in grading DCIS lesions. For example, whereas interobserver reproducibility of distinguishing nuclear grade 3 and 2 lesions has been reported to be superior to that of differentiating nuclear grades 2 and 1, disagreement rates between pathologists still approach 30–40% for nuclear grade 2 and 3 lesions and more than 60% for grade 1 and 2 lesions (23, 24). This interobserver variability may be based on the subjective quality of distinguishing characteristics such as nuclear pleomorphism, nucleolar prominence, and hyperchromasia.

To overcome this problem, we used image analysis to objectively quantify nuclear features and sought features that could distinguish nuclear grade. Not only could this approach provide a more continuous measure, it raised the possibility of detecting subtle nuclear textural changes that even experienced eyes might find difficult to identify in reproducible fashion. The results of this study suggest that the nuclear morphometric feature FA2 correlates well with nuclear grade. This feature combines DNA content, nuclear area, and nuclear texture into one measure. Because different nuclear features were subsequently found to distinguish recurrent and nonrecurrent subjects with nuclear grade 2 or 3 lesions, it was important to define a quantifiable feature that was closely associated with

*Table 4* ORs[a] based on summed features for subjects with high mean FA2

| Summary score | No. of recurrent DCIS subjects (%) | No. of nonrecurrent DCIS subjects (%) | ORs | 95% CI | *P* |
|---|---|---|---|---|---|
| | | Two-feature[b] model | | | |
| 0–1 | 1 (9) | 16 (44) | 1.0 | | |
| 2 | 10 (91) | 20 (56) | 7.06 | 0.74–67.68 | 0.09 |
| | | Three-feature[c] model | | | |
| 0–2 | 5 (45) | 31 (86) | 1.0 | | |
| 2–3 | 6 (55) | 5 (14) | 8.74 | 1.62–47.33 | 0.01 |

[a] ORs are adjusted for age at diagnosis, follow-up intervals, radiotherapy, and nuclear grade.
[b] Features include mean nuclear area and mean var_radi.
[c] Features include mean nuclear area, mean var_radi, and mean freq_high_fft.

*Table 5* ORs[a] based on summed features[b] for subjects with low mean FA2

| Summary score | No. of recurrent DCIS subjects (%) | No. of nonrecurrent DCIS subjects (%) | ORs | 95% CI | *P* |
|---|---|---|---|---|---|
| | | Two-feature[b] model | | | |
| 0–1 | 6 (33) | 27 (73) | 1.0 | | |
| 2 | 12 (67) | 10 (27) | 7.71 | 1.77–33.60 | 0.007 |
| | | Three-feature[c] model | | | |
| 0–1 | 6 (33) | 25 (68) | 1.0 | | |
| 2–3 | 12 (67) | 12 (32) | 6.00 | 1.40–25.75 | 0.02 |

[a] Odds ratios are adjusted for age at diagnosis, follow-up intervals, radiotherapy, and nuclear grade.
[b] Features include variance in nuclear sphericity and variance in freq_high_fft.
[c] Features include variance in nuclear sphericity, variance in freq_high_fft, and variance in var_radi.

nuclear grade so that the whole evaluation could remain objective.

To the best of our knowledge, although similar approaches have been used in other disease settings (34, 43), this is the first case-control study to examine the potential role of image analysis of nuclear morphometry in predicting recurrence of DCIS. Other studies have examined the role for image analytic techniques in objective nuclear grading and distinguishing benign *versus* invasive breast disease. For example, Bacus *et al.* (44) showed in a parallel study using another analytical system that DCIS pathological nuclear grades 2 and 3 could be distinguished from each other and from normal breast epithelium by determination of a mean morphometric nuclear grade based on a standard deviation-derived *Z*-score using four nuclear features (*i.e.*, area, DNA content, entropy, and valley). Nuclear grade 1 samples were not included in their model. It should be noted that these same features are also part of the determination of the FA2 feature used in this study. Another study (45) used image analysis techniques to focus on the normal-appearing epithelial cells in the field of breast lesions in an attempt to distinguish benign and malignant breast disease. The authors defined MACs based on eight nuclear features (including FA2 and absorbance skewness) and found that they could discriminate benign tumor from breast carcinoma in 86% of cases by using a cutoff value of 34% MAC nuclei. In that study, however, DCIS lesions and invasive ductal carcinoma showed considerable overlaps in MAC values, and the relationship of these values to recurrence was not examined.

The population examined in our study was unique in that it included a cohort of patients with DCIS who underwent breast-sparing surgery at a time when most patients were treated with mastectomy. These subjects, therefore, had a longer follow-up. Also unique was the collaboration in histological evaluations of slides by a consensus panel, consisting of expert pathologists from three well-known cancer centers. Although they reached consensus in each case, disagreement remained with regard to grade and presence or absence of necrosis.

The study was limited by several factors. First, the total number of recurrent subjects for inclusion in the study was limited. Although 432 patients diagnosed with DCIS were originally identified as potential candidates, only 102 patients were included in the analysis because of the need for matching recurrent and nonrecurrent subjects and the availability of evaluable tissue blocks and slides for review. This limited our ability to adjust these results for other factors previously shown to be related to a likelihood of recurrence, including histological subtype, body mass index, hormone replacement therapy, or potential responsiveness to subsequent treatment such as radiation and/or tamoxifen (6, 46, 47). Confirmation of these associations await enlarged studies that control for previously described prognostic factors. Nevertheless, despite the relatively small number of recurrent and nonrecurrent subjects, intriguing associations between objective nuclear features and recurrence risk were identified.

A second limitation of the study was that data on margin status, tumor size, and the absence or presence of comedo-type necrosis for the majority of recurrent and nonrecurrent subjects were not readily available from all of the participating institutions. Recent reports suggest that DCIS patients with surgical margins of $>10$ mm (in every direction) have extremely low recurrence rates with or without radiation therapy (2, 14, 15), whereas patients showing $<1$-mm margins have a recurrence rate of 63% without and 29% with postoperative radiation (2). Moreover, prediction of recurrence risk is claimed to be improved if the size of the lesion and nuclear grading are also taken into account (17). Similarly, Cheng *et al.* (14) evaluated the relationship of margin status and likelihood of recurrence among patients with DCIS undergoing breast-conserving surgery and found that tumor size and margin status are independent predictors of residual disease. However, the definition of a

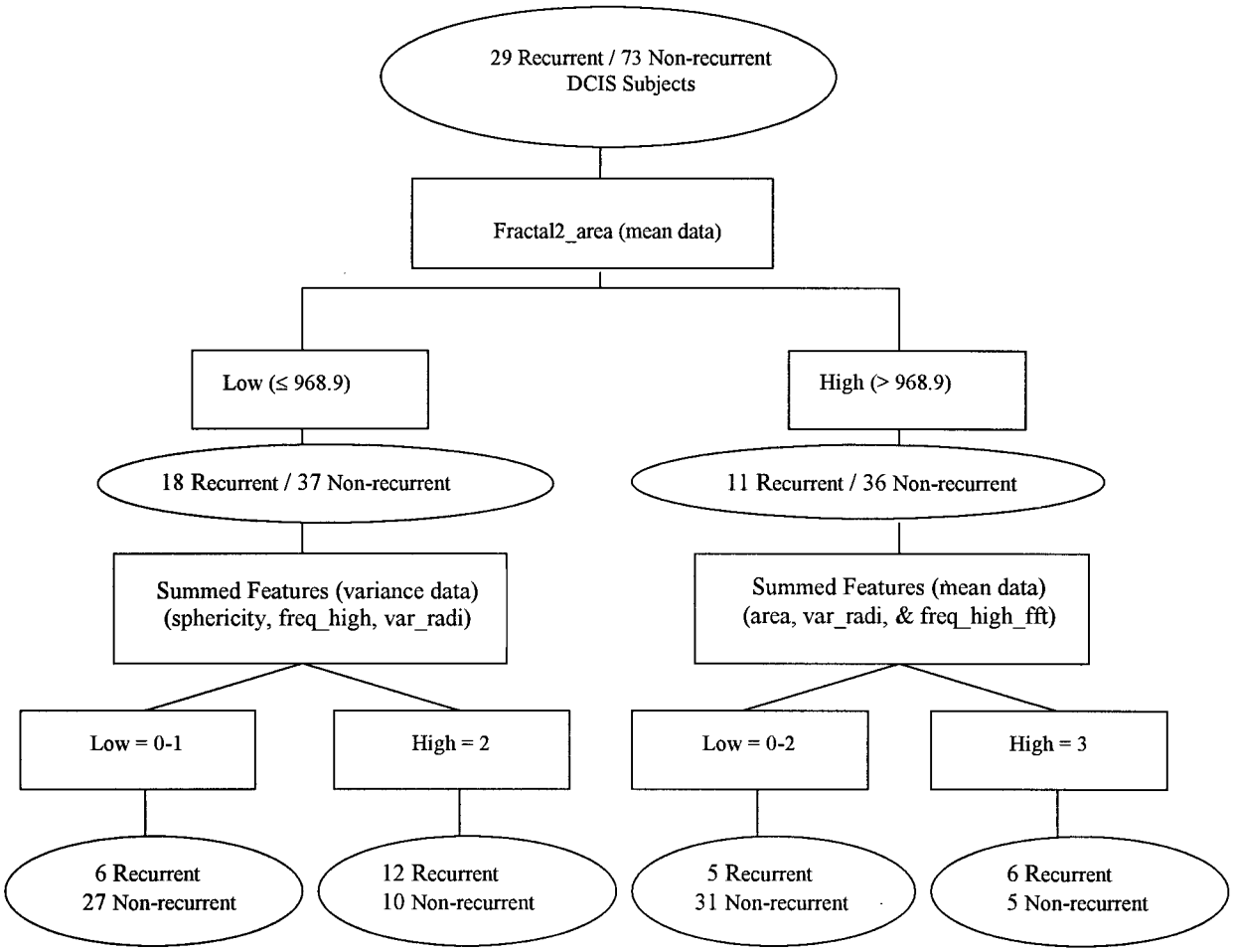


*Fig. 5.* Decision tree for assessing recurrence risk based on FA2, area, var_radi, FQH, and sphericity. □, rectangles show decisions. ⬭, ovals show number of recurrent and nonrecurrent DCIS subjects.

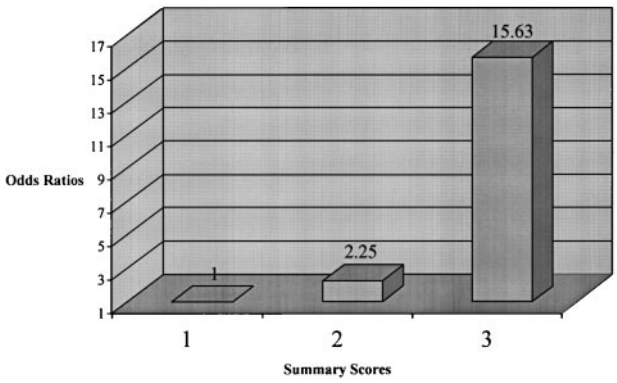


*Fig. 6.* ORs of summary scores of features with high mean FA2.

negative-free margin differs widely among investigators reporting DCIS data, and the results of studies from different institutions are difficult to compare. Thus, in the present study, it was difficult to correlate these nuclear image analysis features with other factors that may be important for predicting the risk of recurrence. Nevertheless, this approach may prove useful for identifying patients with <10-mm margins that may benefit from treatment beyond local excision.

Although the biological mechanisms underpinning local recurrence are not fully understood, two possibilities are usually considered. First, local recurrence may result from the presence of residual tumor cells outside the resected margin. Alternatively, recurrence may be caused by the presence of a multiclonal field. Both possibilities can be supported by the fact that wider resection margins and mastectomy reduce the risk of recurrence significantly. However, the findings that (*a*) DCIS lesions and invasive cancers from the same resection show many common sites of loss of heterozygosity (48), and (*b*) recurrent lesions exhibit many genetic changes in common with the original DCIS lesion (49), favor the first possibility.

Interestingly, lower-grade lesions have been reported to recur as invasive cancer more frequently than higher-grade lesions (50). In this regard, it was interesting that two nuclear features found important for predicting recurrence in subjects with lower FA2 (*i.e.*, lower nuclear grade) were high nuclear elongation and high nuclear eccentricity. Recent studies with mammary cells *in vitro* and *in vivo* have demonstrated that an early event in tumorigenic progression and invasion is increased cell migration associated with a change in cell morphology to an elongated form and a change from epithelial to

mesenchymal features (51–53). The possibility exists, therefore, that the finding of high nuclear elongation in high-risk, lower-grade lesions represents an early stage of epithelial migration, whereby some DCIS cells have developed the ability to migrate along the duct. If these cells migrate beyond the surgical margin without establishing a focus of identifiable DCIS, they may represent the seeds of the future recurrence. To test this hypothesis, we are currently examining the margins of DCIS lesions for the presence of cells showing nuclear features associated with recurrence.

In summary, the current study suggests that nuclear image analysis of DCIS lesions may be an important tool for objective pathological analysis, for both assessment of nuclear grade and determination of features that predict patient outcome. The use of such an approach will overcome the limitations of interobserver agreement and may provide an important adjunct to other clinical procedures for identifying patients with DCIS who are at highest risk of recurrence and may benefit from additional treatment. In the long run, the hope is that the nuclear features discovered to be associated with recurrence will lead to new biological insights regarding the cellular pathways that underlie recurrence and lead to new targeted treatment strategies.

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