Cytometry (Communications in Clinical Cytometry) 38:214–223 (1999)

Nuclear Morphometry as an Intermediate Endpoint Biomarker in Chemoprevention of Cervical Carcinoma Using α-Difluoromethylornithine

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The use of nuclear morphometry as an intermediate endpoint biomarker is described in a Phase I, dose-seeking trial of chemoprevention of cervical cancer, using the agent α-difluoromethylornithine (DFMO). Thirty patients with grade III cervical intraepithelial neoplasia (CIN III) were enrolled, and these received daily doses of DFMO at 0.06–1.0 mg/m^2 for a period of 1 month. Fifteen patients were observed to have a complete or partial regressive response to the agent, as assessed by histopathology. No significant differences in cell feature measurements were found between responders and nonresponders in specimens obtained before treatment, indicating that it may be difficult to predict response on the basis of these measurements. In specimens collected after treatment, large differences in morphometric features were observed between responders and nonresponders, indicating a differential effect of DFMO. Significantly modulated features were considered in terms of their correlations with CIN grade, which was determined from an independent set of measurements from archival tissue. Differences between features were consistent with a deletion of cells with high grade nuclei in the responders, and with the persistence of a more heterogeneous population of high grade cells in the nonresponders. Based on an independent set of measurements from archival material, a morphometric index of progression was derived, yielding a quantitative measure of the degree of nuclear atypia in these lesions. When applied to this trial, the morphometric index was seen to be specifically and consistently decreased in responsive lesions, and unchanged in nonresponders. The study indicates that morphometric features fulfill the requirements for an intermediate endpoint biomarker of cervical cancer chemoprevention. Cytometry (Comm. Clin. Cytometry) 38: 214–223, 1999.

Organized screening for cervical cancer using the Pap smear has been shown to reduce the incidence and mortality figures for this disease by 85% (13), showing that this is a sensitive and effective method for detection of carcinogenesis in the uterine cervix. These screening programs generate an enormous number of specimens each year which are positive or ''not negative'' for pre-invasive lesions, termed cervical intraepithelial neoplasia (CIN).

It is the management of this large volume of pre-invasive lesions that presents the most serious challenge to the health care system. While current models of carcinogenesis identify CIN as potential precursors to invasive cancer, epidemiological studies have indicated that if untreated most of these lesions would never progress to this stage (13,19,29). In the absence of reliable predictors of risk, surgical intervention is a rational standard of care for high grade CIN, although this represents over-treatment for the great majority of patients. Chemoprevention of cervical cancer may present an alternative to the morbidity associated with this over-treatment, and may ultimately have a major impact on the management of this disease.

The most immediate clinical benefits of chemoprevention of cervical cancer may be realized in the treatment of multifocal disease, which is frequently observed to involve the entire lower female genital tract. Multifocal disease has been linked to heterogeneous patterns of human papillomavirus infection, and has been strongly associated with a field effect of transformation and multistep carcinogenesis (15,30). Successful chemoprevention of cervical cancer will provide an urgently needed method for systemic treatment of these lesions.

Grant sponsor: National Cancer Institute; Grant numbers: NO1-CN-25433A, NO1-CN-25433B.

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Received 18 November 1998; Accepted 18 May 1999

The challenge for chemoprevention strategies is clearly to demonstrate that high grade, high risk lesions may be safely treated, and for this reason CIN III may be regarded as the most appropriate initial target for chemoprevention trials. Several Phase I and Phase II trials of chemoprevention of cervical cancer using retinoids and DFMO have shown promising results, and the search for modulable biomarkers has provided some insight into the biology of this disease (6,16,17,31).

DFMO and Chemoprevention

DFMO is an antiproliferative agent, which acts through irreversible inhibition of the enzyme ornithine decarboxylase (ODC). ODC catalyzes a rate-limiting step in polyamine biosynthesis, the conversion of ornithine to putrescine. The central function of polyamines is thought to involve the stabilization of macromolecular interactions in the cell. This may be accomplished through interactions of their multiple positively charged regions with specific anionic regions of proteins and nucleic acids.

Polyamines are known to be intimately associated with DNA and to have critical regulatory interactions: They can induce bends and transitions in DNA from B- to Z- and A-isoforms (28), and have been shown to either promote associations or to cause release of a variety of DNA binding proteins (4). Ultrastructural studies using immunogold labeling have shown that polyamines — primarily spermine and to a lesser extent spermidine — are concentrated in regions of high density chromatin in the interphase nucleus (26). Polyamines may play a critical role in stabilization of histone interactions in formation of the nucleosome and higher order chromatin structures (2,3).

Sufficient levels of polyamines are considered to represent an absolute requirement for cell division, and elevated levels are among the most universal features of neoplastic cells. DFMO is generally regarded as having a cytostatic or differentiating effect in proliferating neoplastic cell populations (9), although there are contexts in which it has also been shown to induce apoptosis (1). There has been intense interest in the use of inhibitors of polyamine synthesis for chemotherapy and chemoprevention of cancer. Unfortunately, the efficacy of DFMO has been limited by several mechanisms of resistance (11,14) in invasive cancer. The rationale for the use of DFMO at moderate to low doses for chemoprevention of cancer comes from several observations, which indicate that upregulation of ODC represents a necessary and sufficient condition for the initiation and promotion of carcinogenesis (20,22,24).

Chromatin Structure as an Intermediate Endpoint

Although histopathologic evaluation has been regarded as the ''gold standard'' for chemoprevention trials, long experience has shown that it is unable to predict the biologic and clinical behavior of CIN lesions. With standardized criteria, histopathology can be reproduced with acceptable accuracy, but it is clear that it provides, at best, a crude measure of the extent of differentiation of the tissue, with cutoff points between different grades defined in a somewhat arbitrary manner. Moreover and more seriously, classical pathologic evaluation of cytologic and nuclear atypia in these lesions is notoriously irreproducible (7,10,12,32). In the diagnostic setting, gradations of nuclear atypia have been dismissed in the Bethesda system, in part due to this uncertainty that is inherent in any subjective visual evaluation scheme. Morphometric analysis provides a quantitative and objective representation of nuclear atypia, and in the chemopreventive setting these characterizations may serve as ideal biomarkers for monitoring regressive response.

In this study, we test the hypothesis that features describing nuclear shape and chromatin structure may be used as surrogate intermediate endpoint biomarkers for chemoprevention studies. In particular, we postulate that response to chemoprevention is associated with the elimination of cells with high grade nuclei, a response that may be related to the induction of cytostasis, differentiation, or apoptosis in the proliferating cell fraction. We further postulate that nuclear morphometry may be used to identify high grade cells and to quantitate this response. In this article we describe a Phase I, dose-seeking trial of DFMO chemoprevention on a group of patients with CIN III. Patients were treated for a period of 1 month with DFMO, and biopsy specimens were obtained before and after administration of the drug.

In order to assess any possible changes in nuclear structure, we have used image cytometry of Feulgen-stained nuclei to characterize the size, shape, DNA content, and patterns of chromatin condensation in these cells. Features extracted from digital cell images represent a quantitative and reproducible assay for classic cytopathologic changes, which have been observed subjectively for over a century. Within the spectrum of aneuploid neoplastic progression, these changes include increases in the size and DNA content of nuclei, increasing irregularities in the shape of nuclei, and most intriguingly dramatic changes in the amount and distribution of highly condensed chromatin. Increased heterogeneity of cell populations is also observed, reflected in an increase in the standard deviations of these features (23,27).

We have previously described the measurement of DNA content as a surrogate endpoint biomarker on the specimens used in this study (5), and have shown that DNA index, 5c exceeding rates, and DNA malignancy grade are modulated in both responsive and nonresponsive lesions. In this report, we show that consideration of nuclear shape and chromatin texture may provide a more complete representation of response to therapy.

MATERIALS AND METHODS

Archival Study

A series of archival specimens was selected in order to provide an independent characterization of progressive changes in CIN. Two hundred fifty cone biopsies containing cervical squamous intraepithelial neoplasia and invasive cancer were reviewed to select specimens in which adjacent normal epithelium was present with intraepithelial neoplasia or microinvasive and invasive cancer. Four micrometer sections were cut from routinely processed

paraffin blocks. These were stained with hematoxylin and eosin (H&E) and were reviewed and mapped by the institutional gynecologic pathologist and I. Boiko. Serial sections were Feulgen stained, and these archival specimens were then measured by diagnostic category. The breakdown of archival tissue specimens were 19 normal, 14 CIN I, 11 CIN II, 21 CIN III, and 5 invasive squamous cancers.

DFMO Study

A Phase I Dose De-escalation trial of DFMO was designed and submitted to the Internal Review Board for approval. Between March, 1994 , and March, 1995: 143 patients with CIN III were screened for eligibility. Of 68 eligible patients, 30 agreed to participate and underwent a complete medical history and physical exam, pelvic exam, Papanicolaou smear, sexually transmitted disease testing, human papillomavirus testing, and colposcopic examination of the vulva, vagina, and cervix. Colposcopically directed biopsies of the cervix for diagnosis were obtained. Biopsies measured $1 \times 2 \times 1$ mm and were of all areas colposcopically suspicious for CIN III. All biopsies were reviewed by the study pathologist to assure eligibility. In total, 25 patients were determined to have adequate tissue for analysis. Details of the study design and results of the polyamine biomarkers can be found elsewhere (18,21). All tissue was processed routinely and sectioned at 4 μm.

Feature Measurements

Digital images of Feulgen-stained cell nuclei were collected interactively using the Cyto-Savant™ imaging system (Oncometrics Inc., Vancouver, B.C., Canada), and feature measurements were performed on digital cell images according to computations as described in detail elsewhere (8). One hundred sixteen features were measured for each cell image. These may be divided into three categories:

1. *Morphological features*. These features serve to characterize increases in size of nuclei, as well the increasingly severe distortions in nuclear shape that are associated with aneuploid progression of CIN. These changes may take the form of increasingly asymmetrical nuclear shapes. Abrupt angular variations in the nuclear contour may be observed and characterized, as well as any appearance of prominences or raggedness of nuclear borders.
2. *DNA content*. The Feulgen-Thionin stain is stoichiometric for DNA and behaves according to the Beer-Lambert law of absorption, so that the integrated optical density (IOD), integrated over the area of the cell, is proportional to the DNA content of the cell. The DNA index for each cell is defined as the IOD divided by a normalization constant, the IOD of internal diploid controls.
3. *Texture features*. Texture features describe the variations in optical intensity over the nuclear image and present an objective and quantitative method for characterization of changes in chromatin appearance. Features are defined which are sensitive to changes in the overall contrast in the nuclear image, to the contrast between chromatin regions, and to the number and density of discrete chromatin particles.

Statistical Analysis

Parameters tested were the mean value of cell feature measurements over the individual slide (slide mean, denoted SLMEAN), as well as the standard deviation of feature measurements within the slide (slide standard deviation, denoted SLSD). The distributions of these slide-based population statistics were examined and compared over the groups of patients in this study.

Wilcoxan matched pair comparisons were performed in order to determine which features are significantly modulated by DFMO. In this test, the matched pair consists of measurements made before and after treatment for a given patient. The difference between feature values before and after treatment is calculated for each patient, and the distribution of this difference over all patients is tested to determine whether it is significantly different from zero.

Patients were grouped into two categories: responders, who showed either a complete (regression to normal histology) or partial response (regression by at least one CIN category) (11 cases), and nonresponders who showed no observable changes in the histology or gross appearance of the lesion (14 cases). Features were tested for significant pairwise differences over each group.

Mann-Whitney *t*-tests were used for unmatched comparisons between responders and nonresponders. These comparisons were made for data obtained before chemoprevention, in order to determine whether it is possible to predict response to DFMO. Comparisons were also made for data obtained from post-treatment biopsies, in order to determine whether there was a differential response to DFMO.

In the archival study, the correlations of feature values with the CIN grade of the lesion were investigated. Normal tissue was assigned CIN grade 0. Gamma and Spearman product correlation analysis were used. The Gamma statistic was used to determine significance. Significantly modulated features in the chemoprevention study were considered in terms of the Spearman correlation coefficients, in order to determine whether response to chemoprevention was associated with an overall reduction of grade.

Stepwise linear discriminant function analysis was performed in order to derive a classification function which would distinguish between low grade and high grade nuclei, based on measurements of individual cells from archival material. In this analysis, feature measurements of at most 100 cells from each slide were pooled to form two groups: (a) normal and CIN I and (b) CIN II and CIN III. A function whose output is a single number for each cell, the discriminant score, was derived by selection of a set of four variables that best separated the groups. Variable selection was based on the multivariate F-statistic. The discriminant score can be viewed as an index of nuclear grade, with higher values corresponding to more severely atypical nuclei. This function was then used to assess the extent and severity of nuclear atypia in individual cells from the chemoprevention study.

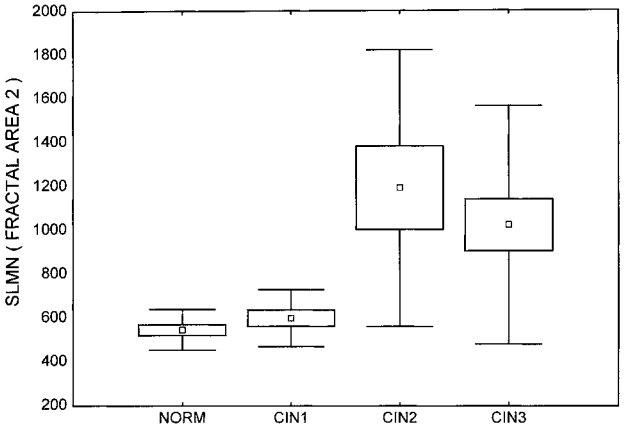

FIG. 1. Archival study. Fractal area2 as a function of CIN grade. Chromatin patterns are more complex and of higher contrast in higher grade lesions.

RESULTS

Archival Study

The archival study was undertaken in order to provide an independent characterization of progressive neoplastic changes in CIN. Figure 1 shows an example of these progressive changes for the parameter fractal area2. From the figure, it is evident that the parameter is modulated in a monotonic manner with increasing CIN grade. The figure also indicates that there is a considerable degree of overlap between the categories of CIN II and CIN III. This can be expected on the basis that histopathologic criteria for dividing CIN II from CIN III are somewhat arbitrary, and may not yield the best correlate for advancing stages of intraepithelial progression.

Changes in morphometric features with advancing nuclear grade may be summarized by their correlation coefficients (denoted r_{arch}), with positive values indicating that the feature is increased with CIN grade, and negative values indicating a decrease with increasing grade. The absolute value of the correlation coefficient indicates the strength of the association; the square of the correlation coefficient describes the fraction of the total variance in the sample that is explained by the association with CIN grade. In discussions of the independent study of chemoprevention, the archival correlation coefficients are used to determine whether features modulated by treatment are related to a reduction of CIN grade. In general, the correlation coefficients seen in the tables below tend to reflect the classic observations that DNA content, irregular shapes, pleomorphism, and chromatin heterogeneity are all increased in higher grade lesions.

Differences Between Response and No Response Groups

Before treatment. Differences between the groups in specimens taken before treatment may indicate that it is possible to predict response to the drug. However, in this study none of the 116 features achieved significance at the 0.05 level using the Mann-Whittney test for slide means and slide standard deviations. The significance values were >0.5 for the majority of features, although a small subset of features were significant at the $P = 0.17$ level, indicating a marginal possibility of significant differences for ten features (not tabulated).

After treatment. Differences between the groups in specimens taken after treatment indicate that there is a differential drug effect, particularly since no differences were found in specimens taken before treatment. Numerous features showed highly significant differences, including DNA index, shape features, and chromatin texture features. These are listed in Table 1 for the slide means, and in Table 2 for slide standard deviations. With few exceptions, the slide standard deviations for responders in Table 2 are smaller for significantly differing features, indicating that the cell populations are more homogeneous in these lesions. In addition, there is a general trend toward a decrease in the group standard deviations for responders, indicating that they are reduced to a more homogeneous group of patients with respect to these parameters.

The correlation with CIN grade from the archival study is listed for each significant feature, with positive values indicating that the feature is increased with CIN grade. Considering the features that are significantly different between responders and nonresponders, we see that all are consistent with the persistence of high grade nuclei after treatment in the nonresponders. The sole exception to this rule is the slide standard deviation of the range_av parameter, which has the weakest and least significant association with grade ($P = 0.04$).

Treatment Effects

Wilcoxan pairwise tests for significant differences in slide means and slide standard deviations were performed for (a) responders (Tables 3 and 4), and (b) nonresponders (Tables 5 and 6). These pairwise tests provide similar information to the analysis of differences between response and no-response groups, but may be the most specific for the detection of drug effects since they will tend to eliminate effects due to individual variations between subjects.

In Tables 3–6, the pairwise differences parameter values were calculated for each patient, and are expressed in terms of the percentage change in the parameter for each case:

$$\%\text{change} = 100\% \times [f(\text{after}) - f(\text{before}))/f(\text{before})],$$

where f(before) and f(after) denote the parameter value either before or after treatment, respectively. The descriptive statistics for the percentage change were then computed over the designated groups in the tables below.

The correlation with CIN grade is again shown for each parameter. We note that if the sign of the percent change is opposite to that of the correlation coefficient, then this change may be considered to represent a reduction of CIN grade. For responders, the only parameter that is inconsis-

Table 1
*Significant Differences in Slide Means Between Response and No-Response Groups After Chemopreventive Treatment**

Feature	*P* (MW)	Response GMEAN	Response GSD.	No response GMEAN	No response GSD	r(arch)
17 radial var	0.009	3.58	0.77	4.74	1.23	0.46
19 eccentricity	0.029	1.46	0.07	1.54	0.09	n.s.
23 elongation	0.036	1.51	0.09	1.59	0.11	n.s.
27 fft_h2	0.029	12.1	1.59	13.57	1.87	n.s.
60 DNA index	0.004	1.19	0.07	1.51	0.25	0.73
62 OD max	0.025	0.56	0.06	0.64	0.10	0.53
69 DNAam_1	0.048	0.11	0.05	0.01	0.03	−0.60
77 av_dist_med	0.038	0.72	0.06	0.76	0.04	0.40
102 fractal_ar1	0.001	5846	1025	7651	1446	0.49
103 fractal_ar2	0.001	995	168	1320	251	0.48

**P*-values are from the Mann-Whittney test. Group means and group standard deviations are listed for each group. The correlation coefficient is the correlation of the feature value with the CIN grade of the lesion. Significant correlations only are shown ($P < 0.05$).

Table 2
*Significant Differences in Slide Standard Deviations Between Response and No-Response Groups After Chemopreventive Treatment**

Feature	*P* (MW)	Response GMEAN	Response GSD	No response GMEAN	No response GSD	r (arch)
15 mean radius	0.025	1.304	0.214	1.615	0.394	n.s.
16 max radius	0.002	2.047	0.282	2.568	0.445	n.s.
60 DNA index	0.029	0.312	0.057	0.475	0.136	0.78
78 av_dist_hi	0.029	0.141	0.053	0.099	0.036	−0.46
94 den_ds	0.029	0.0095	0.001	0.0086	0.0015	n.s.
96 range_avg	0.029	58.54	8.04	50.78	8.65	0.30
102 fractal_ar1	0.012	2332	1643	2833	771	0.53
103 fractal_ar2	0.014	374	142	511	134	0.51
116 grey lev90	0.014	11.44	2.97	14.86	5.26	0.39
124 runpcnt90	0.019	0.055	0.005	0.06	0.007	n.s.

**P*-values are from the Mann-Whittney test. Group means and group standard deviations of SLSD are listed for each feature. Significant correlations with CIN grade are shown ($P < 0.05$).

Table 3
*Response—Slide Means: Distribution of Pairwise Differences in Slide Means Over Cases Responsive to DFMO Treatment**

Feature	*P*	% Change in SLMEAN GMEAN	GMIN	GMAX	GSD	r (arch)
60 DNA index	0.003	−22.77	−44.41	−0.93	15.29	0.73
90 homogeneity	0.021	−5.24	−17.50	10.94	7.42	0.34
101 fractal dim	0.026	1.17	−1.16	3.23	1.33	n.s.
102 fractal_ar1	0.013	−19.36	−64.32	14.83	25.15	0.49
103 fractal_ar2	0.013	−21.39	−65.81	11.05	24.56	0.48
111 long run45	0.016	−11.80	−37.71	16.63	15.22	0.47
125 runpcnt135	0.041	4.22	−6.45	13.83	6.22	n.s.

*Descriptive statistics for significantly different features ($P < 0.05$). The group mean (GMEAN), minimum and maximum values (GMIN, GMAX), and group standard deviation (GSD) are listed. Comparisons with the archival correlation coefficient are listed.

tent with reduction of CIN grade is again the slide standard deviation of range average, similar to the result from Table 2.

Figure 2 shows differences in the parameter fractal area2 for representative cases from the response and no response groups. Representative cases were selected on the basis that they clearly illustrate the trends observed in the tables.

For nonresponders, the significant archival correlation coefficients are also consistent with a reduction in CIN grade for these lesions, suggesting either a partial regressive response, or an effect that may be related to a partial

Table 4
*Response: Slide Standard Deviations**

		% Change in SLSD				
Feature	*P*	GMEAN	GMIN	GMAX	GSD	r (arch)
60 DNA index	0.003	−34.30	−65.21	−8.36	19.41	0.77
83 low dens obj	0.041	−18.95	−50.27	11.87	22.94	n.s.
96 range_avg	0.041	16.03	−31.70	76.36	25.94	0.30
103 fractal_ar2	0.026	−26.31	−74.27	17.24	28.23	0.52

*Distribution of pairwise differences in slide standard deviations over cases responsive to DFMO treatment. Descriptive statistics for significantly different features ($P < 0.05$).

Table 5
*No Response: Slide Means**

		% Change in SLMEAN				
Feature	*P*	GMEAN	GMIN	GMAX	GSD	r (arch)
30 fft_h5	0.030	19.76	−16.35	87.82	28.67	n.s.
32 ffth_h7	0.048	12.86	−21.14	43.86	18.60	n.s.
60 DNA index	0.016	−11.41	−57.04	20.33	17.32	0.73
65 OD kurtosis	0.035	7.86	−16.22	28.67	11.88	n.s.
67 DNAar_med	0.041	41.73	−20.98	305.16	85.37	n.s.
75 comp_high	0.026	17.00	−37.91	62.26	24.79	n.s.
81 DNA_lvh	0.004	−14.07	−49.62	3.20	16.49	0.53
82 DNA_lvmh	0.004	−16.36	−57.29	13.77	18.99	0.54
85 high dens obj	0.041	33.21	−34.18	134.33	47.23	n.s.
93 dens_1s	0.035	100.07	−75.37	332.33	119.3	n.s.
100 av dist high	0.048	81.64	−42.98	691.76	187.9	−0.42
101 fractal dim	0.041	1.13	−6.15	5.22	2.77	n.s.

*Distribution of pairwise differences in slide means over cases not responsive to DFMO treatment. Descriptive statistics for significantly different features ($P < 0.05$).

Table 6
*No Response: Slide Standard Deviations**

Feature	*P*	GMEAN	GMIN	GMAX	GSD.	r (arch)
22 compactness	0.009	42.1	−23.1	169.9	59.7	n.s.
26 fft_h1	0.026	42.7	−27.5	213.3	63.5	n.s.
28 fft_h3	0.041	18.1	−27.2	70.1	26.2	−0.28
30 fft_h5	0.011	25.8	−21.5	87.0	28.4	n.s.
32 fft_h7	0.004	21.9	−9.6	66.8	20.1	n.s.
65 OD kurtosis	0.030	30.2	−55.1	134.0	45.4	n.s.
66 DNAar low	0.006	53.6	−26.3	261.0	79.7	−0.29
69 DNAam low	0.011	155.8	−46.0	994.5	273.3	−0.34
81 DNA lvh	0.026	−16.3	−76.4	36.8	30.0	0.52
82 DNA lvmh	0.019	−17.4	−71.1	20.5	26.8	0.53
85 high dens obj	0.030	156.1	−46.7	924.3	273.3	n.s.
93 dens 1s	0.013	37.5	−47.7	84.7	32.3	n.s.
96 range_avg	0.048	−9.8	−47.1	75.1	29.3	0.30

*Distribution of pairwise differences in slide standard deviations for cases not responsive to DFMO treatment.

cytostatic response. Compared to responsive lesions, there are however many more examples where significantly modulated features are not significantly correlated with grade.

Morphometric Index

In the archival study, stepwise discriminant function analysis identified a set of four variables that distinguished between cells from normal/CIN I biopsies and from CIN II/CIN III biopsies. These variables were: mean radius, mean intensity, DNA index, and contrast. The discriminant function is a linear combination of these variables, and the output of this function is a single number, the discriminant score. Individual cell nuclei are classed as low grade if this number is less than or equal to 0.0; otherwise they are classed as high grade. Figure 3 shows how this index is modulated with advancing nuclear grade on the archival series. Appreciable overlap between CIN II and CIN III lesions is again observed. Application of this function to the DFMO study indicates that it provides a consistent and

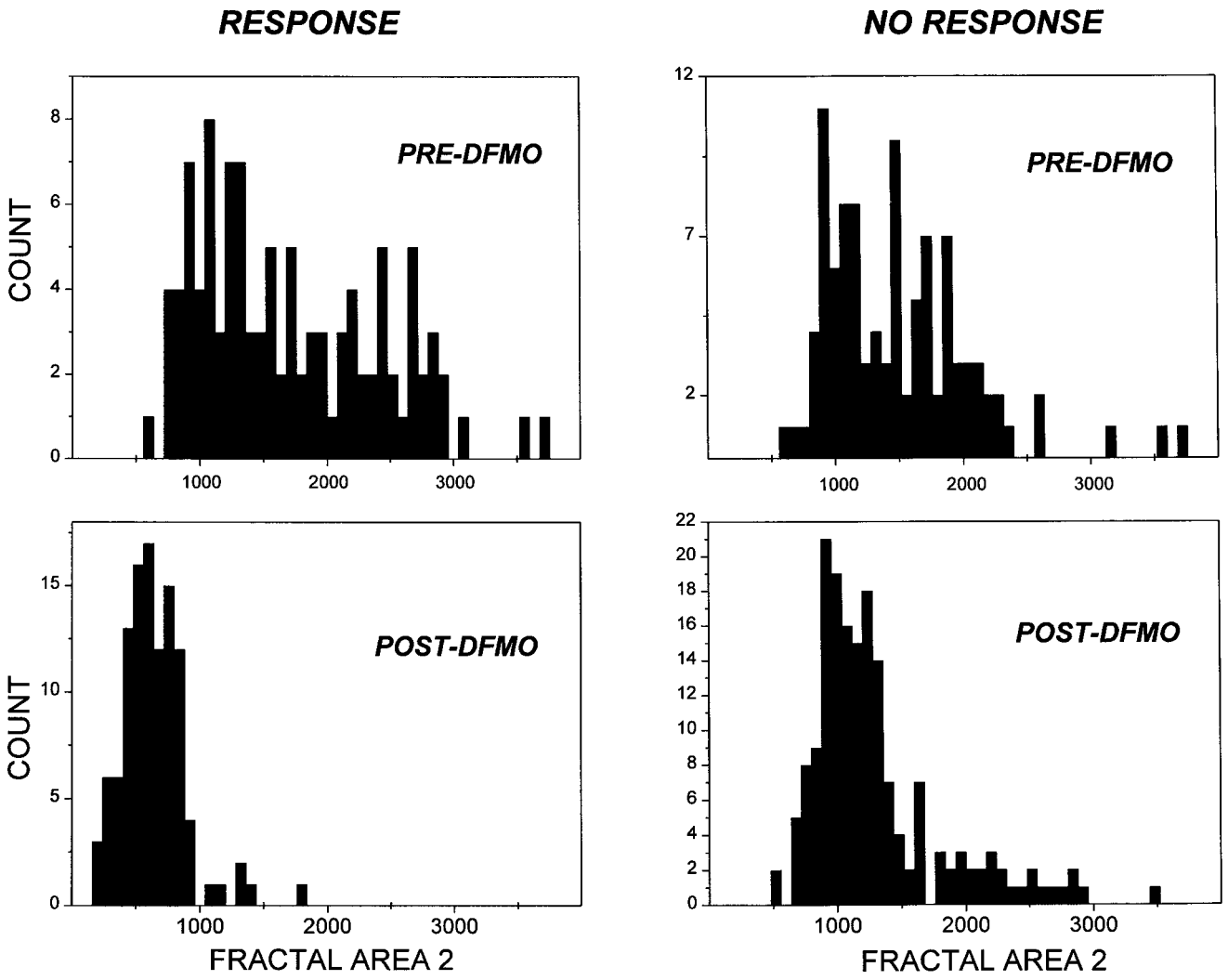

FIG. 2. DFMO study. Histogram of fractal area2 for responsive and nonresponsive lesions. Strong modulation of this parameter is evident in the responsive lesion, indicating the loss of cells with highly complex, highly contrasting chromatin patterns.

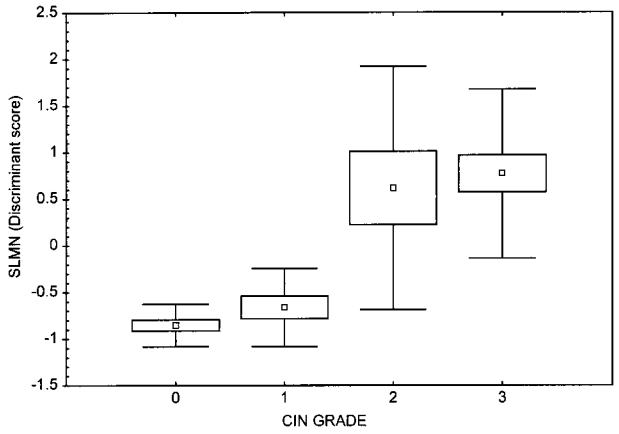

FIG. 3. Modulation of the morphometric index with advancing CIN grade. Overlapping distributions are evident for CIN II and III lesions, indicating that CIN grade is not necessarily the best correlate for advancing stage of the disease.

specific measure of regressive response. Figure 4 shows histograms of the discriminant score for the same representative cases as in Figure 2.

For each slide a morphometric index was defined as the percentage of nuclei classified as high grade (discriminant score > 0.0). In Figure 5, this index is plotted, showing a strong modulation for responders and no significant change for nonresponders. Pairwise comparisons of the index showed a significant difference ($P = 0.0033$) for responders and no significant difference ($P = 0.177$) for nonresponders.

DISCUSSION

Differences Between Response Groups

Before treatment. At the $P = 0.05$ level, no significant differences were detected between responders and nonresponders for features measured on specimens taken before DFMO treatment. A subset of ten features were

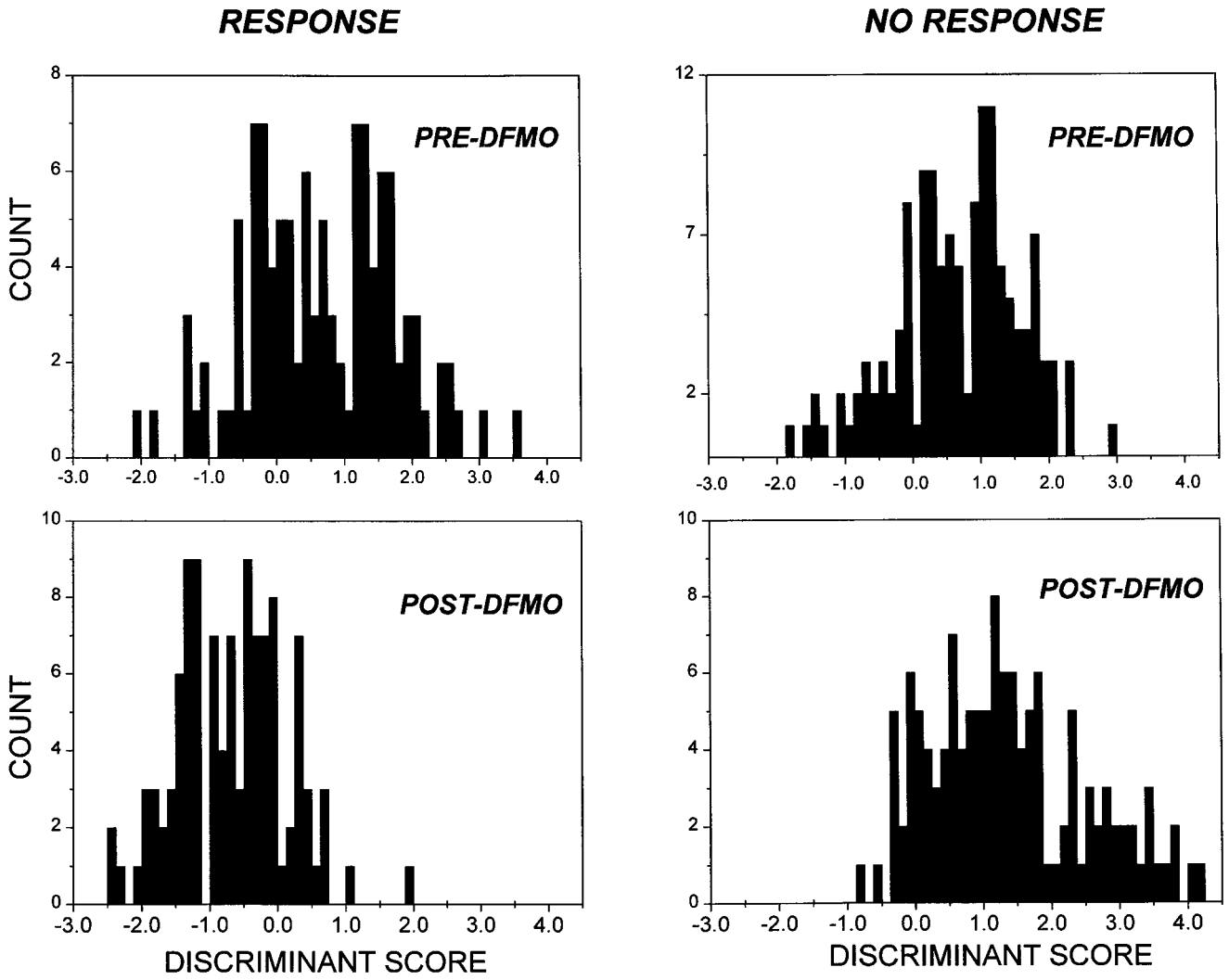

FIG. 4. Histogram of the discriminant score for representative cases of chemoprevention data. Higher values of the function indicate more severe nuclear atypia. Cells with a score greater than 0.0 are classed as high grade.

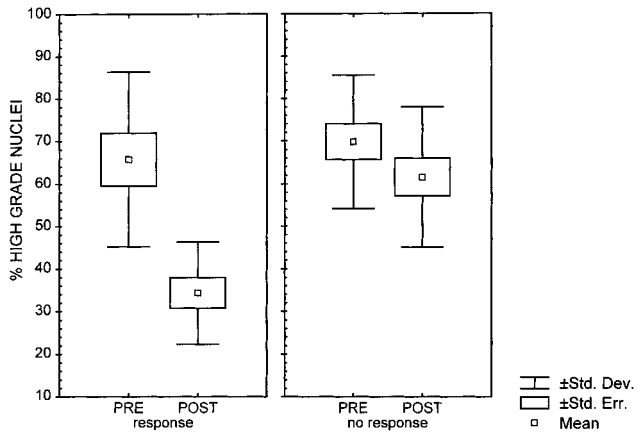

FIG. 5. Distribution of morphometric index (percent high grade nuclei) over chemoprevention series. Pairwise differences in morphometric indices indicate no significant difference for nonresponsive lesions.

significant at the $P = 0.17$ level, but it is clear that the magnitude of these differences is not large. This indicates that in this setting it may be difficult to predict response to DFMO on the basis of these measurements.

Mechanisms of resistance to DFMO in this study are a matter of speculation. One possible explanation is suggested by studies of mouse tumor models, where it has been established that exogenous sources of polyamines contribute significantly to resistance. The antitumor effects of the drug have been shown to be enhanced by dietary restriction and gastrointestinal decontamination (25). In vitro studies have suggested that polyamine transport inhibitors may also be effective in this respect (1).

The alternative hypothesis is that higher grade lesions may be more likely to develop resistance, since these cell populations are more genetically variable, with greater potential for amplifications and deletions of critical signal-

ing and control pathways. To the extent that these measurements reflect CIN grade, this analysis indicates that development of resistance is not correlated with any significant difference in progression of CIN III. If this is the mode of resistance, the analysis suggests that it is a random and probable event at this stage of the disease.

After treatment. Large differences between responders and nonresponders were observed in specimens taken after DFMO treatment. These differences are indicative of a differential response to the drug, particularly since no differences were detected before treatment.

A general feature of neoplastic cell populations is that they tend to be characterized by a progressive increase in the size of nuclei and by increasingly severe irregularities in nuclear shape and chromatin distribution. A conspicuous feature of higher grade lesions is that pleomorphism of cell populations is markedly increased, leading to an increasingly heterogeneous and diverse population of atypical cells. With few exceptions, the standard deviations of shape and texture features in Table 2 are larger in the no-response group, indicating the persistence of heterogeneous cell populations in these lesions.

As has been previously reported (28), the mean DNA index was smaller in the response group, indicating that DFMO has a differential effect. In the previous report, a survey of DNA histograms of all cases indicated that in the responders, DFMO treatment leads to a decrease in the number of cycling cells, and also suggested a reduction in the prevalence of aneuploid cells. This effect was also evident in the slide standard deviations of DNA index, which were also significantly lower in the response group.

Several shape descriptors show significant differences between groups in post-treatment specimens (Tables 1 and 2). The slide means of radial variance, eccentricity, elongation, and fft_h2 are all larger in the no-response group. These features are typically larger for the irregular shapes observed in aneuploid cell populations, although radial variance is the only feature for which this is confirmed by significant archival correlations.

Differences in texture features after treatment are also consistent with the hypothesis of the persistence of a more diverse population of higher grade cells in the no-response group. Differences in fractal areas, homogeneity, DNA_am_low, Odmax, all tend to indicate the presence of more highly condensed, coarsely clumped chromatin patterns before treatment in these lesions. It is intriguing to note the greater asymmetries in medium density chromatin (avg_dist_med) in nonresponsive lesions, which may be associated with amplification processes in particular chromosomal regions. Where archival correlations are significant, the changes are all consistent with a loss of high grade nuclei in the responders. The sole exception is the SLSD of the range_av parameter, which has the weakest association with CIN grade, and which is at borderline significance for this correlation.

The values of the archival correlation coefficients do not all reflect this trend. Many of the significantly different features are not significantly correlated to CIN grade, which raises a number of possibilities: (a) measurements of nuclear atypia may be limited by the biologic variability of lesions in the archival study; (b) it may be difficult to determine accurately the extent of nuclear atypia using CIN classifications; (c) there may be drug effects on shape and chromatin distribution, which are unrelated to progressive neoplastic changes in these parameters.

Pairwise Differences

For the response group, comparison of pairwise differences (Tables 3 and 4) with the correlation coefficients from the archival study again indicate that significantly modulated features are all changed in the direction which would indicate reduction of CIN grade. Similar to the observations from the preceding section, significant changes in texture parameters all strongly support the interpretation that cells with coarse aggregates of high density chromatin are selectively lost from these lesions in response to the drug (DNA index, fractal_areas, homogeneity, long runs, run_percent). Modulations in slide standard deviations also indicate that heterogeneity is reduced with response to the drug. As has been noted previously, the marginally significant parameter SLSD_range_av is the sole exception to the above rules.

For nonresponders, analysis of the archival correlations for significantly modulated features (Tables 5 and 6) indicates: (a) significant correlations are consistent with a reduction of high grade nuclei also in nonresponders, suggesting a partial regressive response, and (b) there are many more features which are significantly modulated but which are not significantly correlated with CIN grade (i.e., the effect is not related to the elimination of high grade nuclei) .

Morphometric Index

The results of the derivation of a discriminant score to classify high grade nuclei is shown in Figures 3–5. The value of the discriminant score is larger for higher grade nuclei. In Figure 4, the severity and extent of nuclear atypia is reduced in the responsive lesion. In the nonresponsive lesion, the main peak is unchanged, and there is a population of higher grade nuclei that appears to be either promoted or selected by treatment. This is a potentially serious concern, but there is a strong possibility that this result is due to differences in sampling between pre- and post-treatment biopsies. These lesions are known to be heterogeneous, and the pretreatment biopsy was quite small. The post-treatment biopsy is selected to be the most advanced part of the entire lesion, and hence the analysis is actually biased against a favorable result.

In Figure 5, the overall distribution of the morphometric index is shown for responsive and nonresponsive lesions, showing clear separation and reduction of grade for responsive lesions and no significant change for nonresponsive lesions. The specificity of this index for changes in responders has implications for the interpretation of a putative partial regressive response for nonresponders, which was suggested by many of the univariate tests. This independent determination of nuclear grade shows no significant modulation in nonresponders, which suggests

either an unrelated drug effect, or an effect that may be related to some degree of cytostasis achieved in these lesions. In any case, the morphometric index indicates that high grade cells are clearly persistent in nonresponsive lesions.

Further refinement of the morphometric index is anticipated. In future we plan to extend these observations with the addition of more cases to the archival study, and with analysis of microinvasive and minimally invasive carcinoma, which we postulate to represent the endpoint of intraepithelial progression. Analysis of invasive carcinoma, in particular the study of associated intraepithelial lesions, may also serve to define the some of the latest stages of intraepithelial progression.

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