Cancer Prevention Research

Nuclear Morphometry Identifies a Distinct Aggressive Cellular Phenotype in Cutaneous Squamous Cell Carcinoma

Evan S. Glazer[3], Peter H. Bartels[1,2], Anil R. Prasad[1,4,5], Michael L. Yozwiak[1], Hubert G. Bartels[1], Janine G. Einspahr[1], David S. Alberts[1], and Robert S. Krouse[1,3,6]

Abstract

By identifying aggressive cutaneous squamous cell carcinoma (cSCC) in patients who are at high risk for recurrences or second primaries after resection, intensive surveillance and therapy may decrease morbidity and mortality. We investigated the role of nuclear morphometry (karyometry) in differentiating between aggressive and nonaggressive cSCC. We retrospectively analyzed cSCC lesions from 40 male patients. Twenty-two patients had evidence of aggressive cSCC (local/regional recurrence or a second primary cSCC), and 18 patients were identified with similar ages and sites of disease as control patients with nonaggressive cSCC (no evidence of recurrence, metastasis, or second primary). We carried out karyometric analysis to identify nuclear features that discriminate between aggressive and nonaggressive cSCC nuclei. We used statistically significant differences (Kruskal–Wallis test, $P < 0.0001$) to compose a quantitative aggressive classification score (proportion of aggressive nuclei from 0% to 100%). For comparisons, we used Fisher's exact test or Student's t test. The mean age was 79 ± 7 years for aggressive cSCC and 80 ± 9 years for nonaggressive cSCC ($P = 0.66$). We analyzed a mean of 96 nuclei in each group. The mean classification score for aggressive cSCC was significantly higher ($69\% \pm 6\%$) than for nonaggressive cSCC ($28\% \pm 5\%$, $P = 0.00002$). Overall, the classification score accurately categorized 80% of our patients ($P = 0.0004$). In most patients, karyometry differentiated between aggressive and nonaggressive cSCC. We found that classification scores, which provide information on individual lesions, could be used for risk stratification. _Cancer Prev Res; 4(11); 1770–7. ©2011 AACR._

Introduction

Each year, more than 200,000 individuals in the United States develop cutaneous squamous cell carcinoma (cSCC); many patients can be adequately treated with local excision (1–3). However in patients with recurrent or metastatic disease (1, 4), large en bloc resection may be necessary to manage such aggressive cSCC. Aggressive lesions often occur on the face and hands, resulting in diminished functional outcomes, especially in cases of recurrence. Recurrence rates may approach 20% for very high-risk patients; metastatic disease generally occurs in 2% to 7.4% of all cSCC patients, but the rate may be as high as 9.9% (3). The risk of multiple (metachronous) cSCC primary lesions is less than 20% for most normal-risk patients during a 2-year interval following diagnosis of the index lesion (5).

Known risk factors for aggressive cSCC include certain tumor characteristics (related to the site, stage, grade, size, location, ploidy, and perineural invasion), patient age, and immunosuppression (e.g., in transplant recipients and older patients; refs. 6, 7). Sun exposure may increase the risk, especially in immunocompromised patients, but this interaction can be difficult to quantify. Furthermore, increasing age is a surrogate maker for both sun exposure and immunosuppression that often confounds the risk assessment. Unfortunately, in individual patients, these risk factors are not easily correlated with aggressiveness because the duration or intensity of sun exposure varies significantly during the lifetime of a patient. The multifactorial biology of cSCC may require tailored treatments toward unique targets.

Clearly, a better method is needed to accurately predict prognosis and to risk stratify patients at the time of diagnosis. To this end, investigating nuclear characteristics has yielded important prognostic information for patients with cSCC. For example, variations in DNA ploidy and increased mitotic figures both portend a poorer prognosis (7, 8).

Authors' Affiliations: [1]Arizona Cancer Center; [2]College of Optical Sciences; and Departments of [3]Surgery and [4]Pathology, University of Arizona; [5]Department of Pathology; and [6]Surgical Care Line, Southern Arizona Veterans Affairs Health Care System, Tucson, Arizona

Corresponding Author: Robert S. Krouse, Arizona Cancer Center and Department of Surgery, The University of Arizona, Surgical Care Line, Southern Arizona VA Healthcare System, 3601 S. Sixth Ave. Tucson, AZ 85723. Phone: 520-792-1450-6145; Fax: 1-520-629-4603; E-mail: robert.krouse@va.gov

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However, with the advent of newer technological modalities, such as karyometry, intensive digital analysis of numerous nuclear features is now possible. Karyometric differences have now been documented in premalignant lesions of multiple types of carcinomas, including cSCC, breast, and colon (4, 9, 10).

In more than 90% of cSCC patients, premalignant and malignant cutaneous lesions can be consistently and accurately classified (4, 11). Successful classification of high-risk cSCC is valuable to allow the best use of more invasive therapy upfront and for the most efficient use of resources during follow-up. For example, high-risk patients may benefit from wider resections with sentinel lymph node biopsy. Likewise, low-risk patients may benefit from more limited resections and may need less follow-up.

In our exploratory study, we hypothesized that aggressive cSCC lesions in high-risk patients have a unique karyometric pattern that is distinct from that of nonaggressive cSCC lesions in low-risk patients. Furthermore, we hypothesized that a discriminant function (DF) created from the most statistically significant nuclear features can accurately differentiate between aggressive and nonaggressive cSCC nuclei.

Materials and Methods

Patient selection

After obtaining Institutional Review Board approval, we identified a total of 46 patients in the Southern Arizona Veterans Affairs Health Care System (SAVAHCS) cSCC database (for 10 years: 2000 through 2009). All cSCC lesions had been surgically excised. We identified cSCC patients who had had clear evidence of aggressive lesions ($n = 23$) as defined by (i) local recurrence, (ii) nodal recurrence, or (iii) multiple primary lesions within 6 months after resection (for our study, we used only the index lesion). From the same database, we identified matched-pair control patients ($n = 23$) with nonaggressive cSCC, that is, no evidence of an aggressive phenotype during a similar follow-up duration. Control patients were matched for age ($\pm$ 5 years) and lesion location. All 46 patients were male.

We prepared tumor samples in a standard fashion. Briefly, all samples were immediately fixed with 10% neutral buffered formalin for at least 24 hours and then, within 1 week, paraffinized. We prospectively entered all clinical data into an extensive database. Then, we cut the histologic sections from the paraffin blocks and, at the same time to minimize preparation variability, stained them with hematoxylin and eosin. Optimal portions of each section were 1,000× imaged with a 100:1 plan apochromatic oil immersion objective (N.A. 1.40, Nikon Corp). An 8-bit, high-resolution, 3-CCD (charge-coupled device) camera digitized the images (Sony Electronics Inc.). To enhance contrast, we utilized a bandpass filter (circa 610 nm).

Karyometric protocols

We measured 95 karyometric features per nuclei per lesion. A total of 100 nuclei per lesion are segmented (identified) via a semiautomated process that robustly identifies the nuclear region with threshold analysis and minor manual correction (12). The sampling rate is approximately 6 pixels per micrometer. The optical resolution is 0.261 micrometers, and the sampling distance is 3.83 pixels/micrometer. We oversample the segmented nuclei to increase overall accuracy (13). The karyometric features are quantitative measurements of features not readily appreciated on histologic examinations. Features can be characterized from the simplest (i.e., zero-order features) to quite complex linear combinations (higher-order functions of features). The zero-order features are based on all of the pixels in a nucleus, such as the total optical absorbance (i.e., hyperchromaticity) or the nuclear area (i.e., size); in addition, they include relative values, mean values, and measures of variance of those features. Second-order functions include similarities or differences between adjacent pixels: a measure of heterogeneity for a given feature. Chromatin patterns are described by higher-order functions that consist of the 2-dimensional relationship between many pixels in a given nucleus (i.e., the linear relationships of specific pixel densities). Finally, third-order features are relationships of features across multiple nuclei in a sample.

Discriminant function

DFs are useful in discriminating between aggressive and nonaggressive nuclei in cSCC. Although any number of features could be used, we decided a priori that no more than 8 features should be included in the DF. We believe that this is the maximum number of features that would produce generalizable results, while remaining statistically significant and biologically diverse, on the basis of previous studies (4, 9, 10). The nuclear features we chose were based on the results after conducting the Kruskal–Wallis test, a statistical test based on ranked values for a given feature (4, 14). Briefly, we ranked values for each nuclear factor according to their assigned group: if the distributions of values were similar, then there was no significant difference; however, if the distributions were unique to each group, then that nuclear feature is significantly different. Due to the large number of nuclear features and comparisons in our study, we set the threshold for statistical significance of the features at $P < 0.0001$. Once we identified statistically significant nuclear features, we used Wilks' lambda to assess the ability to differentiate between aggressive and nonaggressive lesions. We used only those features that maximally reduced Wilks' lambda (i.e., that maximally separated nonaggressive from aggressive cSCC lesions) in a DF.

Classification scores, serving as a metavariable, represented the percent of nuclei in a lesion that fit the criteria of aggressive, per a DF. The classification score is the proportion of aggressive nuclei in each lesion based on the DF comprised of the statistically significant nuclear features. In this way, the classification score describes the percent of nuclei that are phenotypically abnormal.

Within each subgroup, we used approximately half of the lesions for a training set to identify the most statistically significant features to comprise each DF. Only nuclear features with $P < 0.0001$ remained in a DF. The separation into training and testing set was made after the division into subgroups by relative area and total optical density (OD; Fig. 1). The split into training and test sets was done randomly. Then, we applied each DF to the other half of the samples as the test set in each subgroup. Finally, we calculated standardized coefficients for the subgroups as a whole.

Statistical analysis

The DF-related statistics are described above. To analyze differences in means, we used the 2-tailed Student's t test; to analyze proportions, the χ^2 test or Fisher's exact test as appropriate. Other than for the DF, all $\alpha = 0.05$. Uncertainties are expressed as a SD or a SEM. Our sample size calculation required 10 samples in each group (aggressive or nonaggressive) to determine a 50% difference in the classification score, with a SD of 20%, $\alpha = 0.05$, and power = 0.90. For calculations and plotting, we used Stata SE version 11 (StatCorp LP).

Results

Patient characteristics

Of the 46 patient samples identified, 6 were not of sufficient quality to analyze, so we excluded them from both our karyometric analysis and our clinical analysis. In the aggressive cSCC group ($n = 22$), the mean follow-up time was 5.4 years; in the nonaggressive cSCC group, 5.6 years ($n = 18$, $P = 0.80$). The mean patient age was similar in both groups (Table 1). At the time of our analysis, the overall survival rate was 50% in both groups. The site of primary disease was also similar (Table 1). Furthermore, we found no difference in immunosuppression (i.e., in the number of patients on immunosuppressive medications, Table 1).

The aggressive cSCC group included patients with local recurrence ($n = 8$), regional disease ($n = 3$), and a second primary lesion ($n = 11$). Of the second primary lesions, 4 were in the same region (head, extremity, or trunk); the other 7 were in another region (contralateral or alternative region).

In the nonaggressive cSCC group, 1 patient underwent x-ray radiation therapy for a rapidly growing, poorly differentiated scalp cSCC at the directive of the clinician. None of patients in the nonaggressive cSCC group underwent a sentinel lymph node biopsy or lymph node dissection.

Of the 22 patients in the aggressive cSCC group, 6 (27.3%) underwent a sentinel lymph node biopsy or lymph node dissection; 3 (13.6%) underwent x-ray radiation therapy.

Karyometric analysis

In the aggressive cSCC group, we analyzed 96 ± 8 nuclei per patient; in the nonaggressive cSCC group, we analyzed 96 ± 9 nuclei ($P = 0.53$). The ratio of overall
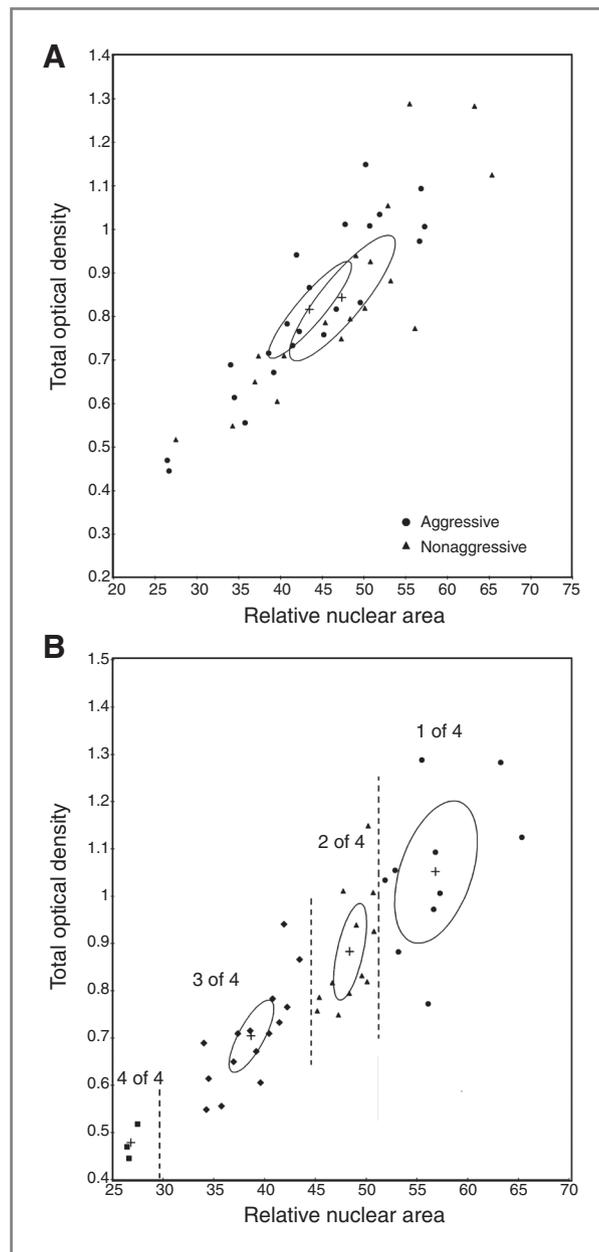

Figure 1. Plot of values for nuclear area and OD of cSCC. We found no statistically significant differences in the average relative nuclear area (units are pixels per nuclei divided by 100) and average total OD between aggressive and nonaggressive cSCC lesions (A). Blinding to aggressive or nonaggressive status resulted in 4 distinct subgroups of lesions (B) that are identified by symbols and separated by dashed line ($P < 0.013$). Ellipses represent 95% confidence limits for bivariate case means. Symbols in Figure 1B represent subgroups (1 to 4) based on separation by relative area and OD without distinction as to aggressive or nonaggressive behavior.

sample size to nuclear feature was approximately 20:1, indicating a sufficient supply of samples per feature analyzed to create DFs. First, we investigated the karyometric equivalents for 2 standard pathologic assessments for poor prognosis: relative nuclear area (i.e., a large

Table 1. Patient characteristics

		Aggressive cSCC (n = 22)	Nonaggressive cSCC (n = 18)	P
Age, y	Mean	78.7	79.8	0.657
Alive	n (%)	11 (50)	9 (50)	1
Site of disease				
	Neck	1	1	
	Scalp	8	9	
	Face	7	4	
	Ear/nose	2	2	
	Extremity	2	2	
	Thorax/abdomen	2	0	
Immunosuppression	n (%)	1 (4.5)	2 (11.1)	0.847

nucleus) and total OD of the nucleus (i.e., hyperchromaticity or high intensity of nuclear staining). On average for each lesion, both nuclear area and density were found to be insignificantly different on karyometric analysis, with significant overlap (Fig. 1A). The distribution of aggressive and nonaggressive cSCC fell into 4 distinct and statistically unique subgroups, independent of the aggressive or nonaggressive quality [Fig. 1B, Beale statistic for best partition fit (ref. 15), $P < 0.013$]. For each of those 4 subgroups, we then compared the remaining 93 nuclear features (excluding total OD and relative nuclear area) according to whether the cSCC nuclei were aggressive or nonaggressive.

We constructed 4 DFs (DF1, DF2, DF3, and DF4, in decreasing nuclear size) to differentiate between aggressive and nonaggressive lesions within each subgroup (Table 2). The relative weighting for each nuclear feature is the standardized coefficient (Table 2). Per the specific DF within each subgroup, we calculated the classification scores (aggressiveness percent). The mean classification score for aggressive lesions was about 2.5 times higher than that of nonaggressive lesions (Fig. 2A, Table 3, $P = 0.00002$). To separate aggressive from nonaggressive cSCC, we used a threshold classification score of 37%. Overall, the classification score accurately categorized 80% of all of our patients ($P = 0.0004$, sensitivity = 86.4%, and

Table 2. Statistically significant nuclear features comprising DFs ($P < 0.0001$)

	Feature description	Standardized coefficient
DF1	Short segment of similar pixel intensities	0.596
	7–8 pixels with OD range of 0.3–0.6	−0.402
	2 pixels with OD range of 0.3–0.6 and 0.9–1.2	0.334
	Frequency of pixels with OD range of 0.4–0.5	0.263
DF2	A number of hyperchromatic pixels	0.261
	Long segment of similar pixel intensities	−0.806
	Frequency of 2 pixels with OD range of 0.3–0.6 and 0.6–0.9	0.25
DF3	OD clumpness	0.571
	OD heterogeneity	−0.597
	Frequency of pixels with OD range of 1.0–1.1	0.381
DF4	OD clumpness	0.565
	Pixel homogeneity	0.556
	Frequency of pixels with OD range of 1.0–1.1	0.473
Homogeneous[a] populations	Heterogenous pixel OD pattern	−0.113
	A long segment of similar pixel intensities	1.465
	A short segment of similar pixel intensities	0.264
	>11 pixels in a run with OD range of 0.3–0.6	−0.873
	A relatively high frequency of 2 pixels with OD range of 0.3–0.6	0.59

[a]Homogeneous refers to the DF that discriminates between nearly homogenous aggressive and nonaggressive population of nuclei.
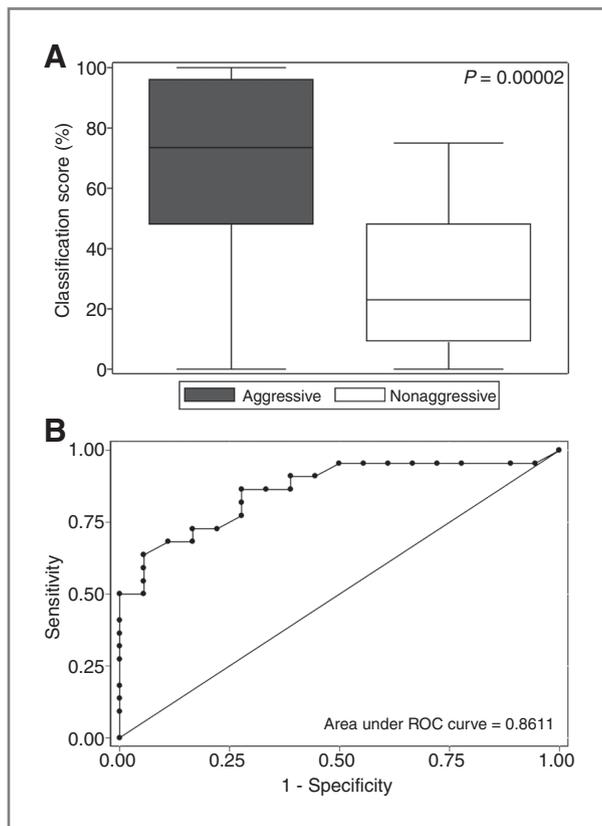
Figure 2. Distribution of classification scores. A, the horizontal line within each box represents the median; the top of the box, the 25th percentile for observation; the bottom of the box, the 75th percentile. The whiskers above and below each box represent the spectrum of scores. Although aggressive cSCC classification scores spanned the spectrum, the vast majority were greater than 37%. The majority of nonaggressive cSCC classification scores were less than 37%. B, the ROC curve showed that the classification score was a good to very good diagnostic test for identifying aggressive cSCC. The area ± SEM under the ROC curve was 0.86 ± 0.06.

specificity = 72.2%). We measured the utility of the classification score with a receiver operating characteristic (ROC) curve, which showed an area under the curve ± SEM of 0.86 ± 0.06 (Fig. 2B). The 95% CI for the area under the curve was 0.74 to 0.98.

Variations in homogeneous nuclei

After assigning classification scores to each lesion, we investigated the karyometric differences between the most aggressive and the most nonaggressive lesions. We com-
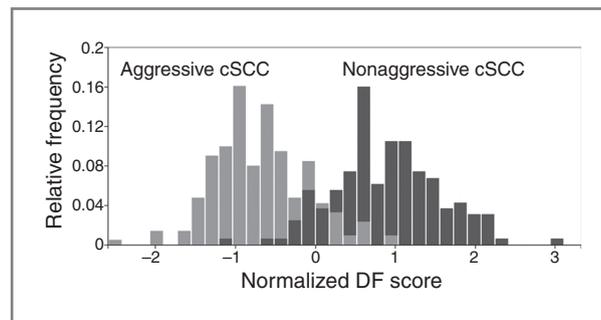

Figure 3. Normalized DFs for nearly homogeneous lesions. We found that 5 nuclear features (Table 2) differentiated between nearly homogeneous populations of aggressive and nonaggressive cSCC with an accuracy of 89.3%, suggesting that cSCC comprises 2 distinct cell populations. The histogram shows the normalized DF score for all nuclei from the nearly homogeneous lesions.

pared nearly homogeneous lesions with classification scores greater than 90% (aggressive, $n = 7$) with lesions with classification scores less than 10% (nonaggressive, $n = 6$). We identified a new DF (Table 2) that properly discriminated nuclei between nearly pure aggressive and nearly pure nonaggressive cSCC with an accuracy of 89.3% (Fig. 3).

Classification discordance

Of the 18 patients with nonaggressive cSCC, 5 were misclassified with high aggression scores. The mean age ± SD of the 5 patients whose nonaggressive cSCC was misclassified as aggressive was 86 ± 3 years, as compared with 78 ± 10 years in the 13 patients whose nonaggressive cSCC was properly classified ($P = 0.02$). We found no association between misclassification and x-ray radiation therapy or lymph node dissection.

Of the 22 patients with aggressive cSCC, 3 were misclassified with low aggression scores. The mean age of those 3 patients (78 years) was similar to that of the 19 patients (79 years) whose aggressive cSCC was properly classified ($P = \sim0.9$). Those 3 patients did, in fact, have aggressive cSCC because of recurrence ($n = 1$), a second primary cSCC in the same region ($n = 1$), and a second primary cSCC in a different region ($n = 1$).

Discussion

In our study, we showed that, in 80% of our patients, cSCC was accurately and efficiently categorized as

Table 3. Classification scores

		Aggressive cSCC ($n = 22$)	Nonaggressive cSCC ($n = 18$)	P
Nuclei analyzed per patient	Mean ± SD	96.0 ± 7.7	95.5 ± 8.8	0.534
Score	Mean ± SEM	68.6% ± 6.0%	28.0% ± 5.4%	0.00002
Misclassifications	n (%)	3 (13.6)	5 (27.8)	0.0004

aggressive or nonaggressive with karyometric analysis of routine histopathologic sections. Though retrospective, our study used extremely stringent statistical methods to identify relevant nuclear features. Furthermore, clinically relevant criteria (nuclear area and hyperchromaticity) resulted in 4 statistically unique subgroups of nuclei with 4 similar, but distinct, DFs. That finding suggested heterogeneity among nuclei size, regardless of how aggressive or nonaggressive the phenotype. Nuclear area and hyperchromaticity act as stratification tools to help determine the classification score on the basis of most statistically relevant nuclear features. An unexpected, but very fruitful, finding was that the proportion of aggressive nuclei (classification score) served as a meta-variable and directly yielded information about the aggressiveness of the clinical phenotype.

The classification score is a very useful tool because it is yields a quantitative probability of recurrence or second primaries. Treatment of recurrences and evaluation of secondary lesions is not without cost and risk. We chose to apply a threshold because this is helpful in discriminating between otherwise similar groups. However, in clinical practice, different thresholds could easily be applied to different patient populations. For example, in sicker patients with significant surgical comorbidities, a higher classification score would be required to balance any potential benefits against risks of further procedures. Likewise, aggressive follow-up may be cost effective at a lower classification score in a younger person with many more years of expected life.

Most cancers are heterogeneous; nonetheless, identifying distinct nuclear phenotypes that correlate with clinical outcomes could prove extraordinarily useful. Other groups have found changes in buccal (oral) epithelium related to lung and breast malignancies (16). We found that the ratio of aggressive to nonaggressive nuclei reflected the probability that a lesion will behave in an aggressive manner clinically. Specifically, if the proportion of aggressive nuclei was less than or greater than 37%, then our model classified lesions at the time of resection with 80% overall accuracy, similar to other karyometric analyses in other organ sites (17). With a sensitivity of 86% and a specificity of 72% in our study, karyometry shows promise as a tool for clinicians to determine adjuvant therapy or follow-up.

The behavior of a given lesion is based on the previously described nuclear phenotypes. If the balance of cancer cells is aggressive (i.e., greater than 37%), then the biology of the entire lesion is shifted toward an aggressive clinical phenotype. Likewise, if the balance of cells in a lesion is nonaggressive, then those lesions behave nonaggressively. This behavior pattern could be an important basis for pathologists to describe cSCC lesions and a relevant descriptor for clinicians to use in discussions with patients. However, karyometry does not indicate when aggressive events will occur. Furthermore, this analytic approach does not indicate whether the behavior pattern is a function of tumor biology (i.e., something we are measuring in a surrogate fashion) or a herald of events that are statistically bound to eventually occur.

We found no statistically significant differences between our 2 groups in the typical histopathologic features of aggressive cSCC—nuclear area and chromatin staining. Ploidy parallels hyperchromaticity (elevated total OD); in our study, because the total OD stratified nuclei regardless of their aggressive status, ploidy alone is likely not a good marker for poor prognosis. However, within our subgroups of similar-sized nuclei, we identified statistically significant karyometric differences. We made no a priori assumptions that nuclear area and chromatin features should or should not be included in a DF. However, our analysis showed that those 2 important features stratified subgroups regardless of cSCC aggressiveness. As such, we controlled for variation in nuclear size without categorization of cancer aggressiveness. Although nuclear size or OD may be important in differentiating cancer from benign cells, its karyometric utility is that it allows identification of aggressive cSCC nuclei after stratification by nuclear size and/or total OD. Thus, we determined relevant karyometric features for each size nucleus and OD.

Our misclassification rates were based on a threshold of 37% for the classification score. Raising the threshold would decrease the false-positive rate and increase the false-negative rate. We felt that it was important to minimize the false-positive rate, to prevent unnecessary invasive procedures. This will become extremely important when applied to larger populations in which the prevalence of aggressive cSCC will be lower. However, we envision the utility of this test will be best applied as a tool to risk stratify very specific patient populations. For example, patients receiving immunocompromising therapies such as transplant recipients are unlikely to tolerate classification scores as high as those individuals who are not immunocompromised. In this way, the quantitative nature of karyometric analysis permits patient-specific and personalized approach to the management of cSCC lesions.

The few aggressive lesions with low classification scores ($n = 3$) represented an interesting subgroup of cancers that clearly were aggressive but did not have all of the nuclear hallmarks. Thus, some phenotypes for aggression (i.e., migration or invasion) may be due to small cellular differences not correlated with nuclear changes measured in karyometry. It is possible (albeit unproven) that invasive characteristics may occur before other measures (e.g., nuclear morphometric variation) that mark progressive disease.

One consequence of the threshold classification score in our study was that 28% of our patients ($n = 5$) may have received more intensive therapies than needed, per the classification score alone. The small numbers in our study did not permit dividing the groups into a low-risk group (patients with a very low classification score), medium-risk group (patients with a classification score in the interquartile range), and high-risk group (patients with a high classification score). But future studies could implement such an approach to provide individual patient risk/benefit

information. Such information could help balance the classification score with known clinical factors such as comorbidities and tumor location, depth, size, and age. The utility of the classification score is that it provides quantitative individualized patient level data in a continuous range.

In addition to the misclassification rate, other limitations to our study merit mention. Because many elderly patients die with cSCC and not directly from it, its overall impact on society may be limited. We sought to identify aggressive lesions in patients who would have the most quality life to gain. But our small study population size, the similar age of the patients in both groups, and their similar mortality rates limit the generalizability of our results. The electronic medical records of the Veterans Affairs health care system and the fact that most patients in the database receive their care within that system make it unlikely, but not impossible, that some patients were lost to follow-up or received care elsewhere; it is possible that a recurrence or death was not captured in the database. Finally, in this study we stained all cells in a single batch. This removed any systemic variation in batch preparation, however, this may be difficult in clinical practice. Solutions to this include background stain normalization to some known standard or normalize the optical measurements to clearly normal tissue in the specimen. Although neither was done here, we believe that these steps would safely permit generalization of this algorithm into clinical practice.

Despite the significant differences we found between aggressive and nonaggressive nuclei and lesions, we cannot unequivocally apply our results to all cSCC patients, given our small study population size ($n = 40$). Likewise, we analyzed nearly 100 nuclei per lesion and combined all nuclei within each subgroup (1 to 4) to have a many-fold increase in the number of nuclei compared with features analyzed. The DF is based on nuclear features at the individual cell level, whereas the classification score is a metavariable at the lesion (patient) level.

Prognostic and predictive markers that portend aggressiveness are very important for patients with cSCC. Although a majority of patients may not need more inten-sive therapy, identifying those that who do will likely prove beneficial. Classification scores may yield subtle differences at the time of resection that carry relevant prognostic information. Predictive markers that help identify patients who are likely to benefit from more invasive treatments for aggressive disease is also extremely important where functional outcomes can be easily diminished.

In conclusion, karyometry successfully differentiated between patients with aggressive and nonaggressive cSCC. The area under the ROC curve suggested that, with minimal refinement, karyometry may be a very good to excellent discriminating tool for aggressive cSCC. The utility of our classification score was that it yielded the proportion of nuclei that were aggressive in a given lesion. It provided data about individual lesions as a continuous function that can be used for risk stratification as part of "personalized therapy". Future studies should prospectively validate the prognostic and potentially predictive utility of karyometric risk stratification in both high- and low-risk groups.

Disclosure of Potential Conflicts of Interest

Acknowledgments

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