Original Article

Study of nuclear morphometry on cytology specimens of benign and malignant breast lesions: A study of 122 cases

ABSTRACT

Background: Breast cancer has emerged as a leading site of cancer among women in India. Fine needle aspiration cytology (FNAC) has been routinely applied in assessment of breast lesions. Cytological evaluation in breast lesions is subjective with a "gray zone" of 6.9–20%. Quantitative evaluation of nuclear size, shape, texture, and density parameters by morphometry can be of diagnostic help in breast tumor.

Aims: To apply nuclear morphometry on cytological breast aspirates and assess its role in differentiating between benign and malignant breast lesions with derivation of suitable cut-off values between the two groups.

Settings and Designs: The present study was a descriptive cross-sectional hospital-based study of nuclear morphometric parameters of benign and malignant cases.

Materials and Methods: The study included 50 benign breast disease (BBD), 8 atypical ductal hyperplasia (ADH), and 64 carcinoma cases. Image analysis was performed on Papanicolaou-stained FNAC slides by Nikon Imaging Software (NIS)–Elements Advanced Research software (Version 4.00). Nuclear morphometric parameters analyzed included 5 nuclear size, 2 shape, 4 texture, and 2 density parameters.

Results: Nuclear morphometry could differentiate between benign and malignant aspirates with a gradually increasing nuclear size parameters from BBD to ADH to carcinoma. Cut-off values of 31.93 μm², 6.325 μm, 5.865 μm, 7.855 μm, and 21.55 μm for mean nuclear area, equivalent diameter, minimum feret, maximum ferret, and perimeter, respectively, were derived between benign and malignant cases, which could correctly classify 7 out of 8 ADH cases.

Conclusion: Nuclear morphometry is a highly objective tool that could be used to supplement FNAC in differentiating benign from malignant lesions, with an important role in cases with diagnostic dilemma.

Key words: Atypical ductal hyperplasia; benign breast disease; breast; carcinoma; nuclear morphometry; Papanicolaou

Introduction

Breast cancer constitutes a major public health problem worldwide. Cytological diagnosis in breast lesion is based on the subjective evaluation of features such as cellularity, cellular morphology, abnormal chromatin pattern, and mitotic activity.[1] Fine needle aspiration cytology (FNAC) results can be inaccurate in areas of "gray zone" such as atypical ductal hyperplasia, ductal hyperplasia, and even in some benign and malignant cases with false positive or false negative results. The incidence of these reports varies in literature from 6.9–20%.[2]

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Alterations in nuclear structure is the morphological hallmark of cancer diagnosis. Nuclear morphometry is the measurement of various nuclear parameters by image analysis. Direct scanning techniques generally analyze only size and texture features whereas analysis of digitized images also allows the quantization of shape features. At the same time, these systems provide rapid and reproducible analysis of cell and tissue samples for objectively evaluating morphological features. Nuclear morphometry is capable of detecting changes that are too small to be visually perceived and could be used effectively to classify the inconclusive breast FNACs.[3] When combined with cytology impression, morphometry can improve distinction between benign and malignant lesions, especially in areas of "gray zone," and thus help resolve cases with diagnostic dilemma.

Till now, majority of image analysis studies have been performed on histological sections.[3-8] There are limited reports of computer-aided image analysis in breast FNAC.[3,9-16] Nuclear morphometric analysis is better performed on cytology smears than histological tissue samples because cells are intact and well-preserved on FNAC smears as compared to histologic sections, in which processing cuts cells at various planes.

In our present study, nuclear morphometry was performed on breast FNAC smears. The present study aims at using nuclear morphometry on aspirates of histologically confirmed breast lesions to assess their role in diagnosis and delineating benign from malignant lesions with derivation of suitable cut-off values between the two groups.

Materials and Methods

The present study was conducted in our institution from 2011 to 2013. Fifty-eight benign and 64 malignant cases were studied. FNAC was performed on 720 patients who presented with breast lump during this period. Out of these 720 cases, 670 (93.1%) were satisfactory and 50 (6.9%) unsatisfactory due to inadequate material, poor cellularity, nuclear overlapping, degenerated nuclei, or poor staining. Out of 670 cases, trucut/excision biopsy/mastectomy specimen was available only for 122 cases (18.2%), which were included in the study. Cytological and histopathology examination were performed separately by single experienced pathologists.

Nuclear morphometry was performed on Papanicolaou-stained FNAC slides, and 50 nonoverlapping nuclei were evaluated per case by a trained pathologist who was blinded of cytology and histopathology diagnosis. Images were captured at 100× magnification under oil immersion using Nikon digital camera (Nikon DS-Ri - Nikon Instruments Inc., America) at same settings of microscopic light intensity, iris diaphragm, and condenser position. Images were saved in the attached computer in JPEG 2000 format. The saved images were opened in Nikon Imaging Software-AR (ver 4.00), and a background correction was done for uniformity of image intensity. Calibration was performed before each measuring session. Images of selected nuclei were outlined using the mouse of the computer at the same zoom settings. These outlines were refined automatically and automatic measurements were made by the software NIS Element ver. 4.00. A distance was assigned to 1 pixel (by calibration) and automatic measurement was done by comparing objects of different images. For intensity, brightness, and density measurement, image color layer was used by the software and measurement was done in pixel. After measurement, the data were transferred to MS-Excel sheet for further analysis.

Nuclear morphometric parameters analyzed included nuclear size, nuclear shape, nuclear texture, and nuclear density parameters [Table 1]. These parameters were compared between benign and malignant groups, and cut-off values for nuclear size parameters derived between the two groups.

Statistical analysis was performed using the SPSS (Statistical Package for the Social Sciences) program for Windows, version 17.0. Chicago. The mean values of each sample with standard deviation (SD) were calculated for all cases. Data were checked for normality before statistical analysis using Shapiro Wilk test. Normally distributed continuous variables were compared using analysis of variance test (ANOVA). If the *P* value was significant and variance was homogeneous, Bonferroni multiple comparison test was used to assess the differences between the individual groups; otherwise, Tamhane's T2 test was used. The Kruskal–Wallis test was used for variables that were not normally distributed, and further comparisons were done using Mann–Whitney U test. A receiver operating characteristics (ROC) curve was evaluated for nuclear size parameters to determine the optimal cut-off values between benign and malignant cases. For all statistical tests, a *P* value of less than 0.05 was considered to indicate a significant difference.

Results

Out of the 720 cases for which FNAC was performed during the period of study, 122 histologically confirmed cases with satisfactory cytology smears were included in the study.

Based on cytology features such as cellularity, cell morphology, nuclear margin, nuclear chromatin, nucleoli etc., 50/122 (40.9%)

cases were categorized as BBD [Figure 1], 8 (6.5%) as ADH [Figure 2], and 64 (52.4%) as carcinoma [Figure 3]. On histopathology, 58 cases were found to be benign which included 50 BBD (including fibroadenoma, fibrocystic disease, fibroadenosis, benign papillary lesion, and ductal hyperplasia without atypia), 8 ADH cases, and 64 were malignant (including invasive ductal carcinoma, lobular, papillary, apocrine, and medullary carcinoma). Age of the patients in the study ranged from 14 to 91 years. Median age of benign (28.43 years) was lower than malignant cases (48.07 years).

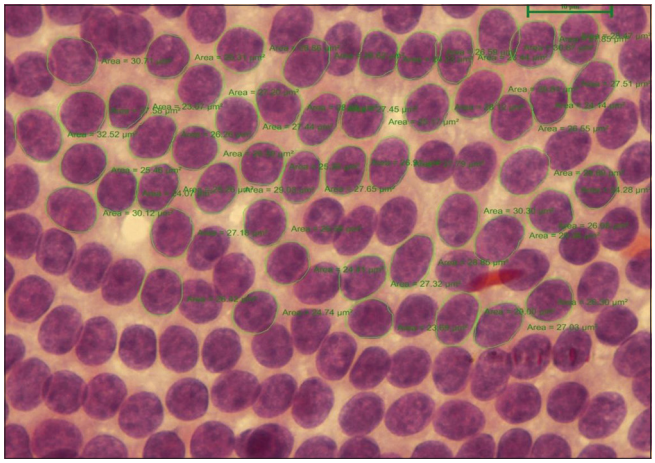

Figure 1: Benign breast disease (Pap, x1000)

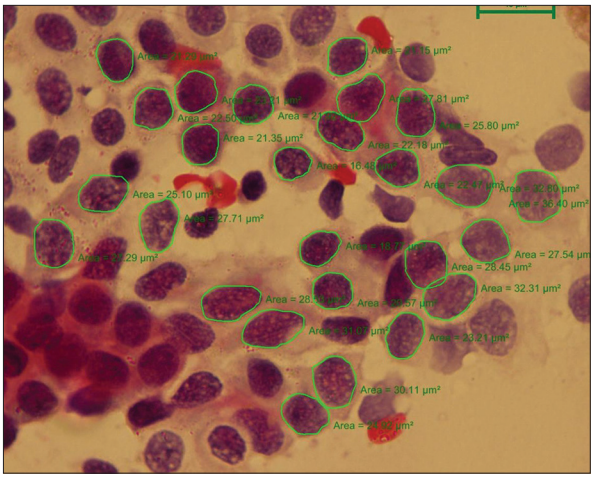

Figure 2: Atypical Ductal Hyperplasia (Pap, x1000)

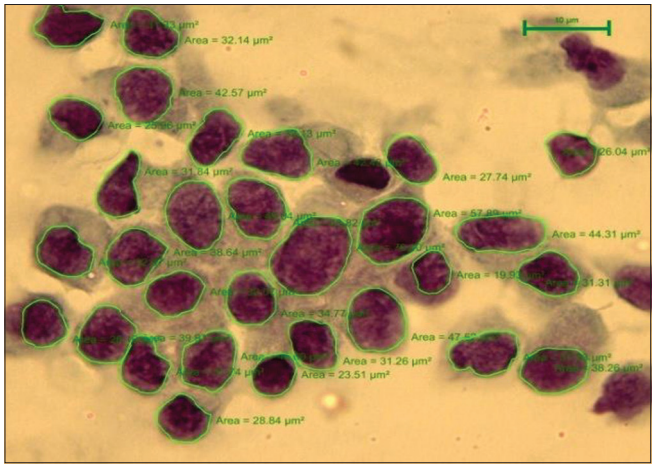

Figure 3: Carcinoma (Pap, x1000)

Nuclear morphometry parameters were analyzed and compared between benign and malignant groups [Table 2]. Nuclear size, nuclear texture, and nuclear density parameters were highly significant in differentiating between benign and malignant cases ($P < 0.001$ for all of them). Among nuclear shape parameters, only the shape factor was statistically significant in differentiating between benign and malignant cases ($P < 0.001$). On comparing the three groups on cytology, all nuclear size parameters showed an increasing trend from BBD to ADH to carcinoma [Table 3], with a significant difference between ADH and carcinoma for all nuclear parameters except roughness ($P < 0.495$), mean intensity ($P < 0.11$), mean brightness ($P < 0.98$) and mean density ($P < 0.341$).

ROC analysis was done to determine the optimal cut-off values for various variables between benign and malignant groups [Figure 4]. The area under the curve and its sensitivity and specificity were calculated using statistical methods to analyze the diagnostic value of all these variables. Our cut-off values for mean nuclear area, equivalent diameter, minimum feret, maximum ferret, and perimeter between benign and malignant cases were found to be 31.93 μm², 6.325 μm, 5.865 μm, 7.855 μm, and 21.55 μm, respectively. In our study, using histopathology diagnosis as the final diagnosis, the combined specificity of the derived cut-off values between the two groups was excellent (ranging from 98.3 to 100%) with a good sensitivity (ranging from 79.6 to 81.2%). Seven out of 8 ADH cases had nuclear size below the cut-off value and could be correctly classified as benign on morphometry.

Discussion

FNAC is a simple and cost effective method, however, it is based on a subjective visual evaluation of features such as cellularity, cell morphology, and chromatin, which can be associated with interobserver and intraobserver variability. It has a further disadvantage in differentiating among cases with morphological overlap (precancerous group to frank carcinoma). This study emphasizes on morphometry as an objective tool to supplement subjective cytology evaluation in differentiating benign from malignant lesions for crucial preoperative assessment.

Table 1: Nuclear morphometric parameters used in study

Nuclear size parameters	Nuclear area	Area	Real area of the nucleus
	Nuclear diameter	Equivalent diameter	Diameter of a circle with the same area as the measured nucleus
	Nuclear feret	Maximum feret	Maximum value of the set of feret's diameter (equals the projected length of object at an angle)
		Minimum feret	Minimum value of the set of feret's diameter
	Nuclear perimeter	Perimeter	Total boundary measure
Nuclear shape parameters (measures nuclear shape pleomorphism and nuclear membrane irregularity)		Shape Factor (Shape factor of "1" means a perfect circular nucleus, and any value <1 shows nuclear shape pleomorphism)	4π Area/(convex hull perimeter)^2: Defines whether the nucleus is rough or not (measures nuclear shape pleomorphism)
		Roughness (Roughness of "1" means minimal roughness or irregularity of nuclear membrane)	Convex hull perimeter/perimeter: Indicates how rough/irregular is the nuclear membrane
Nuclear texture parameters (measure of granularity of nuclear chromatin)	Nuclear Intensity parameters	Mean Intensity	Mean of intensity values of pixel
		Sum Intensity	Sum of intensity in every pixel of object
	Nuclear Brightness parameters	Mean Brightness	Mean of brightness values in pixel
		Sum Brightness	Sum of brightness in every pixel of object
Nuclear density parameters (measure of nuclear chromasia)		Mean Density	Mean of density values of pixels
		Sum Density	Sum of individual optical densities of each pixel in the area being measured

Table 2: Nuclear morphometric parameters in benign and malignant lesions

Histopathology	Cytology		*P*
	Benign (*n*=58)	Malignant (*n*=64)	
	50 BBD 8 ADH	64 Carcinoma	
Area (μm^2)	25.49±3.88	51.43±20.47	<0.001
Equivalent diameter (μm)	5.66±0.44	7.87±1.50	<0.001
Minimum feret (μm)	5.11±0.41	7.09±1.29	<0.001
Maximum feret (μm)	6.61±0.57	9.26±1.81	<0.001
Perimeter (μm)	18.39±1.49	25.69±4.99	<0.001
Shape factor*	0.95±0.02	0.94±0.01	<0.001
Roughness†	1.0032±0.003	1.0033±0.003	0.401
Mean intensity	96.37±18.14	80.56±10.63	<0.001
Sum intensity	683101.7±158116	1153390.65±532738	<0.001
Mean brightness	37.82±7.14	31.53±4.08	<0.001
Sum brightness	267910.74±62013.03	452250.92±20883.2	<0.001
Mean Density	0.45±0.10	0.54±0.07	<0.001
Sum Density	3232.86±950.35	7519.18±2819.28	<0.001

Values are given as mean±standard deviation; *Shape factor of "1" means a perfect circular nucleus and any value <1 shows nuclear shape pleomorphism; †Roughness of "1" means minimal roughness or irregularity of nuclear membrane

Nuclear morphometry is the quantitative analysis of morphological changes in the nucleus by nuclear size parameters (measures nuclear enlargement), nuclear shape parameters (measures nuclear shape pleomorphism and nuclear membrane irregularity), nuclear texture parameters (measures coarseness of nuclear chromatin), and nuclear density parameters (measures nuclear chromasia). It can differentiate between benign and malignant cases and help in the definitive diagnosis of cytologically borderline cases.[17]

In our study, all nuclear size, nuclear texture, nuclear density parameters, and 1 out of 2 nuclear shape parameters (shape factor) were highly significant in differentiating between benign and malignant cases ($P < 0.001$). The lack in significant difference in roughness might be due to an inherent problem in tracing irregular nuclear outlines in malignant cases. Minor nuclear membrane convolutions and indentations could be overlooked during the process of digitization of nuclear images.

Kalhan *et al*. found similar results in their study with all parameters, except shape and intensity, to be significant in differentiating benign from malignant lesions.[18] Aggarwal *et al*. in their study regarding intraoperative imprint smears and histopathological sections of various breast lesions observed a significant difference in the mean nuclear area, mean nuclear perimeter, mean nuclear diameter, and nuclear-to-cytoplasmic ratio between benign and malignant lesions ($P < 0.05$).[19] However, in their study, shape factor was not found to be significant in differentiating between benign and malignant lesions unlike the finding in the present study. Wolberg *et al*., in their study on breast cytology, showed significant difference between benign and malignant cases for all the nuclear parameters except mean of fractal dimension, standard error of texture, standard error of smoothness, standard error of symmetry, and standard error of fractal dimension which is very similar to our study.[20]

Cut-off value between benign and malignant lesion derived in our study was 31.93 μm^2 for nuclear area and 21.55 μm for nuclear perimeter. This is not comparable to other studies by Boruah *et al*. (cut-off of 60.61 μm^2 for nuclear area and 27.81 μm

Table 3: Nuclear morphometry: Progression pattern

Nuclear morphometric parameters	BBD (n=50)	ADH (n=8)	Malignant (n=64)	ADH vs Carcinoma (P)
Area (μm^2)	24.86±3.59	29.44±3.51	51.43±20.47	0.0036
Equivalent diameter (μm)	5.59±0.41	6.08±0.36	7.87±1.50	0.0013
Min feret (μm)	5.07±0.40	5.39±0.43	7.09±1.29	0.0005
Max feret (μm)	6.51±0.3	7.26±0.42	9.26±1.81	0.0028
Perimeter (μm)	18.15±1.40	19.91±1.18	25.69±4.99	0.0018
Shape factor*	0.95±0.02	0.93±0.02	0.94±0.01	0.022
Roughness†	1.0033±0.002	1.0025±0.004	1.0033±0.003	0.495
Mean intensity	97.69±17.06	88.16±23.50	80.56±10.63	0.11
Sum intensity	677628.44±153651	717309.59±191745	1153390.65±532738	0.025
Mean brightness	38.35±6.73	34.57±9.21	31.53±4.08	0.098
Sum brightness	265768.80±60264.39	281297.88±75194.00	452250.92±20883.2	0.0001
Mean density	0.45±0.09	0.51±0.16	0.54±0.07	0.341
Sum density	3087.85±772.72	4139.20±1444.22	7519.18±2819.28	0.0014

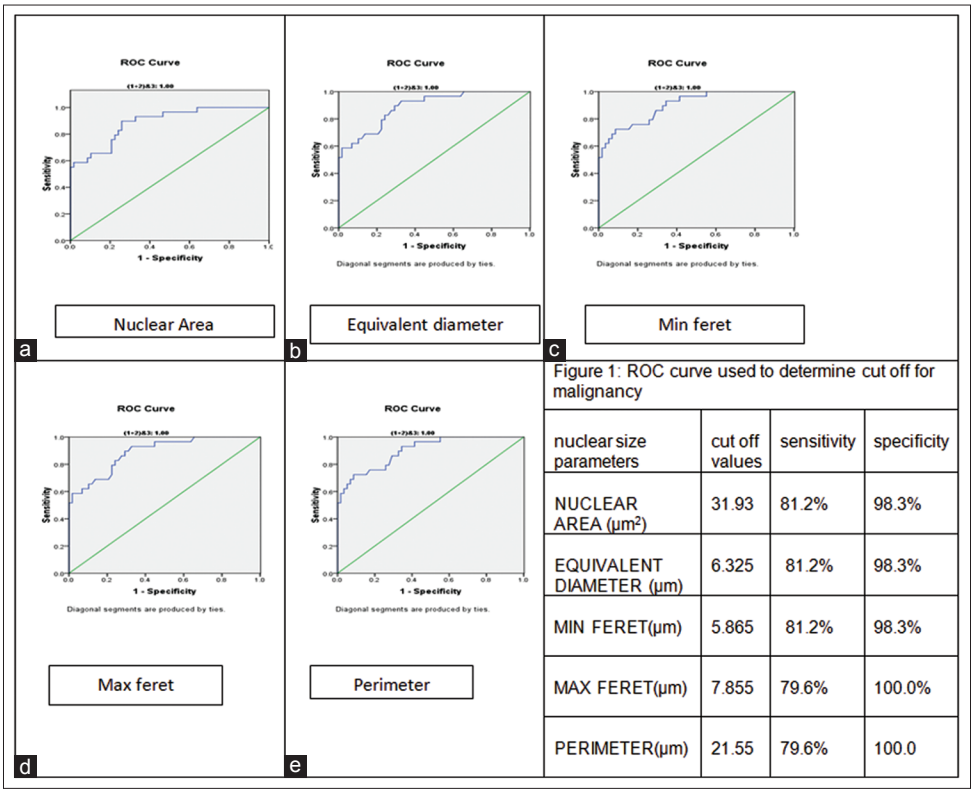

Figure 1: ROC curve used to determine cut off for malignancy

nuclear size parameters	cut off values	sensitivity	specificity
NUCLEAR AREA (μm^2)	31.93	81.2%	98.3%
EQUIVALENT DIAMETER (µm)	6.325	81.2%	98.3%
MIN FERET(µm)	5.865	81.2%	98.3%
MAX FERET(µm)	7.855	79.6%	100.0%
PERIMETER(µm)	21.55	79.6%	100.0

Figure 4: (a-e) Receiver operating characteristics curves and cut-off values between benign and malignant cases

for nuclear perimeter) and Abdalla *et al*. (mean nuclear area cut-off of 60.5 µm²).[16,21] This difference could be due to the difference in the software used in other studies, along with difference in other analytical factors (patient ethnicity, slide preparation and staining differences, etc.).

Our study showed a gradual increase in nuclear size parameters from BBD to ADH to carcinoma cases. The progression pattern of nuclear morphometric parameters from benign to atypical, Ductal Carcinoma in Situ (DCIS), and further to invasive carcinoma, has been emphasized in various studies on histopathology.[3,4,8,16,22-24] Although this study had only 8 ADH cases, 7 out of 8 cases had nuclear size below the cut-off value and could be correctly classified as benign on morphometry. One of the ADH cases had higher nuclear size parameters, which could be due to overriding of nuclear/cytoplasmic contours during tracings, magnifications used, and speed of conducting analysis.

This study emphasizes on morphometry as an objective tool to supplement subjective cytology evaluation in differentiating benign from malignant lesions for crucial preoperative assessment and decision on patient management. However, errors can occur due to technical problems in nuclear morphometry, which can lead to false positive and false negative results. This can be overcome by training, internal calibration, and standardization by an expert observer.

Conclusion

Nuclear morphometry has proved to be very useful in differentiating benign lesions from malignant ones on cytology in this study. Despite small sample size of borderline cases in our study, nuclear morphometry has proved to be a useful tool in classifying 7 out of 8 ADH cases. However, a study on a large number of inconclusive cases is needed for further evaluation of its role in the "gray zone" in cytology.

Thus, after training, internal calibration, and standardization, nuclear morphometry can prove to be a very useful tool in supplementing FNAC in differentiating between benign and malignant lesions for crucial decision on patient management, especially in cases with diagnostic dilemma.

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Conflicts of interest
There are no conflicts of interest.

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