Original Article

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An ImageJ Based Semi-Automated Morphometric Assessment of Nuclei in Oncopathology

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Abstract

Introduction: Nuclear morphology is an important determinant in the diagnostic and prognostic interpretation of tumors. Incorporation of morphometric analysis of the nucleus makes such interpretations more objective and precise. In this study, we have used a semi-automated image analysis method to analyze tumor nuclei in breast and cervical cancer.

Methods: Using ImageJ and three of its plug-ins - Kuwahara filter, Bi-exponential edge preserving smoother filter, and Mexican hat filter - We developed an image processing algorithm. We used this to analyze the nuclear parameters in three grades of invasive ductal carcinoma and cervical neoplastic lesions including cervical intraepithelial neoplasia 1 (CIN1), CIN2, carcinoma *in situ* (CIN3), and squamous cell carcinoma. The parameters analyzed were a nuclear area, perimeter, and circularity. The results obtained were statistically analyzed.

Results: Mean and standard deviation values of area and perimeter measurements showed statistically significant differences between the three grades of breast carcinoma. Within the cervical neoplasia, there were statistically significant differences between invasive carcinoma and intraepithelial neoplasia of all grades. In addition, median values for area parameter was much lower than mean values suggesting a skewed distribution of tumor cells.

Conclusions: This study suggests that morphometric analysis of nuclear parameters is helpful in the grading of the tumors and in assessing their prognosis. In higher grade tumors, the median value for the nuclear area is markedly lower than the mean value suggesting a right skewed distribution. The latter feature may be an important characteristic of aggressive tumors.

Key words: Analysis, Breast neoplasms, Carcinoma, Squamous cell, Uterine cervical neoplasms

INTRODUCTION

In the diagnostic histopathological evaluation of tumors, nuclear morphology plays a central role. The nature of the tumor and its aggressiveness are mainly determined by nuclear features. With the coming of digital age and the easy availability of several image analysis softwares, histomorphometry is being increasingly used in oncopathology for diagnostic, prognostic, and research purposes.[1] Among tumors that have been studied include those of colon, breast, ovary, skin, kidney, etc.[1-4] One of the most popular image analysis softwares is ImageJ, an open source software developed by Rasband at National Institute of Health.[5] It has a simple interphase and numerous free plugins. In an earlier study,[6] we used ImageJ and three of the plugins, for the first time, to develop an image processing and analysis algorithm to analyze papanicolaou (PAP) smears. In this study, we have modified the processing algorithm to make it capable of analyzing nuclear morphology in histopathological sections. Using that we have analyzed breast carcinoma and cervical neoplasia.

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MATERIALS AND METHODS

Nuclear measurements were carried out on invasive ductal carcinomas of breast of all the three grades and cervical lesions including cervical intraepithelial neoplasia 1 (CIN1), CIN2, CIN3 (carcinoma *in situ* [CIS]), and squamous cell carcinoma. Tissue samples from all these cases were processed to prepare 3 micron sections. These sections

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were stained with hematoxylin and eosin (H and E) stain. Digital images of representative areas were taken using 8 mega pixel Olympus SP 350 compact Zoom Camera attached through a microscope adopter to Olympus CX 41 trinocular microscope. The photographs were taken using ×40 objective and ×10 ocular. The images were processed in a photo-editing software to improve the contrast. In all images, three representative areas, each equivalent to 1600 × 1200 pixel crop (1, 92,000 pixels), were used for nuclear analysis. In breast carcinoma, a minimum of 340 nuclei (and up to 1785) were analyzed. In cervical neoplasia, a minimum of 165 nuclei (and up to 441) were analyzed. ImageJ image analysis software was used in this study to carry out nuclear measurements.[5] In an earlier study,[6] we had developed an analysis algorithm using three ImageJ plug-ins to analyze PAP smears: Kuwahara filter,[7] Bi-exponential edge preserving smoother (BEEPS) filter,[8] and Mexican hat filter.[9] That algorithm was modified and adapted to analyze histological sections (Figure 1). The Kuwahara filter reduces the noise and gently homogenizes the area of interest while preserving the edges; BEEPS filter blurs the distracting background without adversely influencing the edges; and the Mexican Hat Filter applies Laplacian of Gaussian filter to isolate signal from the noise.

Processing Algorithm

After the images were separated into individual colors using color deconvolution plug-in,[10,11] "color one," which represents hematoxylin component, was selected (when using this plug-in, vector "H and E" was selected). On "color one," "Kuwahara filter" was applied with sampling window showing an odd number between 5 and 9. BEEPS filter was applied twice (iteration = 2) at the default value. A radius between 3 and 5 was chosen for Mexican hat filter. The quality of sections (thinner sections yield better results) and the image contrast should be good to ensure optimal results. The "analyze tool" in ImageJ was configured to measure Area, Perimeter, and Circularity. The perimeter is the boundary length of a region of interest (ROI, in this case, is nucleus). Circularity value indicates how close to a circle the object is. The settings for "Analyze tool" were as follows: Size = 1000 to infinity (to exclude structures <1000 pixels); circularity = 0.20-1 (to ignore elliptical and linear structure); show = Overlay outlines or masks. The unit of measurement was pixel (default in ImageJ). Cellular density was calculated for 100000 pixels.

The processing of the images was partially automated by creating two macros. First macro executed the steps from color deconvolution to application of Mexican hat filter; then thresholding and binary conversions were done manually; this was followed by the second macro to execute the final steps of processing and analysis (Figure 2). The report (results and summary) is generated automatically as a spreadsheet.

The results obtained were analyzed statistically. The mean value and standard deviation of different samples were compared using *t*-test (for means) and *F*-test (for standard deviations). A $P < 0.05$ was taken as significant.

RESULTS

Breast Carcinoma

Nuclear density (i.e., number of cells per 100000 pixels) increased with the grade of the tumor: Grade 1 = 59; Grade 2 = 77.4; and Grade 3 = 89.3. More aggressive tumors were associated with higher density (Table 1).

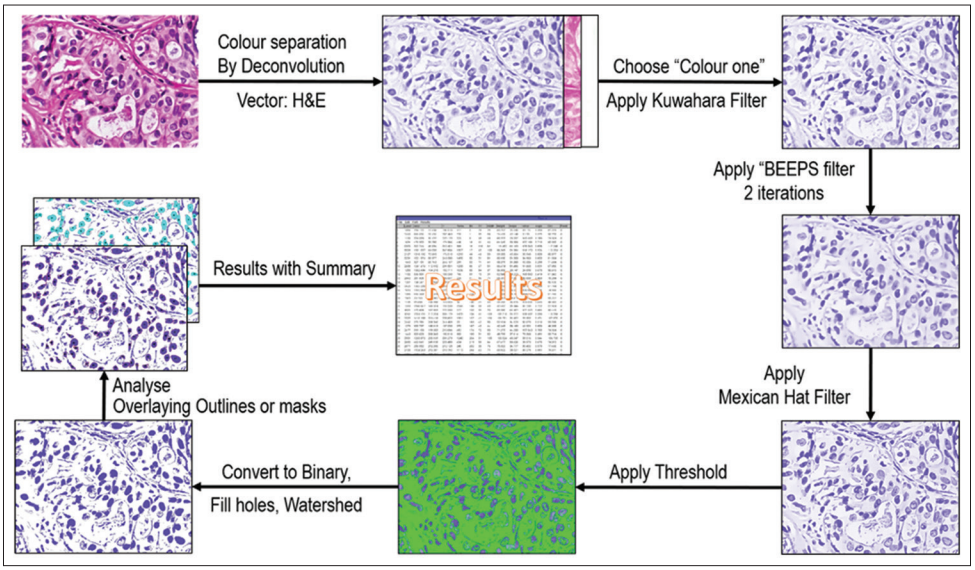

Figure 1: The processing algorithm

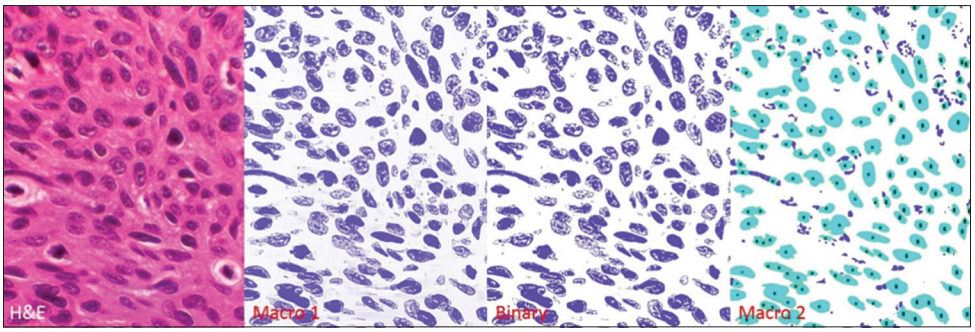

Figure 2: Processing of image analysis was done in three steps involving the application of two macros and manual binary conversion (original H and E image taken at ×40; the image size is 1600 × 1200 pixels)

Table 1: Mean, median, and SD values for the measured parameters in three grades of breast carcinoma

Lesion	Count (density)	Mean±SD (median)		
		Area	Perimeter	Circularity
Grade 1	340	1673.24±581.82	180.20±37.73	0.655±0.138
	(59)	1553.5	175.5095	0.6715
Grade 2	1785	2118.88±960.65	200.79±48.18	0.652±0.137
	(77.5)	1912	192.024	0.664
Grade 3	1543	2299.15±1211.06	211.89±59.32	0.635
	(89.3)	1966	201.196	0.651±0.142

Count: Number of nuclei counted; Density (in parenthesis in the second column): Number of nuclei/100000 pixels, SD: Standard deviation

Table 2: Comparison of mean and SD values for the measured parameters within three grades of breast carcinoma with statistical significance

Parameters	Values	Grade 1	Grade 2	Grade 3
Area				
Grade 1				
Mean	1673.24	-	*P*<0.001	*P*<0.001
SD	581.82	-	*P*<0.001	*P*<0.001
Grade 2				
Mean	2118.88	*P*<0.001	-	*P*<0.001
SD	960.65	*P*<0.001	-	*P*<0.001
Grade 3				
Mean	2299.15	*P*<0.001	*P*<0.001	-
SD	1211.06	*P*<0.001	*P*<0.001	-
Perimeter				
Grade 1				
Mean	180.2	-	*P*<0.001	*P*<0.001
SD	37.738	-	*P*<0.001	*P*<0.001
Grade 2				
Mean	200.799	*P*<0.001	-	*P*<0.001
SD	48.18	*P*<0.001	-	*P*<0.001
Grade 3				
Mean	211.89	*P*<0.001	*P*<0.001	-
SD	59.32	*P*<0.001	*P*<0.001	-
Circularity				
Grade 1				
Mean	0.655	-	*P*=0.815	*P*<0.05
SD	0.139	-	*P*=0.727	*P*=0.571
Grade 2				
Mean	0.653	*P*=0.815	-	*P*<0.001
SD	0.137	*P*=0.727	-	*P*=0.115
Grade 3				
Mean	0.635	*P*<0.05	*P*≤0.001	-
SD	0.142	*P*=0.571	*P*=0.115	-

SD: Standard deviaiton

Morphometric Analysis

Results of nuclear measurements in breast carcinoma and cervical neoplasia are summarized in tables (Tables 1 and 2).

Two of the parameters measured, i.e., area and perimeter showed a very good correlation with the grade of the tumor. The mean, standard deviation (SD) and median values for these two parameters were much higher in grade 3 tumors compared to the tumors of lower grades (Table 1). The differences in mean and SD were statistically significant ($P < 0.001$) (Table 2). These results are consistent with the view that nuclear enlargement and pleomorphism (higher SD) are characteristic of higher grade tumors. There was also a statistically significant difference in the values obtained for these two parameters when we compared Grade 2 tumors to Grade 1 tumors. As far as circularity was concerned, only Grade 3 tumors showed a significantly lower value ($P < 0.05$ to 0.01) than the other grades implying that the tumor cells were less circular and more irregular. One other significant observation was that the median value for area parameter was much lower than the mean value suggesting a skewed distribution of tumor cells particularly in tumors of a higher grade.

Cervical Neoplasia

Nuclear density varied with the type of the lesion. There was a progressive increase in nuclear density as intraepithelial neoplasia progressed from CIN 1 to CIN3: CIN1 = 28.6; CIN2 = 65; and CIN3 = 73. However, full blown squamous cell carcinoma exhibited lower density than CIN 2 and CIN 3:57.4 (Table 3).

The mean and SD of all the measured parameters showed significant differences between squamous cell carcinoma and different types of intraepithelial neoplasia ($P < 0.05$ to 0.0001) (Table 4). In addition, the standard deviation of area parameter was markedly high implying a high degree of pleomorphism. The median value for area parameter was much lower than the mean (2245 vs.

3350.99 pixels). This is indicative of markedly skewed distribution of tumor cells. Within intraepithelial neoplasia, the mean values and SD for the area were not significantly different between CIN2 and CIN3 (CIS). However, the values for measured parameters for CIN1 were significantly lower than CIN2 ($P < 0.05$).

DISCUSSION

Study of nuclear morphology is an important part of the histological evaluation of tumors. Even under routine light microscopic evaluation, nuclear characteristics provide the crucial information necessary to determine the tumor's biological behavior and aggressiveness. With the advent of digital age, image analysis techniques have been increasingly applied to study the nuclear morphology as such evaluations are likely to be less subjective and more precise. Many proprietary (Imagepro Plus,[12] Pax-It,[13] Olympus Stream,[14]) and open source (ImageJ, Cell-Profiler,[15]) softwares are available to do the same. Of these, ImageJ is quite popular as it has large number plugins and is relatively simple to use.

In an earlier morphometric study,[6] done on PAP smears, we used three of its plugins (BEEPS, Kuwahara Filter, and Mexican hat filter) in a newly designed algorithm. In the present study, we modified that algorithm to suit the assessment of histological sections. The histological sections, in general, tend to be slightly thicker than cytological smears with cohesive cells dispersed in a distracting stromal background. Besides that, the excessive clumping of chromatin makes nuclei less homogeneous. So, our design goals when developing the processing algorithm were to render the nucleus more homogeneous while preserving its edges and to isolate it from the distracting background. To achieve the former effect, the median filter is commonly used. It achieves homogeneity by softening the details. However, it causes blurring of the edges. So, we used Kuwahara filter for achieving this. It removes the noise and renders the target area more homogeneous but preserves the edges. Both BEEPS filter and Mexican hat filter help in isolating the ROI in their separate ways; the former blurs the background while preserving the edges; the latter enhances the signal by applying Laplacian of Gaussian filter. Our method worked consistently when the nuclei were well stained with adequate contrast.

Commonly measured parameters include nuclear area, its perimeter, circularity, and diameter. Of these, the diameter is in most cases a derived parameter as most cells/nuclei in histological material are not circular. In the morphometric analysis, the mean and median values of these parameters and the standard deviation (reflecting the extent of

Table 3: Mean, median, and SD values for the measured parameters in cervical neoplasia including squamous cell carcinoma

Lesion	Count (density)	Mean±SD (median)		
		Area	Perimeter	Circularity
SCC	441	3350.99±2730.39	274.70±114.27	0.512±0.132
	(57.4)	2245	242.83	0.495
CIS				
CIN 3	281	2877.97±1652.19	232.84±71.10	0.635±0.103
	(73)	2423	222.066	0.64
CIN 2	252	2972.76±1521.26	238.74±62.76	0.631±0.127
	(65)	2590.5	236.6435	0.647
CIN 1	165	2647.85±1246.95	220.74±55.54	0.666±0.137
	(28.6)	2319	213.966	0.692

SCC: Squamous cell carcinoma; CIS: Carcinoma *in situ*, CIN 1, 2, 3: Cervical intraepithelial neoplasia 1, 2, 3, Count: Number of nuclei counted, Density (in parenthesis in second column): Number of nuclei/100000 pixels, SD: Standard deviation

Table 4: Comparison of mean and SD values for the measured parameters within cervical neoplasia including squamous cell carcinoma with statistical significance

Parameters	Values	SCC	CIS	CIN2	CIN1
Area					
SCC					
Mean	3350.995	-	*P*<0.01	*P*<0.05	*P*<0.005
SD	2730.389	-	*P*<0.001	*P*<0.001	*P*<0.001
CIS					
Mean	2877.968	*P*<0.01	-	*P*=0.5	*P*=0.122
SD	1652.193	*P*<0.001	-	*P*=0.181	*P*<0.001
CIN2					
Mean	2972.766	*P*<0.05	*P*=0.492	-	*P*<0.05
SD	1521.263	*P*<0.001	*P*=0.181	-	*P*<0.01
CIN1					
Mean	2647.855	*P*<0.005	*P*=0.122	*P*<0.05	-
SD	1246.95	*P*<0.001	*P*<0.001	*P*<0.01	-
Perimeter					
SCC					
Mean	274.701	-	*P*<0.001	*P*<0.001	*P*<0.001
SD	114.273	-	*P*<0.001	*P*<0.001	*P*<0.001
CIS					
Mean	232.842	*P*<0.001	-	*P*=0.312	*P*=0.061
SD	71.104	*P*<0.001	-	*P*<0.05	*P*=0.001
CIN2					
Mean	238.747	*P*<0.001	*P*=0.312	-	*P*<0.005
SD	62.763	*P*<0.001	*P*<0.05	-	*P*=0.090
CIN1					
Mean	220.742	*P*<0.001	*P*=0.061	*P*<0.005	-
SD	55.537	*P*<0.001	*P*<0.001	*P*=0.090	-
Circularity					
SCC					
Mean	0.512		*P*<0.001	*P*<0.001	*P*<0.001
SD	0.132		*P*<0.001	*P*=0.498	*P*=0.550
CIS					
Mean	0.635	*P*<0.001		*P*=0.688	*P*<0.01
SD	0.103	*P*<0.001		*P*<0.001	*P*<0.001
CIN2					
Mean	0.631	*P*<0.001	*P*=0.688		*P*<0.01
SD	0.127	*P*=0.498	*P*<0.001		*P*=0.280
CIN1					
Mean	0.666	*P*<0.001	*P*=0.01	*P*=0.01	
SD	0.137	*P*=0.550	*P*<0.001	*P*=0.280	

SCC: Squamous cell carcinoma, CIS: Carcinoma *in situ*, CIN 1, 2, 3: Cervical intraepithelial neoplasia 1, 2, 3, SD: Standard deviation

variation and distribution) are collected. The nuclear area and perimeter measurements have been shown in several studies to have good correlation with prognosis in breast carcinomas,[3] and melanomas.[16] They have also been shown to be useful in distinguishing benign from malignant lesions. Some studies have claimed that standard deviation has a better predictive value than the mean value.[1,16]

In our study, we measured area, perimeter, and circularity. Circularity is a measure of how close to a circle the ROI is. If it is close to 1, the ROI is nearly circular; if it is close to 0, ROI is linear or markedly elliptical. The mean values for the area, perimeter, and standard deviation for the three grades of breast carcinoma showed statistically significant differences. However, the values for circularity did not exhibit statistically significant variation between all the grades.

Within cervical neoplasia, morphometry proved extremely useful in differentiating squamous cell carcinoma from cervical intraepithelial neoplasia of all grades. Differences in the means and standard deviations of all the measured parameters (area, perimeter, and circularity) were statistically highly significant. The latter observation is in agreement with the results of an earlier study.[1] Within the intraepithelial neoplastic lesions, only CIN1 showed statistically significant differences with CIN2 and CIN3 in the mean and SD values of the measured parameters. However, differences between CIN2 and CIN3 were not significant. Rightly, the latter two entities should be treated as one (as done in PAP cytology).

We measured median values of all the parameters. There was noticeable to marked differences between the values for median and mean of area parameter. The median values were consistently lower than mean values suggesting a right (positive or upward) skewed distribution. This was borne out when we did distribution fitting (Figure 3). This was more marked in higher grade lesions.

We also determined the nuclear density (number of nuclei/100000 pixels) in all the lesions. In breast carcinoma, nuclear density was much higher in Grade 3 lesions (89.3) compared with lower grade lesions (59 and 77.4) (Table 1). However, among cervical lesions, the highest nuclear density was observed in CIS (CIN3 - 73). Squamous cell carcinoma had much lower nuclear density (57) than CIN2 (65) and CIN3 (Table 3).

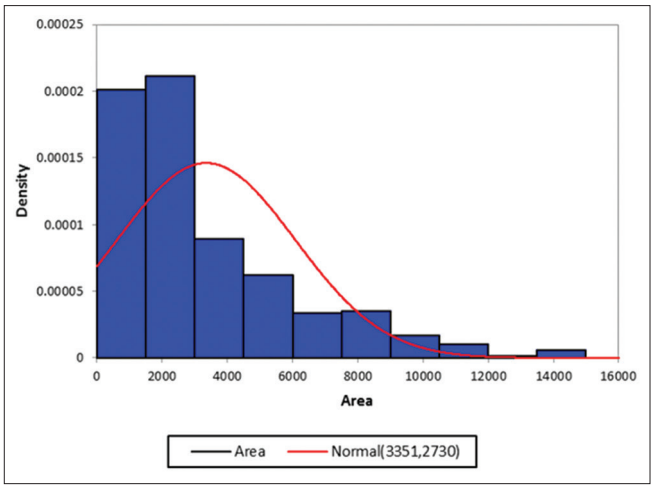

Figure 3: Distribution fitting of the results of squamous cell carcinoma showing right (positive or upward) skewed distribution

CONCLUSION

A newly designed image analysis algorithm was employed to analyze nuclear morphology in breast and cervical carcinomas. The nuclear parameters analyzed include area, perimeter, and circularity. The mean, median, and standard deviations of all the measured parameters were determined. In addition, nuclear density was also found out. The following conclusions were drawn:

- The mean and the standard deviation values of the nuclear area and perimeter showed statistically significant differences between the three grades of breast carcinoma. The nuclear morphometry can be employed usefully in assessing the grade.
- Similarly, there were statistically significant differences between squamous cell carcinoma and all grades of cervical intraepithelial neoplasia.
- The Median values for the area parameter, in particular, was significantly lower than the values for the mean in higher grade lesions including squamous cell carcinoma, suggesting a right skewed distribution. The latter may be an important characteristic of carcinomas and may be used in distinguishing it from benign lesions that mimic carcinoma. However, further observations are necessary.
- The nuclear density showed a direct correlation with the grade of breast carcinoma. In cervical lesions, CIS (CIN3) had the highest nuclear density.

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