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Original Research

# Utility of nuclear morphometry in serous ovarian carcinoma and its correlation with grades

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## ABSTRACT

**Objective:** The main objectives of this study were: To evaluate nuclear major axis (MAJX) and minor axis (MINX), nuclear area (NA), nuclear perimeter (NP), nuclear aspect ratio (NAR), and nuclear roundness (NR) with their variability using morphometric techniques in malignant and benign ovarian tumors and to correlate them with histological grades. **Methods:** Morphometric parameters were evaluated in 8 low-grade (LG) and 22 high-grade (HG) serous ovarian carcinoma and 30 benign grade (BG) cases by digital image morphometric technique using histological sections. **Results:** The mean of the size-related nuclear parameters: MAJX, MINX, NA, NP, and their variability were statistically significantly greater ($P < 0.01$) in malignant cases than benign, whereas mean NAR and its variability were significantly lower in malignant cases. Mean NR was significantly higher but its variability (standard deviation - NR) was significantly lower in malignant cases. Histological grade exhibited strong positive correlation with MAJX ($\rho = 0.864$), MINX ($\rho = 0.882$), NA ($\rho = 0.875$), NP ($\rho = 0.859$), and moderate positive correlation with NR ($\rho = 0.682$); unlike NAR ($\rho = -0.794$). Except NR, all other parameters showed positive correlation with their variability. Mean MINX and NA in HG tumor were statistically significantly higher ($P < 0.05$). For all malignant cases: MINX > 5.03 $\mu$m and NA > 30.44 $\mu m^2$ can be used to differentiate from benign with 100% efficiency. **Conclusion:** Morphometric parameters related to nuclear size and their variability were significantly larger in malignant cases than the benign and showed strong positive correlation with the grades. Nuclear shapes of the malignant nuclei were rounder than the benign. Nuclear morphometry can be gainfully exploited in the diagnosis of ovarian carcinoma quantitatively.

KEY WORDS: Morphometric analysis, nuclear parameters, serous ovarian tumor

## INTRODUCTION

Ovarian cancer is the seventh most common cancer and the eighth most common cause of death from cancer in women. It is more common in North America and Europe than Africa and Asia [1]. In India, cancer-based registries show that ovarian cancer is the third leading site of cancer among women, behind cervix and breast cancer. The age-adjusted incidence rates of ovarian cancer vary between 5.4 and 8.0 per 100,000 in different parts of the country [2,3]. Certainly, the malignancies are detected only in the late stages. In histopathological examination of tumor tissue, the multitude of patterns and borderline morphology often present a gray area in accurate typing and grading.

Histopathological and cytological variables in correlation with clinical findings are helpful in forecasting the clinical outcome of cancer patients. Nuclear features are the cornerstone in diagnosis and grading of suspected neoplastic lesions. In malignant tumors, the nuclear size is usually larger and more irregular in high-grade (HG) tumors as compared to benign lesions and is a reflection of DNA content, ploidy, and active proliferation. Nuclear morphometric study is one of the techniques to evaluate the tumors and their grades [4,5]. Computer-assisted image morphometry provides high-precision measurement of several variables, characterizing the size and shape of nuclei. Thus, a number of these nuclear parameters, in addition to adding accurate diagnosis, appear to be useful as prognostic predictors in various human malignancies [6-9].

Quantitative measurement of nuclear parameters in ovarian tumors, expressed in universally accepted unit, is very important for standardization, to understand and assess such neoplasm. Such results are then applicable worldwide for healthcare and research. In this study of serous ovarian tumors, the main objectives were:

a. To evaluate major axis (MAJX), minor axis (MINX), nuclear area (NA), nuclear perimeter (NP), nuclear aspect ratio (NAR), and nuclear roundness (NR) using morphometric techniques and calculate variability of the nuclear parameters (standard deviation [SD]- MAJX, SD-MINX, SD-NA, SD-NP, SD-NAR, and SD-NR),
b. To study the correlation of studied parameters with histological grades,

c. To study the distribution pattern of nuclear parameters in benign and malignant cases.

## MATERIALS AND METHODS

The present study included 30 cases of serous ovarian carcinoma and 30 cases of benign ovarian tumor that were managed during 2010-2014. There was 8 low-grade (LG) and 22 HG serous ovarian carcinoma among the malignant cases. The samples were divided into three groups: Benign grade (BG), LG, and HG. The histological type was confirmed by reviewing hematoxylin and eosin(H and E)-stained slides. Tumor grading was done according to the scoring system recommended by Malpica *et al.* where a two-tier grading system was introduced, in which tumors are subdivided into low-grade and HG [10].

### Nuclear Morphometry

Morphometric analysis was performed on H and E-stained histological sections of 5 $\mu$m thickness of formalin-fixed paraffin-embedded tissue, having optimal histological detail [Figure 1a-c].

A computerized digital photomicrograph system (Dewinter Optical Inc. with Digi Eye 330 digital photomicrography camera and Biowizard 4.2 Image analysis software) was used for image analysis. The measuring scale of the image analysis software was properly calibrated. For each sample, five high-power fields (400×), having maximum cellularity with active tumor, were recorded for the study. The fields having necrosis, inflammation, or calcification were not included. For each case, 200 nuclei, clearly separated from others, were chosen to evaluate nuclear shape and size. The nuclei were outlined using a mouse attached to the computer and then separated from others before the determination of their nuclear parameters using the software [9]. After measurement, the data were transferred to an MS Excel sheet for further analysis. Nuclei were analyzed for MAJX, MINX, NA, NP, NAR, and NR. NAR was defined as the ratio of the long axis to the short axis of a nucleus; an elongated nucleus takes larger value of NAR. The NR is expressed as $[(4\pi\ NA)/(NP)^2] \times 100$ in percentage. Thus, for a perfectly round nucleus its value is the maximum, that is, equal to 100. A nucleus having irregular shape had smaller value of NR. The MAJX, MINX, NA, and NP are related to the nuclear size, whereas NAR and NR are related to nuclear shape.

### Statistical Analysis

MAJX, MINX, NA, NP, NAR, and NR for each nucleus of every sample were analyzed and mean of each parameter was considered as the value for the sample. SD of the nuclear parameters: SD-MAJX, SD-MINX, SD-NA, SD-NP, SD-NAR, and SD-NR were considered as the variability of that parameter for a sample. The mean values of the parameters with SD and range were calculated for BG, LG, and HG. Analysis of variance (ANOVA) with *post hoc* Tukey's honest significant difference (Tukey's HSD) test was performed to assess the differences in all the studied parameters for three groups ($n$ = 60) and the $P$ values were determined. The statistical correlations of the analyzed for all parameters with grades and Spearman's rank correlation coefficients ("ρ") were calculated. In the correlation study, benign samples are designated as Grade 0, LG and HG carcinomas are designated as Grade I and Grade II, respectively. Correlations of the nuclear parameters with their variability were investigated and Pearson's correlation coefficients ('r') with linear regression were determined. Distribution patterns of NA, NAR, and NR for benign and malignant were also studied.

## RESULTS

The mean values of nuclear parameters; variability of the nuclear parameters; and age with SD and range for BG, LG, and HG groups with $P$ values of ANOVA test are presented in Table 1. Spearman's rank correlation coefficient "ρ" between the grades and parameters are also showed in Table 1. Table 2 shows $P$ values of multiple comparisons for the parameters between the pairs of groups using *post-hoc* Tukey's HSD test. All the morphometric parameters and their variability were significantly different for benigns than the malignants. However, MINX, NA, SD-NAR, and SD-NR showed significant difference between LG and HG. All studied parameters showed significant correlations with grades.

### Correlation with Grades

Spearman's rank correlations were studied between the histological grades and studied parameters. Strong positive correlations with tumor grade were observed for size-related nuclear morphometric parameters: MAJX ($\rho$ = 0.864), MINX ($\rho$ = 0.882), NA ($\rho$ = 0.875), NP ($\rho$ = 0.859), SD-MINX ($\rho$= 0.780), and SD-NA ($\rho$ = 0.855). Whereas, NR ($\rho$ = 0.682) and SD-NP ($\rho$ = 0.491) exhibited moderate- and mild-positive correlations, respectively, NAR ($\rho$ = −0.794), SD-NAR ($\rho$ = −0.793), and SD-NR ($\rho$ = −0.702) exhibited strong negative correlation with grade. Figure 2a-c shows the scatter plots of the NA, NAR, and NR of the samples with their grade to demonstrate the correlations, scatter plots of other three size-related parameters (MAJX, MINX, and NP) were similar to the NA.

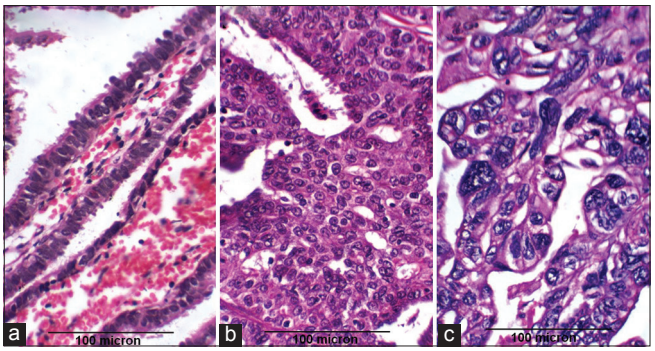


**Figure 1:** (a-c) Photomicrograph of benign (a), low-grade (b), and high-grade (c) ovarian tumor (×400)

**Table 1: The mean values of age and studied nuclear morphometric parameters with SD and range for BG, LG, and HG malignant ovarian tumors with *P* values obtained by ANOVA test. Spearman's rank correlation coefficient "$\rho$" between grades and the parameters are also showed**

| Parameter (unit) | BG ($n$=30) | LG ($n$=8) | HG ($n$=22) | *P* value (ANOVA) | Spearmen "$\rho$" with grades |
|---|---|---|---|---|---|
| Age (year) | 43.0±15.3 (20-73) | 51.9±4.4 (46-57) | 56.1±10.3 (37-76) | 0.002* | 0.419* |
| MAJX ($\mu$m) | 7.03±0.90 (5.23-8.64) | 9.58±0.96 (8.70-11.82) | 10.50±1.18 (8.51-12.90) | <0.001* | 0.864* |
| MINX ($\mu$m) | 4.09±0.60 (2.93-5.03) | 6.30±0.62 (5.12-7.28) | 7.30±0.81 (5.77-8.75) | <0.001* | 0.882* |
| NA ($\mu$m$^2$) | 21.32±5.52 (11.03-30.44) | 44.87±8.46 (33.05-63.21) | 57.50±12.65 (38.15-83.15) | <0.001* | 0.875* |
| NP ($\mu$m) | 21.59±2.94 (15.66-26.84) | 29.79±3.11 (27.15-37.10) | 32.77±3.99 (26.26-40.92) | <0.001* | 0.859* |
| NAR | 1.80±0.18 (1.56-2.20) | 1.57±0.13 (1.43-1.76) | 1.47±0.08 (1.32-1.65) | <0.001* | −0.794* |
| NR (%) | 57.78±5.19 (47.94-65.71) | 63.70±4.50 (57.58-69.30) | 66.34±3.78 (59.67-72.34) | <0.001* | 0.682* |
| SD-MAJX ($\mu$m) | 1.56±0.34 (1.11-2.39) | 1.97±0.42 (1.40-2.57) | 2.13±0.69 (1.39-4.30) | 0.001* | 0.490* |
| SD-MINX ($\mu$m) | 0.97±0.21 (0.60-1.49) | 1.37±0.19 (1.14-1.65) | 1.58±0.39 (1.12-2.90) | <0.001* | 0.780* |
| SD-NA ($\mu$m$^2$) | 8.12±2.50 (4.04-12.79) | 16.75±3.98 (11.92-22.27) | 22.88±9.92 (11.72-56.02) | <0.001* | 0.855* |
| SD-NP ($\mu$m) | 5.13±1.14 (3.67-7.87) | 6.57±1.37 (4.59-8.42) | 7.11±2.32 (4.45-14.30) | <0.001* | 0.491* |
| SD-NAR | 0.48±0.09 (0.36-0.66) | 0.37±0.09 (0.28-0.48) | 0.29±0.05 (0.20-0.40) | <0.001* | −0.793* |
| SD-NR (%) | 14.27±0.92 (12.78-16.15) | 13.26±1.23 (11.72-14.98) | 12.12±1.17 (9.86-14.23) | <0.001* | −0.702* |

BG: Benign group, LG: Low-grade carcinoma, HG: High-grade carcinoma, SD: Standard deviation, MAJX: Major axis, MINX: Minor axis, NA: Nuclear area, NP: Nuclear perimeter, NAR: Nuclear aspect ratio, NR: Nuclear roundness, ANOVA: Analysis of variance. Values are expressed as: Mean±SD (minimum value-maximum value), *n*: Number of sample, *Significant ($P$<0.05)

**Table 2: *P* values of multiple comparisons for the parameters between the pairs of groups using *post hoc* Tukey's HSD test considering all three groups**

| Parameter (unit) | *P* value (BG vs. LG) | *P* value (BG vs. HG) | *P* value (LG vs. HG) |
|---|---|---|---|
| Age (year) | 0.192 | 0.001* | 0.695 |
| MAJX ($\mu$m) | <0.001* | <0.001* | 0.082 |
| MINX ($\mu$m) | <0.001* | <0.001* | 0.003* |
| NA ($\mu$m$^2$) | <0.001* | <0.001* | 0.004* |
| NP ($\mu$m) | <0.001* | <0.001* | 0.093 |
| NAR | 0.001* | <0.001* | 0.276 |
| NR (%) | 0.006* | <0.001* | 0.357 |
| SD-MAJX ($\mu$m) | 0.006* | <0.001* | 0.729 |
| SD-MINX ($\mu$m) | <0.001* | <0.001* | 0.182 |
| SD-NA ($\mu$m$^2$) | <0.001* | <0.001* | 0.063 |
| SD-NP ($\mu$m) | 0.005* | <0.001* | 0.722 |
| SD-NAR | 0.002* | <0.001* | 0.044* |
| SD-NR (%) | 0.014* | <0.001* | 0.030* |

BG: Benign group, LG: Low-grade carcinoma, HG: High-grade carcinoma, SD: Standard deviation, MAJX: Major axis, MINX: Minor axis, NA: Nuclear area, NP: Nuclear perimeter, NAR: Nuclear aspect ratio, NR: Nuclear roundness, HSD: Honest significant difference. *Difference is significant

### Correlation with Variability

Variability of the parameters exhibited strong positive correlation with their mean value for MAJX ($r$ = 0.701), MINX ($r$ = 0.788), NA ($r$ = 0.881), and NAR ($r$ = 0.952) but showed moderate positive correlation with NP ($r$ = 0.680) and moderate negative correlation with NR ($r$ = −0.640). Scattered plot with linear regression of NA, NAR, and NR of the samples with their SD are presented in the Figure 3a-c. We have seen two different clusters of benign and malignant samples in the first scatter plots because values of X-axis parameters (NA) for all benign samples were lower than the malignant samples. Similar result was seen for MINX.

### Distribution of Nuclear Parameters in a Sample

Figure 4a-c stands for distributions of NA, NAR, and NR for five benign and five malignant cases, respectively. For NA distribution, malignant samples have smaller peak with right toward shift and larger range than the benign. Whereas for NAR and NR distribution, malignant samples have larger peak and smaller range than the benign; the peaks of the malignant sample in NR distribution were shifted toward right unlike NAR distribution. Distribution patterns for other nuclear size-related parameters (MAJX, MINX, and NP) for benign and malignant cases were similar to the NA.

## DISCUSSIONS

Serous ovarian carcinomas have a wide biological spectrum ranging from benign, innocuous tumors to highly aggressive ones associated with poorer prognosis. This heterogeneity even among the subgroups is reflected in the varied prognosis, treatment modalities, and outcomes in different studies. Differentiating between atypical benign tumors and borderline malignancies are at times difficult. Over the years, various ancillary techniques have been used to grade or differentiate these tumors [11]. Nuclear morphometry is one such technique that relies on definite measured parameters of the nucleus of the cell [5,7,12]. Although morphometry has been used to successfully differentiate between borderline and malignant cases previously, the tool was limited by cumbersome technique [11]. Currently, rapid advancements in image analysis and computerized morphometry combined with high-throughput automation makes this technique a distinct possibility for regular use with the added advantage of objective evidence and better interobserver agreement. These measurements also reflect true biological events in tumor pathology such as increased nuclear content (ploidy), turnover, and degree of differentiation [13,14]. Hytiroglou *et al*. showed that interactive morphometric analysis of nuclear features, combined with appropriate statistical methods, could be used to distinguish between borderline and invasive serous ovarian tumors [15]. Palmer *et al*., in their evaluation of 132 serous ovarian tumors found that nuclear parameters strongly correlated with extent of disease residuum, tumor grade, and FIGO stage [5].

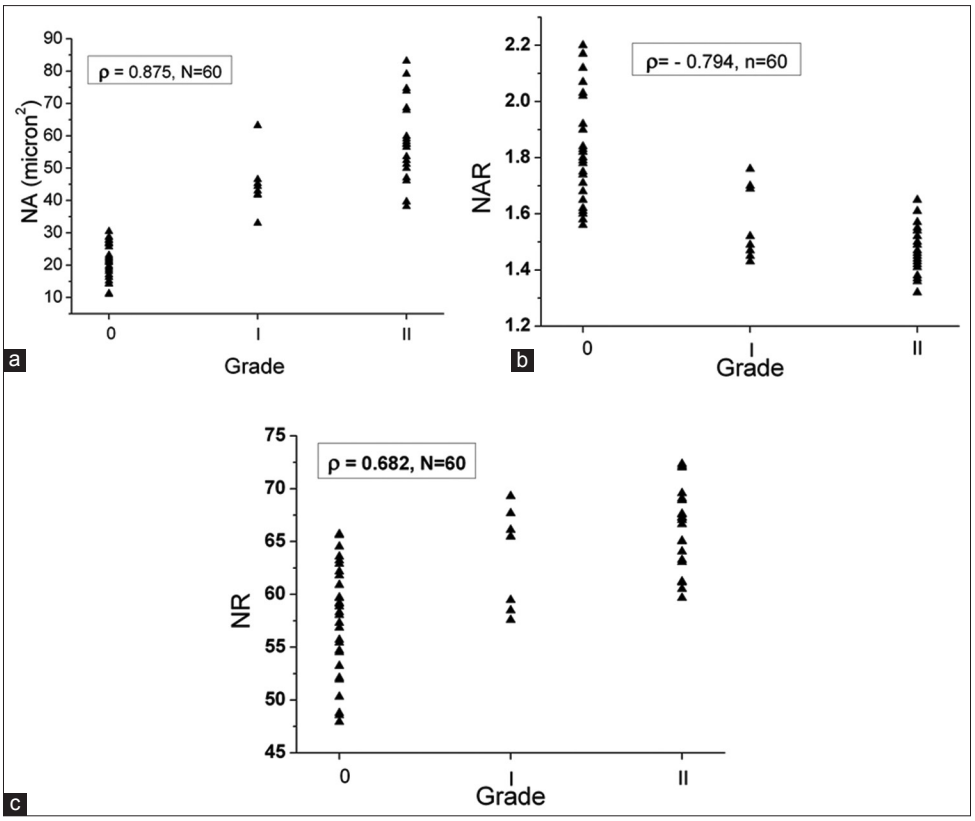


**Figure 2:** (a-c) Scatter plot of mean: (a) Nuclear area, (b) nuclear aspect ratio, and (c) nuclear roundness with their histological grade; Spearman's rank correlation coefficient "ρ" with histological grade shown in the respective plot. Grade 0 = benign, Grade I = low grade, and Grade II = high grade

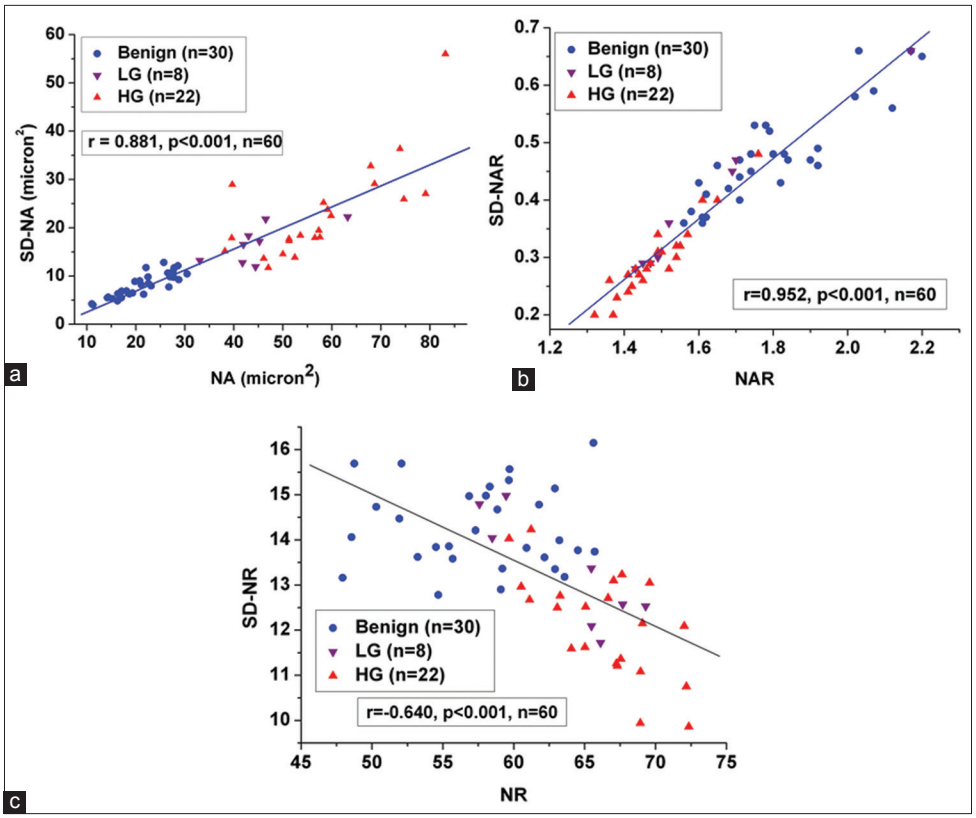


**Figure 3:** (a-c) Scatter plot of mean: (a) Nuclear area, (b) nuclear aspect ratio, and (c) nuclear roundness with their standard deviation (SD) (variability) for all samples (benign and malignant); linear regression of these parameters with their SD is shown by the solid lines in their respective plot with Pearson's correlation coefficient ('r')

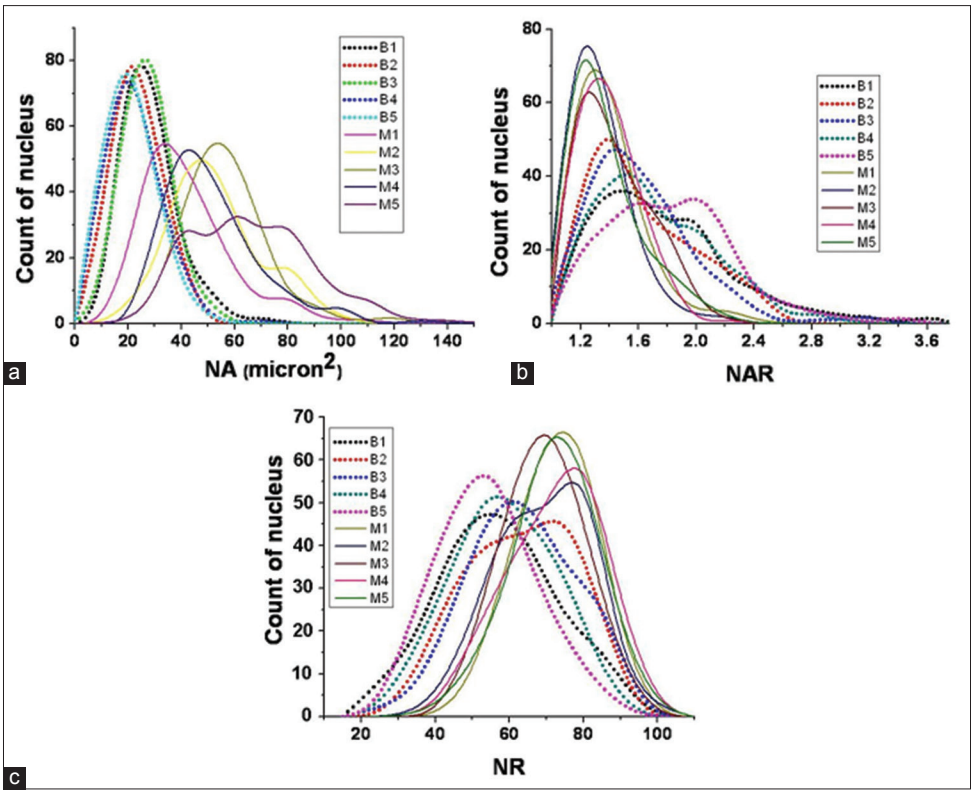


**Figure 4:** (a-c) The measured distribution of nuclear area (a), nuclear aspect ratio (b), and nuclear roundness (c) of five benign samples (B1, B2, B3, B4, B5) and five malignant samples (M1, M2, M3, M4, M5). For each sample, 200 nuclei were assessed

In this study, we evaluated the usefulness of nuclear morphometry on a small sample size as a pilot study. We have standardized the measurements to universally accepted units ($\mu$m or $\mu m^2$) so that comparisons can be made irrespective of the imaging system or magnifications used. This was a major shortcoming in many earlier studies which we have tried to address. It will help in calculating cutoff in the future studies with larger number of cases.

Here, we found all the size-related nuclear morphometric parameters to be significantly larger in malignant groups compared to the benign group. Strong positive correlations with tumor grade were observed for these parameters: MAJX ($\rho = 0.864$), MINX ($\rho = 0.882$), NA ($\rho = 0.875$), and NP ($\rho = 0.859$). Furthermore, the sizes of benign nuclei tend to be more homogenous as compared to the malignant nuclei. Variability of nuclear size (SD-MAJX, SD-MINX, SD-NA, and SD-NP), which is the quantitative measure of nuclear pleomorphism, was significantly wider in malignant groups than the benign. The HG malignant tumors showed the higher variability in size than the LGs. These details, correlate well with the fact that, as the chief driver of malignancy, the nuclei are more active and rapidly multiplying, thus they have larger size in tumor cells and exhibit pleomorphism.

Distinct cluster separation, without overlap, of benign and malignant samples was seen in the scatter plots of mean NA [Figure 3a] and MINX versus their variability. The mean NA value in malignant tumors was almost double of their benign counterparts with no overlap. Mean MINX and NA at cut-off values of (a) MINX > 5.03 $\mu$m and (b) NA > 30.44 $\mu m^2$ were able to differentiate malignant cases from benign with 100% efficiency.

NR and NAR of the malignant groups were significantly different from the benign ($P < 0.05$). The malignant nuclei were more rounded, whereas the benign nuclei tended to be more oval with a smaller short axis. The variability of the shape-related nuclear parameters (i.e., SD-NAR, SD-NR) in benign cases was significantly higher than the malignant cases. This may be due to oval shape of the benign nuclei; the possibilities of their appearance in a histological section widely vary from circular to elliptical depending on the orientation of sectioning. Whereas the malignant nuclei are more or less spherical, hence the possibility of their appearance to be nearly circular in a histological section is more.

We attempted to differentiate between histologically categorized LG and HG tumors based on the measured parameters. Although all the size-related parameters and their variability in the HG were larger than the LG, only MINX and NA showed significant difference. HG nuclei were rounder than LG nuclei; NAR was significantly lower in HG than LG. Further variability of nuclear shape (SD-NAR and SD-NR) was significantly lower in HG than LG. However, as we analyzed only eight cases of LG tumor, a more robust study would be required to comment on the discriminating value of these values. In our study, the mean NA in HG tumor was 57.50 $\mu m^2$ (range: 38.15-83.15 $\mu m^2$) which is comparable with the values obtained by Hsu *et al*. in their study [7]. They showed that tumors with a value >46 $\mu m^2$ had a poorer prognosis and more importantly mean NA was

an independent prognostic factor for HG serous carcinoma. Brinkhuis *et al.* in their study of quantitative pathological variables as prognostic factors in advanced ovarian carcinoma, found the SD of the NA to be very significant parameter in a multivariate analysis [16]. Similarly, Baak *et al.* and Katsoulis *et al.* concluded that nuclear size is an important predictor of the sensitivity of tumor cells to cisplatin chemotherapy [14,17].

In this study, distribution of nuclear parameters of malignant samples was distinctly different from the benign ones. Malignant samples have larger range and smaller peak than the benign for NA distribution. Other size-related nuclear parameters in malignant samples showed similar pattern of distribution. Whereas, the distribution pattern of shape-related nuclear parameters (NAR and NR) of the malignant samples showed narrower range and higher peak than the benign samples. Thus, distribution of nuclear size and shape in a given sample can also be utilized for diagnosis purpose. Therefore, our study also showed that the morphometric evaluation of nuclear parameters with their SD and distribution of nuclear size and shape can be used to make decision in diagnostics with good precision.

## CONCLUSION

In conclusion, the nuclear morphometric parameters related to nuclear size and their variability were significantly larger in malignant than the benign serous ovarian lesions and they showed strong positive correlation with tumor grades. MINX showed the best correlation with the grades. Malignant nuclei were found rounder than the benign; hence they had larger NR and smaller NAR. These parameters can be gainfully exploited as an adjuvant measure in diagnosis and grading, either as a composite score or a calculated cutoff. These results can be used for the automatic screening of malignancy, with the help of image analysis software where the identification and measurement of nuclear parameters can be automated once the screened fields are marked. These values also facilitate a better understanding of the tumor biology and can be utilized for further research. In this pilot study, we have demonstrated the usefulness, method of standardization, and robustness of these measurements. The need is, however, for a larger study with more cases where precise cut-off values can be obtained for identifying borderline cases correctly and in prognostication, especially with regards to current neoadjuvant chemotherapy.

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