DOES MICRONUCLEUS SCORE REALLY INDICATE DYSPLASIA IN CERVICAL PAP SMEARS? IF SO, HOW FAR?
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BACKGROUND
The grade of dysplasia on cervical pap smears may be indicated by micronucleus (MN) scoring, much like cancers of oral cavity, urinary bladder and esophagus.

METHODS
This is a cross sectional study. MN scores of 106 subjects comprising of all major diagnostic categories included in 'The Bethesda system, 2014 for reporting cervical pap smears' were taken. High grade squamous intraepithelial lesion (HSIL) and invasive carcinoma (IC) were further grouped as 'high-risk' and the rest, 'low-risk' to construct Receiver Operating Characteristic (ROC) curve to seek a cut-off delineating the two classes. Analysis of variance was used to determine significance of differences in MN scoring between the various groups.

RESULTS
Difference of mean MN scores of HSIL (9.4) and IC (10.7) was significant from the low-risk group but not within themselves. A huge difference in MN scores between low grade squamous intraepithelial lesion and HSIL is notable. The difference of mean age was significant between high and low risk groups. ROC curve delivered a cut-off of 5.15 to distinguish between the two categories with 85.7% sensitivity, 97.2% specificity and 93.3% accuracy.

CONCLUSIONS
Sequential and significant increase of MN score from low to high grade dysplasia is established by current study. A cut-off of 5.15 MN score adequately detects HSIL and IC. Despite its performance, MN scoring is time-consuming, labour intensive and strenuous process, which might make it difficult to impose on laboratories and pathologists.

KEYWORDS
High Grade Squamous Intraepithelial Lesion, Invasive Cancer, Receiver Operating Characteristic Curve, Cut-Off


BACKGROUND cancer
Cervical cancer is fourth most common cancer among women worldwide representing 7.9% of all female cancers and it is the second leading cause of cancer mortality in Indian women. The incidence of cervical cancer varies widely and about 90% of deaths due to cervical cancer occur in developing nations.1 In a report published in 2017 from International agency for research on cancer, 122844 cases with 67,477 deaths from cervical cancer were reported. In women aged 15-44 years, cancer cervix holds the second position in cancer incidence among women. The highest age standardized incidence of cervical cancer is 22% in India, compared to 19.3 in southern Asia and 14.0 in the world.2

Despite its severity, cervical cancer responds favorably to secondary preventive measures when detected early by effective screening & early diagnostic methods.1

HPV testing and p16 immunostaining are applied to detect cervical intraepithelial neoplasia (CIN), which may or may not progress to invasive cancer thus conducing to the reduction of incidence of cervical carcinoma. However, the cost of HPV testing and p16 immunostaining are high, so, in developing countries, cervical cancer goes undetected at higher frequencies than in developed countries. Hence a simple procedure, the 'Micronucleus test (MNT)' may be used in conjunction with the cervical pap. It has been proven to identify the menace of malignancies of cervix, oral cavity, oesophagus and urinary bladder.3

A micronucleus (MN) is an additional small nucleus in the cytoplasm consequent upon chromosomal breakage or chromosomal loss, formed when chromosomes or chromosomal fragments fail to be incorporated into the nucleus during cell division.4 Micronucleus serves as a potential biomarker of genotoxicity.5 Their frequency appears to increase in carcinogen-exposed tissues long before any clinical symptoms are evident and therefore, the MNT predicts cancer risk.6

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A few studies have been done on MN scoring in cervical pre-neoplastic and neoplastic conditions. The current study evaluates the MN score in the entire gamut of Pap smear outcomes from Negative for Intraepithelial Lesion or Malignancy (NILM) to invasive cancer (IC) including all the pre-neoplastic and neoplastic diagnoses mentioned in The Bethesda system, 2014.7

METHODS
A total of 106 cases consisting of non-neoplastic, pre-neoplastic and neoplastic cases were studied. The smears were collected from the archives of the cytology section of the pathology department of our institution from 2012 to 2014 spanning 2 years.

As a part of routine check-up, the pap smears were taken in the outpatient department by the gynecologists and sent to the cytology laboratory where 30 minutes were allocated for fixation in 95% ethanol prior to staining with Papanicolaou’s stain. Microscopic examination followed to evaluate for cytoplasmic and nuclear characteristics so that the smear could be categorized into one of the diagnostic classes as propounded by ‘The Bethesda system, 2014’. Review was conducted for all smears for micronuclei scoring. The original diagnoses were taken into account while calculating the statistics.

Cells tangled in clusters and overlapped cells where their delineation of cytoplasmic and nuclear borders were compromised were debarred, just individual cells lying in a clean background were included to be counted for MN. Cytoplasmic remnants, apoptotic and degenerated cells also made the exclusion list while counting and scoring MN. Zigzag method was used to screen the slide.8

The MN diameter varied from 1/16 to 1/3 nuclear diameter while retaining the nucleus’ shape, colour and texture with similar or sometimes, trifile lower staining intensity. The round to oval shaped MN was found to lie near the nucleus without attaching to it and could be focused on the same plane. A score of 1 was bestowed upon cells harboring single/multiple MN. After being screened first by two pathologists, it was subjected to review by a third observer and the final scores decided in consensus. A total of 2,000-3,000 cells were counted per smear and the MN score was conveyed per 1,000 cells.9

The results were then subjected to statistical evaluation using ‘SPSS software for WINDOWS v.20, IBM, New York. The significance of variance between the mean MN scores of the diagnostic groups was analysed by One Way Analysis Of Variance (ANOVA). The Least Significant Difference/least square deviation (LSD) test was used to calculate the p-value as shown in Table 3. The diagnostic groups were divided into two classes – the low-very low and the high risk. The low risk comprised of NILM, REA, ASC-US and LSIL. The HSIL and IC together defined the high-risk class because a significant 33-50% of HSILs progress to IC. In contrast, most LSILs revert to normal, just 16-25% advance towards HSIL.10

In yet another reference, we find that 30% of the HSILs regress, 60% persist and only 10% progress to frank invasive carcinoma.11 Nevertheless, patients were found to be 25 times at risk of cancer on a long-term (5-year) follow up after initial detection and conservative therapy of HSIL.12

A receiver operating characteristic (ROC) curve was constructed to test the efficacy of MN score to distinguish between the two groups or rather, detect HSIL and IC. The sensitivity added to specificity subtracted from 100 constitutes the Youden’s index (YI), i.e. YI = (sensitivity + specificity) - 100. The cutoff with the highest YI is accepted as the best achievable with any particular ROC curve.

RESULTS
Of the 106 patients, 20 were diagnosed cytologically as Negative for intraepithelial lesion/ malignancy (NILM) having mean age of 41 years, 20 as ‘Reactive cellular changes associated with inflammation’ (REA) with Mean age of 43 years and 16 as Atypical Squamous Cell of Undetermined Significance (ASC-US). The LSIL comprised of 15 cases, the HSIL encompassed a further 17 cases with mean age of 48.4 years and the ‘Invasive cancer’, 18 cases with mean age of 52 years (Table 1). The difference of mean age was insignificant with ANOVA except between high risk and the low risk groups since most of the low risk groups were in the early 40s. The HSIL patients were in the late 40s and IC, in early 50s. Again, the mean age difference between IC and HSIL cases was not significant.

We received biopsy specimens for four cases of ASCUS and all HSIL and IC cases for histological correlation. Biopsy was not available in NILM, REA and LSIL categories. While the biopsy of the HSIL cases showed either a Cervical intraepithelial neoplasia (CIN) II/III, the IC cases showed invasive squamous cell carcinomas in all of their biopsies. Thus, no discordance between cytological and histological findings was found in the HSIL and IC categories. Of the four available biopsies of the ASC-US cases, three showed chronic cervicitis and one, (CIN) I. Two observers separately and independently counted the number of micronucleated cells (MNC) per 1,000 of epithelial cells in oil immersion magnification (×100 objective). Mean MN Score was found to be 0.5069 ± 0.3501 in NILM cases, 0.5186 ± 0.4324 in REA, 1.9607 ± 0.9698 in ASCUS, 3.6585 ± 1.3708 in LSIL, 9.3931 ± 4.550 in HSIL and 10.7008 ± 4.1946 in invasive cancer (Table 2). The MN score went gradually and steadily uphill from NILM to ASC-US, a slight step-up to LSIL and thence a huge leap to HSIL, followed by a casual canter to IC as shown in Figure 1.

The key facts revealed from MN scores found in the different diagnostic groups are obtained by ANOVA (Table 3). They are-

1. The MN score was significantly higher in IC compared to all the groups except HSIL (p = 0.143).
2. The difference in MN score between that of HSIL and other groups was significant except IC (p = 0.143).
3. The MN score of LSIL was significantly different from that of other groups except ASC-US (p = 0.204).
4. The difference of MN scores between that of NILM and REA was insignificant (p = 0.989).

The Area under the curve (AUC) is 0.979. The MN count
of 5.15 detects HSIL + IC with 85.7% sensitivity and 97.2% specificity yielding a YI of 82.9. At a lower MN count of 3.86, with sensitivity and specificity of 94.3% and 88.7%, the YI was 83. Since the distribution was skewed with 71 cases in the low risk group and 35 in the high-risk group, accuracy came to play a bigger role. At MN score of 5.15, the accuracy was 93.3% while a 3.86 cut-off achieved just 90.5% accuracy. Thus, an MN score of 5.15 was considered to be the cut-off in the current study.

Figure 2 demonstrates the micronucleus across all the Bethesda, 2014 diagnostic categories. The nuclear enlargements in ASCUS, mild nuclear irregularity in LSIL contrast well with the marked nuclear abnormalities in HSIL and IC.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Cases</th>
<th>Age Range (Yrs.)</th>
<th>Mean Age (Yrs.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NILM</td>
<td>20</td>
<td>24 - 57</td>
<td>41</td>
</tr>
<tr>
<td>REA</td>
<td>20</td>
<td>20 - 62</td>
<td>43</td>
</tr>
<tr>
<td>ASCUS</td>
<td>16</td>
<td>24 - 64</td>
<td>45</td>
</tr>
<tr>
<td>LSIL</td>
<td>15</td>
<td>23 - 55</td>
<td>43.2</td>
</tr>
<tr>
<td>HSIL</td>
<td>17</td>
<td>26 - 69</td>
<td>48.4</td>
</tr>
<tr>
<td>IC</td>
<td>18</td>
<td>35 - 70</td>
<td>52</td>
</tr>
<tr>
<td>Total</td>
<td>106</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 1. Age Distribution of Cases Selected for MN Scoring

<table>
<thead>
<tr>
<th>Group</th>
<th>NILM</th>
<th>REA</th>
<th>ASCUS</th>
<th>LSIL</th>
<th>HSIL</th>
<th>IC</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of Cases</td>
<td>20</td>
<td>20</td>
<td>16</td>
<td>15</td>
<td>17</td>
<td>18</td>
</tr>
<tr>
<td>Mean Age</td>
<td>41</td>
<td>43</td>
<td>45</td>
<td>43.2</td>
<td>48.4</td>
<td>52</td>
</tr>
<tr>
<td>MN Score ± SD</td>
<td>0.5069 ± 0.3501</td>
<td>0.5186 ± 0.4324</td>
<td>3.6585 ± 1.3708</td>
<td>9.3931 ± 4.550</td>
<td>10.7068 ± 4.1946</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Stepwise Gradual Increase in MN Score from Inflammatory to ASC-US to LSIL to HSIL Group, Followed by a Slight Increase in IC

<table>
<thead>
<tr>
<th>Group</th>
<th>NILM</th>
<th>REA</th>
<th>ASCUS</th>
<th>LSIL</th>
<th>HSIL</th>
<th>IC</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of Cases</td>
<td>0.989</td>
<td>0.015</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Asymptotic Sig. a</td>
<td>0.016</td>
<td>0.004</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Asymptotic 95% Confidence Interval</td>
<td>0.204</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>IC</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.143</td>
<td></td>
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<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.143</td>
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<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.143</td>
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<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.143</td>
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Table 3. Result of ANOVA (p Value) (Post Hoc test): p Value Significant at ≤0.05

DISCUSSION

Studies involving oral squamous cell carcinoma (OSCC) reveal that MN in oral mucosal with OSCC was threefold to fourfold more frequent than that of controls. Exfoliated cervical cells exposed to few risk factors for cancer cervix are more likely to express a greater prevalence rate of MN than those without exposure.

Yet, within a diagnostic group, MN scores are disparate because of variation in lifestyle, contact with environmental genotoxic agents, micronutrient deficiency, genetic constitution, inherent incidence of MN and other factors related to carcinogenesis and damage to chromosomes. From previous studies, we know that MN indicates chromosomal damage or aberrations rather than risk of cancer and since, chromosomal defects underlie most cancers, the MNT is used as a biomarker for predicting cancer risk. MN scoring has been used to assess the risk of malignant transformation in uterine cervix.

Guzmán et al. in 2003 noted that HSIL smears had the highest MN frequency of 33% followed by LSIL at 18%. IC showed a modest 16%. The MN frequency of irradiated cells were expected to bear the highest magnitude of chromosomal aberrations induced by radiation itself and thus were considered positive controls. However, the MN frequency of HSIL was the highest but not significantly higher than that of LSIL. The LSIL, HSIL and IC showed a significantly higher MN frequency than smears within normal limits. In contrast, the present study showed that the MN score of HSIL was significantly higher than LSIL (P<0.000).

A similar study by Gayathri et al. in 2012 also found that there was a gradual increase in MN frequency from NILM to LSIL as in our study and there is also a significant difference in MN frequency between LSIL and HSIL. Their MN scores were comparable to ours in the precancerous and cancerous lesions i.e. 8.03 and 10.05 in HSIL and IC respectively vs. 9.39 and 10.7 correspondingly in our study. The quantum leap in the trend of MN scores from LSIL to
HSIL found in our study is similar to that of Gayathri and coworker’s study. Their conclusion that determination of MN scores serves as an ancillary tool for cancer screening, earnestly begs for additional or buttressing evidence since the score at which cancer or high-risk cases may be suspected has not been determined, nor any method about how the scores should be used, described. Gayathri and coworkers went merely as far as to establish that grade of dysplasia is proportional to the MN score and nothing else.

A study by Bueno et al18 in 2014 showed that the MN frequencies in the different groups were 0.95 ± 1.12 (n = 223) in the control group (NILM), 2.98 ± 1.20 (n = 50) in ASCUS, 4.04 ± 1.45 (n = 52) in Cervical intraepithelial neoplasia (CIN) I, 5.97 ± 1.83 (n = 30) in CIN II, 7.29 ± 1.55 (n = 17) in CIN III and 8.64 ± 1.55 (n = 25) in cervical cancer.18

These frequencies were significantly higher in pre-cancerous and cancer groups than that of the control group (p<0.001). It should however, be noted that CIN is a diagnosis on histopathological sections. The definition of CIN on cytological pap smears is at best sketchy and therefore, the outcome purported in the said study is speculative. Compared to our study, their MN score was tad low (9.39 and 10.7 in HSIL and IC respectively) vs 7.29 (CIN III) and 8.64 correspondingly in Bueno and coworkers’ study.

Samanta et al19 in 2011 performed a study correlating MN scores with the different cytologic diagnostic groups proposed by the Bethesda system, 2001. They also found that a gradual increment of MN scores with an upward trend was noted from the NILM to LSIL. A huge leap was evidenced from LSIL to HSIL as observed in the current study. However, the MN scores of IC (18.5±9.5) were lower than that of HSIL (19.7±17.18), tad different from our study but similar to that of Guzmán et al.16 MN greater than one were rarely witnessed, occasionally in ASC-US and LSIL but a little higher in the IC-MN antigen (MnAg) expression by immunohistochemistry was studied by Liao et al in SIL as well as adenocarcinoma-in-situ (AIS) cases. All dysplastic cells in both the categories stained positively. However, in SIL, endocervical columnar and reserve cells which were not dysplastic expressed affinity for the antibody against MnAg, whereas in AIS, the same morphologically undisturbed cells were negative. They proposed that MnAg expression in conjunction with routine cytology will discriminate between REA and dysplastic cells, both of which might be morphologically atypical.19

In yet another study by Shi et al20 (2015), MN scoring was correlated with cytologic diagnoses as well as high-risk HPV infection. They used Kruskal-Wallis test as their statistical tool to determine the mean rank of MN count. The mean rank showed significant difference among the diagnostic groups. The diagnostic groups HSIL and IC correlated significantly with the mean rank of MN counts whereas age, extent of married cohabitation, pregnancy frequency did not. Positivity for high-risk HPV was very high in IC and HSIL and moderately high in LSIL yielding statistical significance. From their ROC curve, they determined that an MN count of 7.5 could determine HSIL and IC with 85% and 66% sensitivity and specificity respectively. The MN scores displayed similar tendency in the current study. However, our cut-off was tad less, 5.15 versus their 7.5 to distinguish between the high and the low risk groups. We achieved a sensitivity and specificity of 85.7% and 97.2% respectively yielding a YI of 82.9 whereas their YI was 51 only. At a cut-off of 7.53, our study generates sensitivity approximately 50% and specificity of 100% and accuracy of 88.6%.

In this context, it should be noted that some oncogenic strains of HPV (16, 18, 31, and 33), which predispose to cervical cancer elicit HPV proteins namely E6 and E7 that disrupt cytokinesis and centrosome duplication thereby resulting in chromosome instability. The chromosomal abnormalities arising therein produce the micronuclei.21,22 Since the chromosomal aberrations accrue as the dysplasia progresses in stages to invasive carcinoma, the HSILs and ICs may exhibit a higher MN frequency. Our results were similar to that of other studies.

While performing our study, it was observed that MN scoring puts strain on available time for diagnostics and is immensely labor intensive. It takes three pathologists to arrive at any concluding score to be effective on any particular case. Moreover, counting 1000 available cells per case is stressful to psyche and sight, particularly to pathologists with weaker eyesight. For all the labor it begets, MN scoring by no way is confirmatory. It may indicate an increased risk, much the same way as morphology does. Conventional smears, with its attendant background debris renders many of the cells unaccountable. Thinprep smears, in contrast is much more suited to the study of MN, obviously because of its cleaner background. Still, thinprep, with its accompanying cost of the instrument and disposables elevates the cost of the smear, a disadvantage we sought to counteract in the first place by encompassing MN scoring instead of HPV and p16 testing in routine screening for cervical precancerous and cancerous lesions. Ergo, MN scoring requires strict regulations, from either government or international societies to get implemented in laboratories to be a feasible option to adopt in routine pap screening.

We reviewed approximately 12 articles pertaining to cervical pap smears and 26 articles that concern buccal cells and urothelial cells, published within a span of 8 years between 2018 and 2011 and indexed in PubMed/ MedLine. All the studies documented a comparison of Micronucleus (MN) expression/ frequency between controls and malignant/ premalignant lesions or subjects exposed to known risk factors for malignancy. None of the studies published and indexed in Medline used MN expression in routine reporting. Very occasionally, prospective studies were conducted but then, it was never proved that a higher MN count at the outset actually resulted in a higher chance of developing cancer in future.

In a nutshell, we have worked upon the whole spectrum of cervical lesions to achieve their MN scores in the current study. A significant difference was observed between MN
scores of the high-risk group comprising HSIL (9.4) and IC (10.7) and the low risk group. The MN scores of LSIL and ASCUS also differed significantly from normal and inflammatory lesion but not between themselves. Similarly, HSIL and IC also reveal insignificant difference. The sequential and significant increase of MN score from the low-grade dysplasia to higher grade is well established in other studies\(^6,17,20\) and also endorsed by the current study. A cut-off of 5.15 MN score detects HSIL and IC with 85.7% sensitivity and 97.2% specificity. Only one other study\(^19\) mentioned a cut-off of 7.5 to discriminate between the two groups, albeit with much less sensitivity and specificity. Outwardly, though MN scores seem to perform well in predicting risk of dysplasia and carcinoma, it is time-consuming, labor intensive and strenuous process, that might make it difficult to impose on laboratories and pathologists.

**CONCLUSIONS**

Sequential and significant increase of micronucleus score from low to high grade dysplasia in Pap smears is established by the current study. A cut-off of 5.15 MN score adequately detects HSIL and IC with accuracy of 93.3%, sensitivity of 85.7% and specificity of 97.2% yielding a Youden Index of 82.9. Despite its performance, MN scoring is time-consuming, labor intensive and strenuous process, which might make it difficult to impose on laboratories and pathologists alike.

**REFERENCES**


