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Micronucleus in Cervical Intraepithelial Lesions and Carcinoma

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Key Words
Cervical smear · Cervix · Micronucleus · Screening

Abstract
Aims and Objectives: To score and compare micronucleus (MN) in the whole spectrum of cervical lesions including normal, inflammatory, abnormal squamous cell of undetermined significance (ASC-US), low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL) and invasive cancer (IC) and to evaluate the role of MN as a biomarker in different pre-neoplastic and neoplastic lesions. ***Materials and Methods:*** A total of 224 slides, comprised of normal (40), inflammatory (40), ASC-US (30), LSIL (38), HSIL (30) and IC (46), were studied. All the cases of HSIL, IC and ASC-US had histopathology. The LSIL, normal and inflammatory smears were again reviewed by 2 experienced cytopathologists independently. Two observers separately and independently counted the number of micronucleated cells per 1,000 of epithelial cells in oil immersion magnification (×100 objective) which was expressed as MN score per 1,000 cells. ***Results:*** The mean MN scores ± SD in normal, inflammatory, ASC-US, LSIL, HSIL and IC cases of cervical lesions were 1.02 ± 1.59, 0.4250 ± 0.71208, 2.87 ± 2.21, 4.7368 ± 5.62179, 21.30 ± 17.18 and 18.50 ± 9.54, respectively. MN scores of IC and HSIL were significantly high compared to the normal ($p<0.000$), the inflammatory ($p<0.000$), the ASC-US ($p < 0.000$) and to the LSIL ($p < 0.000$) group (analysis of variance test). LSIL showed significant difference with the normal ($p = 0.043$), the inflammatory ($p = 0.019$), the HSIL ($p<0.000$) and the IC ($p<0.000$) group but not with the ASC-US ($p = 0.342$) group. ***Conclusions:*** MN scoring on the epithelial cells of cervix could be used as a biomarker in cancer screening. This is an easy, simple, reliable, reproducible and objective test which can be performed on routinely stained smears.

Introduction

Micronucleus (MN) is formed by chromosomes or chromosome segments that fail to be incorporated in cell nuclei during cell division. MN formation represents a measure of both chromosome breakage as well as chromosome loss and is a sensitive indicator of chromosomal damage [1]. High frequencies of MN have been observed in epithelial cells in direct contact with carcinogens such as ionizing radiation [2, 3], arsenic [4] or tobacco smoke [5] and also in patients with genetic diseases characterized by increased chromosome instability [1, 6]. For years MN has been used for measurement and bio-monitoring

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Table 1. Cases selected for the micronucleus scoring in cervix

Group	n	Age range	Mean age	Biopsy outcome
Normal	40	22–56	44	
Inflammatory	40	20–60	40	
ASC-US	30	26–76	51	Inflammatory/reactive
LSIL	38	22–70	46	
HSIL	30	25–67	46	CIN II/CIN III
IC	46	25–70	47.5	SCC, adenocarcinoma, adenosquamous carcinoma
Total	224	20–76	48	

SCC = Squamous cell carcinoma.

of the genotoxicity of various carcinogens [7, 8], heavy metal poisoning [4, 9], anti-neoplastic drugs [10–12] and pollutants [7, 8, 13]. Indeed, for the last few decades, MN has generally been used as a biomarker of chromosomal damage, genome instability and cancer risk, integrating acquired mutations and genetic susceptibility towards mutations [14, 15]. Therefore, increased MN frequency is expected in pre-neoplastic conditions and that has also been proved by several investigators [16–20]. The role of MN in various steps of carcinogenesis has been substantiated by investigators and it has clearly been shown that the level of baseline chromosome damage in untreated cancer patients is much higher than in cancer-free controls [14]. Therefore, MN scoring could be used as a biomarker to identify different pre-neoplastic conditions much earlier than the manifestations of clinical features and might specifically be exploited in the screening of high-risk population for a specific cancer [2, 21]. For these reasons, the prevalence of MN in epithelial cells has been considered a potential tissue-specific indicator of cancer risk [2, 21]. Occasional studies have shown increased MN frequency in invasive cervical cancer and researchers have suggested that the MN score in exfoliated cervical cells may be an additional criterion for establishing cervical cancer risk [16, 22]. However, there is only limited number of studies on MN scoring in cervical pre-neoplastic and neoplastic conditions [23, 24].

In the present study, we scored and compared MN in the whole spectrum of cervical lesions including normal, inflammatory, atypical squamous cell of undetermined significance (ASC-US), low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL) and invasive cancer (IC) to evaluate the role of MN as a biomarker in different pre-neoplastic and neoplastic lesions of cervix.

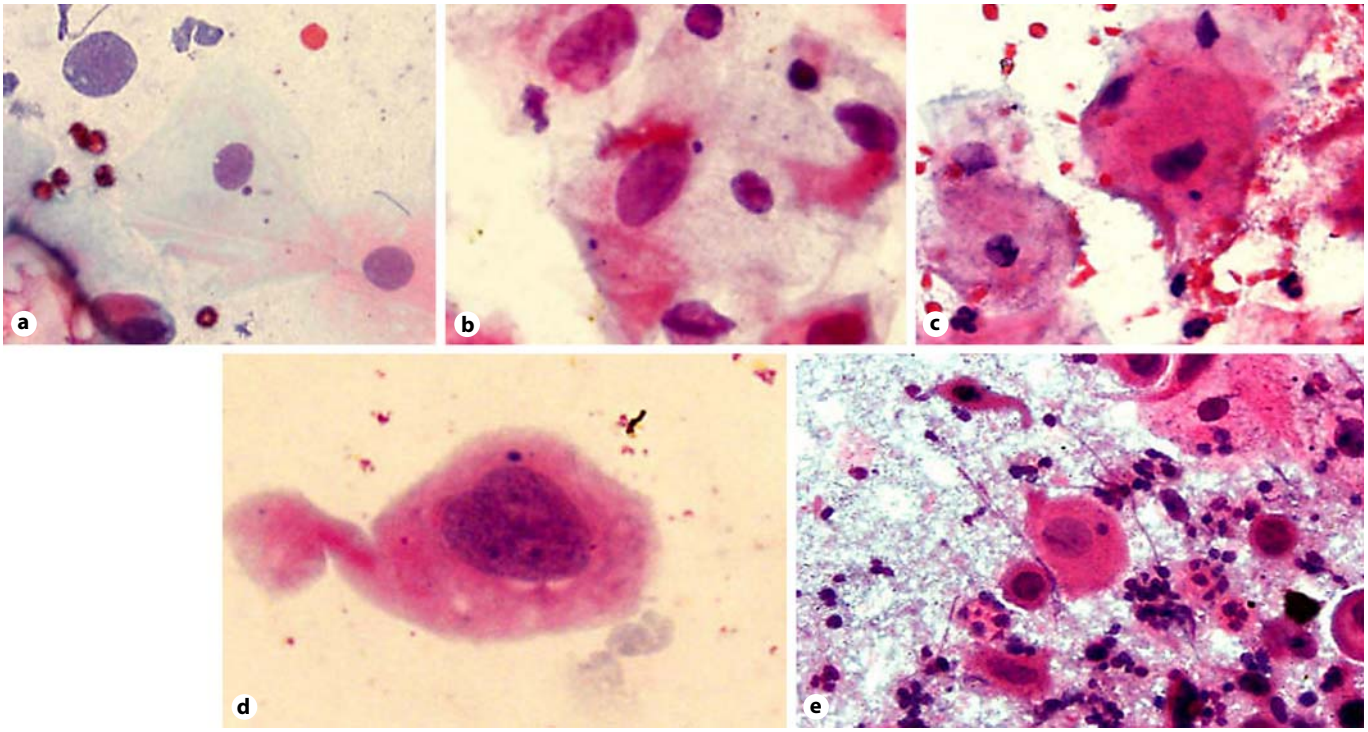
Materials and Methods

We studied MN in the whole spectrum of cervical lesions comprised of 6 different groups: normal, inflammatory, ASC-US, LSIL, HSL, and IC. These cases have been reported in the period between 2005 and 2009. Histopathology outcome of ASC-US, HSIL and IC cases were traced out and the cytology slides or the cases were reallocated accordingly. All ASC-US slides with cervical intra-epithelial neoplasia (CIN) outcome were compromised for various reasons, such as scanty cellularity, severely obscured background due to dense inflammation, poor staining quality and preservation related changes compromising visualization of MN. Therefore we could not score ASC-US slide with CIN outcome and these cases were eliminated from the study. We only scored the ASC-US slides with inflammatory or reactive outcome. We studied a total of 224 slides comprised of normal (40), inflammatory (40), ASC-US (30), LSIL (38), HSIL (30) and IC (46). Cervical smears were taken in the gynecology out patient department as a routine technique by gynecologists. All the smears were fixed in 95% ethanol for 30 min. Subsequently, routine Papanicolaou's stain (Pap stain) was done in the department of cytology.

The detailed age distribution and biopsy outcome of these cases are highlighted in table 1. All the cases of HSIL, IC and ASC-US had available histopathology. The LSIL, normal and inflammatory smears were again reviewed by 2 experienced cytopathologists independently (P.D. and R.N.).

MN Scoring

Two observers separately and independently counted the number of micronucleated cells (MNC) per 1,000 of epithelial cells in oil immersion magnification (×100 objective). Clumps of cells with obscured nuclear or cytoplasmic boundaries and overlapping of cells were avoided and separated or cells lying singly were preferred for counting of MNC. Criteria for MNC were followed as mentioned by Garcia et al. [25]. Cells with double or multiple MN were given a score of 1. After observation by first 2 persons (P.D. and S.S.), a third observer (R.N.) reviewed the slides and final scores were given only after overall consensus. Thus, for each smear a total of 2,000–3,000 cells was counted and the numbers of MNC in each case were expressed per 1,000 cells (MN score). Each slide, on an average, took 15–20 min for the first observer and 8 to 14 min for the second and third observer.



Color version available online

Fig. 1. Micronucleus in normal (**a**), ASC-US (**b**), LSIL (**c**), HSIL (**d**) and IC (**e**). Pap stain, ×1,200.

MN was non-refractile, round to oval in shape with a smooth perimeter suggestive of a membrane. Diameter of MN was variable from 1/16 to 1/3 the diameter of the main nucleus and the shape, color, and texture of MN were similar to those of nucleus (fig. 1). Degenerated cell, apoptotic cell, cytoplsamic fragments and overlapping cells were exempted from counting and scoring [1].

Results

The mean age of the patients in normal, inflammatory, ASC-US, LSIL, HSIL and IC cases of cervical lesions was 44, 40, 51, 46, 46 and 47.7 years and the mean MN scores ± SD were 1.02 ± 1.59, 0.4250 ± 0.71208, 2.87 ± 2.21, 4.7368 ± 5.62179, 21.30 ± 17.18 and 18.50 ± 9.54, respectively (table 2). There was a stepwise gradual increase in MN score from inflammatory to ASC-US to LSIL to HSIL, followed by a slight decrease in IC (fig. 2). The increase of MN score is most significant in the LSIL to HSIL group (fig. 2). Analysis of variance (ANOVA) along with least square deviation test was applied to analyze the differences in mean values of MN scores among different groups (table 3). The statistical analysis revealed significant difference of MN score in different groups. MN score of IC was significantly high as compared to normal ($p < 0.000$), inflammatory ($p < 0.000$), ASC-US ($p < 0.000$) and LSIL ($p < 0.000$) groups but not to HSIL ($p = 0.139$). HSIL showed significant difference of MN score when compared with normal ($p < 0.000$), inflammatory ($p < 0.000$), ASC-US ($p < 0.000$) and LSIL ($p < 0.000$) but not with IC ($p = 0.139$). LSIL showed significant difference of MN score with normal ($p = 0.043$), inflammatory ($p = 0.019$), HSIL ($p < 0.000$)

Table 2. Mean micronucleated cell score in cervical lesion

Group	n	Mean age years	MN score
Normal	40	44	1.0250 ± 1.59305
Inflammatory	40	40	0.4250 ± 0.71208
ASC-US	30	51	2.8667 ± 2.20866
LSIL	38	46	4.7368 ± 5.62179
HSIL	22	46	19.73 ± 17.18490
IC	46	47.5	18.5000 ± 9.54463

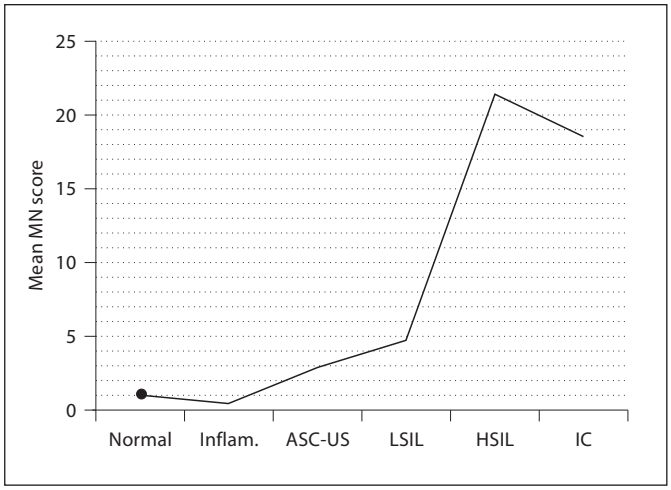


Fig. 2. Trends of micronucleus score in cervical lesions.

Table 3. Result of analysis of variance test (p value)

	Normal	Inflammatory	ASC-US	LSIL	HSIL	IC
Normal		0.739	0.344	0.043	0.000	0.000
Inflammatory	0.739		0.210	0.019	0.000	0.000
ASC-US	0.344	0.210		0.342	0.000	0.000
LSIL	0.043	0.019	0.342		0.000	0.000
HSIL	0.000	0.000	0.000	0.000		0.139
IC	0.000	0.000	0.000	0.000	0.139	

p value is significant at the level of ≤0.05.

and IC ($p < 0.000$) groups but not with the ASC-US ($p = 0.342$) group. ASC-US showed significant difference of MN score only with HSIL ($p < 0.000$) and IC ($p < 0.000$) but not with LSIL ($p = 0.342$), inflammatory ($p = 0.210$) or normal ($p = 0.344$) groups. No significant difference of MN score was noted in the normal versus the inflammatory group ($p = 0.739$; table 3). Two or more MNs were relatively rare or occasional. Multiple MNs were noted in IC (14/46), HSIL (7/30), ASC-US (3/30) and LSIL (2/38) cases.

Discussion

In this study we have done MN scoring in the full spectrum of cervical lesions. We noted significant difference of MN score in HSIL and IC with all other groups. We also noted significant differences of MN score in LSIL and ASCUS with normal and inflammatory lesion. There are limited studies of MN scoring on Pap stained smears. Guzma'n et al. noted that HSIL smears had the highest frequency of MNCs [23]. However, the frequency of MNC in HSIL and LSIL smears was not significantly different in their study. In contrast, the present study showed the gradual increase in MN scores from normal to inflammatory, ASCUS, LSIL and to HSIL. The MN score of LSIL versus HSIL was statistically highly significant ($p < 0.000$) in our study.

Liao et al. [26] studied the expression of MN antigen (MnAg; detectable by monoclonal antibody by immunohistochemistry) in decolorized Pap smears with cytological diagnoses of squamous intra-epithelial lesion (SIL) and adenocarcinoma in situ (AIS). In the SIL cases, MnAg protein expression was seen in dysplastic and morphologically normal endocervical columnar and/or reserve cells in the Pap smears. All the AIS cases also were MnAg positive. Virtually all of the dysplastic and/or atypical endocervical glandular cells expressed diffuse strong plasma membrane staining for MnAg. In contrast to SIL cases, the normal columnar and reserve cells were negative for MnAg. It was postulated that MN antigen might serve as an early biomarker of cervical neoplasia. The combination of cytology and MnAg immunostaining may be helpful to decrease the false negative cases and also to discriminate between cellular atypia due to benign reactive changes versus cellular atypia due to dysplasia in the category of ASCUS and AGUS [26]. The present study was done on routine Pap smears and indirectly showed similar findings because the cases with biopsy proven diagnosis of CIN II, CIN III and IC had the highest MN scores.

The wide variation in the MN scores among different individuals in the same group may be attributable to environmental exposure to genotoxic agents, lifestyle factors, micronutrient deficiency, genetic make up, baseline MN frequencies, ethnicity and other factors associated with carcinogenesis and chromosomal damage. This could also suggest that MN formation is not exclusively induced in carcinogenesis, rather it is a multifactorial event and attributed to several other factors like environment, genetic make up, baseline MN frequencies, ethnicity and other factors associated with carcinogenesis and chromosomal damage (which are still unknown need further study). We can assume that increased MN frequency is more suggestive of increased chromosomal damage rather than neoplasia. However, neoplastic and pre-neoplastic conditions might show significant MN

frequencies because cancer cells generally have acquired chromosomal abnormality. So MN is a non-specific biomarker which should be considered in a proper clinical setting.

Three mechanisms may contribute towards the formation of micronucleus: metabolic stress caused by tumor growth, clastogenic products released from tumor cells and the presence of HPV [23, 27]. Chromosomal instability, particularly in chromosomes 1, 3, 5, 11 and 17, is associated with the development of cervical carcinoma [28]. It was demonstrated that the presence of micronucleus correlated with malignancy. The researchers further concluded that the micronuclei are indicative of numerical and/or structural chromosome aberrations during cell mitosis. Andrew et al. [24] evaluated micronucleus frequencies in cervical cells from 74 women and a pancentromeric DNA probe was used to discriminate between MN that had formed through chromosomal loss and breakage. There was a good number of cervical cells with both chromosome loss (centromere positive micronucleus) and breakage (centromere-lacking micronucleus) in the LSIL and HSIL categories. In fact, many researchers have observed the higher frequencies of micronucleated cells among cancer patients compared to healthy individuals [29, 30]. The ease and low cost of the detection of MN may allow it as a prognostic indicator during the planning and validation of programs for cancer monitoring and prevention.

We encountered a few difficulties while scoring smears with keratohyaline granules, nuclear debri, bacterial colonies and stain deposits. A DNA-specific dye could be used for MN score on the smear. Possibly liquid-based cytology can be used for multiple slide preparation and to do the special stains.

In brief, MN scoring on the epithelial cells of cervix could be used as a biomarker in cancer screening. This is an easy, simple, reliable, reproducible and objective test and can be done on routinely stained smears.

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