Original Article

Apoptosis and Micronucleus in Cervical Pap Smears: Promising Assays to Increase the Diagnostic Value of The Test

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ABSTRACT

Background: The micronucleus (MN) test on exfoliated cells has been successfully used to screen population groups at risk for cancers of cervix, oral cavity, esophagus and urinary bladder. MN originates from chromatin fragments or whole chromosomes; their presence in cells is a reflection of chromosomal aberration. Their frequency rises in carcinogen-exposed tissues much earlier than the symptoms.

Apoptosis is easily discernible in cervical Pap smears in the form of karyorrhexis, karyolysis and condensation of chromatin.

Very few studies are available in literature have studied the significance of MN scoring and apoptosis in cervical non-neoplastic, pre-neoplastic and neoplastic conditions.

We compared the MN score and apoptosis in the whole spectrum of cervical lesions and also evaluated the role of MN score and apoptosis as biomarkers in different non-neoplastic, pre-neoplastic and neoplastic lesions.

Aim: This is a retrospective study to evaluate the significance of apoptosis and micronucleus counts in the spectrum of lesions in cervical pap smears.

Methods: It was a retrospective study conducted using Papanicolau stained cervical smears archived in the department of pathology. We evaluated a total of 230 smears, which included 63 normal smears, 106 inflammatory smears, 15 smears of Atypical squamous cells of undetermined significance, 20 smears of Low grade squamous intraepithelial neoplasm, 12 smears of high grade squamous intraepithelial neoplasm, and 14 smears of invasive carcinoma.

Two pathologists separately and independently counted the number of cells with micronucleus and the number of apoptotic cells per 1,000 squamous epithelial cells.

Results: Micronucleus score was higher in invasive carcinoma and preneoplastic conditions of cervix than the normal and inflammatory conditions. Apoptotic cells were more in preneoplastic lesions and invasive carcinomas as compared normal and inflammatory conditions except in case of inflammatory atrophic smears and erosions of cervix. There was a positive correlation between micronucleus score and apoptosis in the study groups.

Conclusions: Micronucleus score and apoptotic cells increases with increasing grade of dysplasia. It is maximum in malignancy and minimal in normal and inflammatory smears. There is a positive correlation between increased apoptosis and high micronucleus score. These two additional features increase the accuracy of reporting.

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Introduction

Approximately 20 per 100,000 Indian women are likely to suffer from carcinoma of cervix. [1] The common age group is 35-65 years. [2] Cervical Pap smears are the most commonly used screening tests in detecting cancers of cervix.

The micronucleus (MN) test on exfoliated cells has been used to screen population at risk for cancers of cervix, oral cavity, esophagus and urinary bladder in the past. MN originates from DNA fragments or whole chromosomes; their presence is a reflection of chromosomal aberration. They occur in high numbers in carcinogen-exposed tissues much earlier than the symptoms. [1] Apoptosis is easily discernible in cervical Pap smears in the form of karyorrhexis, karyolysis and pyknosis. Their frequency of occurence increases with increase in the severity of the premalignant and malignant lesions of cervix. [3,4] These features are not routinely included in cervical Pap smear reports.

Very few studies are available in literature which have studied the association between MN scoring and apoptosis in cervical non-neoplastic, pre-neoplastic and neoplastic conditions.

We compared the MN score and apoptosis in the whole spectrum of cervical lesions and also evaluated the role of MN score and apoptosis as biomarkers in different non-neoplastic, pre-neoplastic and neoplastic lesions

Aim

This is a retrospective study to evaluate the significance of apoptosis and micronucleus counts in the spectrum of lesions in cervical pap smears.

Materials and Methods

We retrospectively evaluated 230 cervical smears stained with Papanicolau stain received during the period of 3 years from January 2012 to December 2015 that fulfilled the inclusion and exclusion criteria mentioned below. The study material consisted of 15 smears of ASCUS, 20 smears of LSIL, 12 smears of HSIL, 14 smears of squamous cell carcinoma, 106 smears of specific and nonspecific inflammatory lesions and 63 normal smears.

Cytological smears were obtained on microscopic slides using Auer spatula from the uterine cervix. These smears were fixed with commercially available spray fixative (available with the RAPID PAP ™ KIT) for 15 min and stained by PAP technique using a commercially available staining kit RAPID PAP.

MN scoring: Slides were examined under optical microscope at 400×, magnification in a zig-zag method. Two thousand cells were included in each slide following criteria[5-8]:

(a) Intact cytoplasm and relatively flat cell position on the slide with little or no overlap with adjacent cells

(b) Nucleus normal and intact, with smooth and distinct nuclear perimeter and

(c) Little or no debris

Criteria used for identification of micronucleus were,

(a) Regular rounded structure with perimeter suggestive of a membrane located within inner half of the cytoplasm.

(b) Diameter variable from 1/16 to 1/3 the diameter of the main nucleus without any overlap or a bridge to the nucleus.

(c) Staining intensity and texture similar to that of the nucleus

(d) Same focal plane as nucleus

Cells with single or multiple MN were given a score of one. All slides are screened by two pathologists. Thus for each smear a total of 2,000 cells were counted and the numbers of MNC in each case were expressed per 1,000 cells (MN score) (Figure 1a and b). The frequency of MN was expressed as mean count among the study groups and comparison was done statistically.

Criteria for Apoptosis: Presence of karyorrhexis, karyolysis and pyknosis in the squamous cells are considered as features of apoptosis [4] and the count is expressed in terms of number of apoptotic cells per 1000 squamous epithelial cells after screening 2,000 squamous cells (Figure 1c,d,e).

Inclusion Criteria

- Squamous cells lying singly will be preferred for counting of MN and apoptotic cells.
- Squamous cells not obscured by blood, inflammatory] cells, debris and organisms.

Exclusion Criteria

- Clumps of cells with obscured nuclear or cytoplasmic boundaries and overlapping cells were avoided.
- Degenerated cells, cell covered with debris, mucus, WBC, bacteria and RBC were exempted from counting and scoring.
- Anucleate squames, endocervical and endometrial cells were avoided.
- Inadequate and faded slides were excluded.

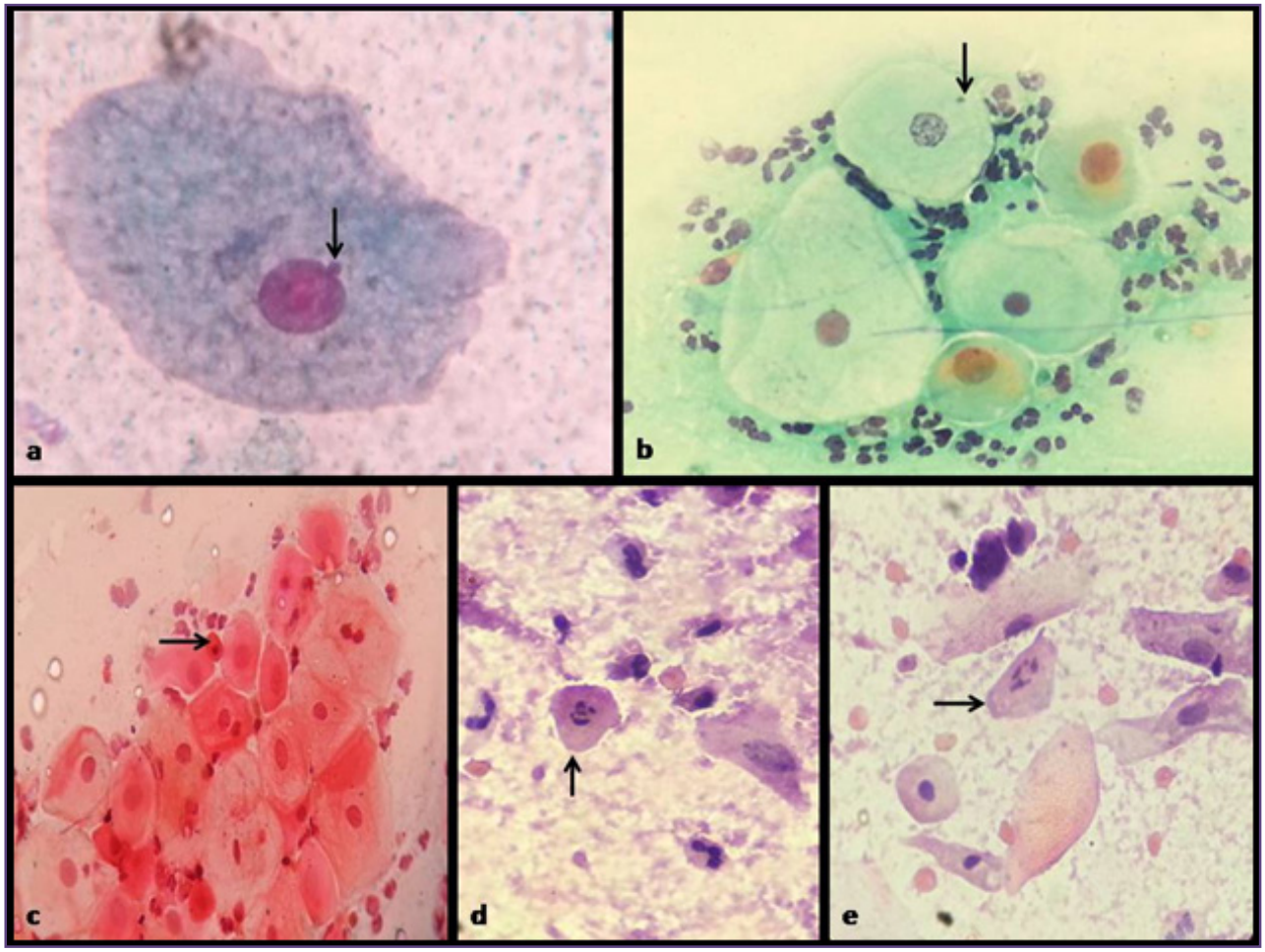

Fig.1: Photomicrographs showing a) and b) squamous cells with micronucleus (black arrow) (Papanicolau stain; x1000); c) Squamous cell with pyknotic nucleus (black arrow); d) and e) squamous cells with karyorrhectic nuclei (black arrow) (Papanicolau stain; x400).

Statistical Analysis: The data was analysed using SPSS software version 19.0. Mean±SD values of micronucleus scores and apoptotic counts were calculated. The micronucleus scores and apoptotic counts of various study groups were compared using Chi square test. This test was applied after combining the smear type, micronucleated and apoptotic cell counts until not less than 20% of expected values were less than 5. Pearsons' correlation test was performed to discern the relationship between micronucleus and apoptosis in various smear types.

Results

In this study we found that majority of the normal and inflammatory smears along with those associated with atrophic changes, reactive atypia, erosions and polyps were showing low scores for micronucleus, LSIL and HSIL and invasive carcinomas showed higher micronucleus scores. This was statistically significant. Chi-square = 126. 77 Degree of freedom [DF]= 4, $p < 0.001$(Table 1&2).

Also apoptotic cells were found to be more in invasive carcinomas, LSIL and HSIL than in normal non specific inflammatory smears and inflammatory smears with reactive atypia. However inflammatory smears associated with atrophic changes, polyps and erosisons of cervix showed more number of apoptotic cells in the smears. This was statistically significant. Chi-square = 74.2 Degree of freedom [DF]= 4, $p < 0.001$.(Table 3&4).

Mean micronucleus scores and mean number of apoptotic cells [Mean+/-SD] in case of normal smear, inflammatory smear, ASCUS, LSIL, HSIL and invasive carcinoma are shown in table 5 along with Pearson correlation values. There was gradual increase in micronucleus scores and apoptosis in the study groups from normal to the invasive carcinoma group. There was a positive correlation between micronucleus score and apoptosis in the study groups.

Table 1: Distribution of micronucleus in various study groups.

Smear Type	Micronucleated cells /1000squamous cells				Total
	< 1	1 - 2	2 – 3	3 & above	
Normal smear	52	11	0	0	63
	82.5%	17.5%	0.0%	0.0%	100.0%
Inflammatory smear	61	31	13	1	106
	57.5%	29.2%	12.3%	.9%	100.0%
ASCUS	6	8	1	0	15
	40.0%	53.3%	6.7%	0.0%	100.0%
LSIL	0	7	8	5	20
	0.0%	35.0%	40.0%	25.0%	100.0%
HSIL	0	2	3	7	12
	0.0%	16.7%	25.0%	58.3%	100.0%
Invasive carcinoma	0	1	4	9	14
	0.0%	7.1%	28.6%	64.3%	100.0%
Total	**119**	**60**	**29**	**22**	**230**
	51.7%	**26.0%**	**12.6%**	**9.5%**	**100.0%**
Chi-square = 126. 77 DF = 4, p < 0.001					

Table 2: Distribution of micronucleus in various types of inflammatory smears.

Micronucleated cells /1000squamous cells in various subtypes of inflammatory smear					
	< 1	1 - 2	2 – 3	3 & above	Total
Inflammatory Atypia	13	5	0	0	18
	72.2%	27.8%	0.0%	0.0%	100.0%
Erosions	6	5	0	0	11
	54.5%	45.5%	0.0%	0.0%	100.0%
Atrophic smear	4	6	0	0	10
	40%	60%	0%	0%	100%
Polyps	1	2	0	0	3
	33.3%	66.7%	0%	0%	100%

Table 3: Distribution of apoptotic cell count in various study groups.

Smear Type	Apoptotic cells/1000squamous cells					Total
	< 1	1 - 2	2 – 3	3 - 4	4 & above	
Normal smear	43	10	10	0	0	63
	68.3%	15.9%	15.9%	0.0%	0.0%	100.0%
Inflammatory smear	65	14	5	13	9	106
	61.3%	13.2%	4.7%	12.3%	8.5%	100.0%
ASCUS	4	9	2	0	0	15
	26.7%	60.0%	13.3%	0.0%	0.0%	100.0%
LSIL	0	2	5	12	1	20
	0.0%	10.0%	25.0%	60.0%	5.0%	100.0%
HSIL	1	3	4	3	1	12
	8.3%	25.0%	33.3%	25.0%	8.3%	100.0%
Invasive carcinoma	0	0	2	10	2	14
	0.0%	0.0%	14.3%	71.4%	14.3%	100.0%
Total	**113**	**38**	**28**	**38**	**13**	**230**
	49.2%	**16.5%**	**12.2%**	**16.5%**	**5.6%**	**100.0%**
Chi-square = 74.2 DF = 4, p < 0.001						

Table 4: distribution of apoptosis in various types of inflammatory smears.

Apoptotic cells /1000squamous cells in various types of inflammatory smears						
	< 1	1 - 2	2 – 3	3 -4	4 & above	Total
Inflammatory Atypia	11	3	0	3	1	18
	61.1%	16.7%	0.0%	16.7%	5.6%	100.0%
Erosions	0	0	0	3	8	11
	0.0%	0.0%	0.0%	27.3%	72.7%	100.0%
Atrophic smear	0	0	0	2	8	10
	0%	0%	0%	20%	80%	100%
Polyps	0	0	0	2	1	3
	0%	0%	0%	66.7%	33.3%	100%

Table 5: Mean micronucleus score and apoptotic cell count with Pearson correlation in various study groups.

Type of smear	Micronucleus score [Mean±SD]	Apoptotic cells [Mean±SD]	Pearson Correlation
Normal smear	0.17±0.38	0.47±0.75	0.0024
Inflammatory smear	0.56±0.74	1.10±1.79	0.0818
ASCUS	0.76±0.59	0.9±0.38	0.8690
LSIL	2.02±0.73	2.25±1.01	0.7048
HSIL	2.79±1.01	2.95±1.45	0.0479
Invasive carcinoma	3.14±0.94	3.57±1.10	0.0200

Discussion

Cytogenetic markers like chromosomal aberrations (CAs), sister chromatid exchanges (SCEs), and micronuclei (MN) are sensitive indicators of genetic damage. [9] The mean prevalence of cells with micronucleus in the general populations is 0.0 to 0.9%. Higher range of micronucleus counts can be the result of chromosomal alterations. [10] The analysis of MN in the epithelial cells has been shown to be a sensitive assay for monitoring chromosomal damage in human populations. [9,10]

Three most commonly hypothesized mechanisms responsible for the formation of MN are metabolic stress, clastogenic substances released from neoplastic cells and the oncogenic viruses like HPV. [11,12] Chromosomal instability, involving chromosomes 1,3,5,11 and 17 is associated with the development of carcinoma of uterine cervix. The presence of MN correlates with malignancy. The MN are indicative of numerical and/or structural chromosome aberrations during cell division. [13]

MN monitoring must be considered as an additional criterion for the early detection of cytogenetic damage in routine gynaecological examinations. The genetic instability caused by human papilloma virus involves the expression of viral oncogenes. E6 expression may result in failed cytokinesis, E7 expression may uncouple centrosome duplication from cell division. Also the absence of a functional TP53 gene, makes the cells aneuploid. False negatives account for 10-50% of Pap test results as a result of limited sensitivity in detecting precancerous lesions of cervix, coupled with the subjective interpretation of results. Complementary methods that increase the sensitivity of screening for cervical cancer include high-risk HPV test and micronucleus (MN) counts. The people from low socioeconomic strata cannot afford HPV testing in many developing and poor countries. [8]

MN is a biomarker of chromosomal aberration which has increased risk of cancer.[14] The combination of cytology and Mn Ag immunostaining may be helpful to decrease the false negative cases. Differentiation between cellular atypia due to benign reactive changes versus cellular atypia due to dysplasia in the category of ASC-US and AGUS is also possible. [15]

The MN test is a simple, rapid, inexpensive, practical, and noninvasive screening technique that is well accepted by the patients for management of subjects under carcinogenic risks after exposure to genotoxic agents like tobacco, ionizing radiation and oncogenic viruses. Increased micronuclei frequency in the grossly normal appearing buccal mucosa of the high risk individuals is associated with greater risk of carcinogenesis. [16]

Micronuclei (MN) are small chromatin bodies that appear in the cytoplasm by the condensation of acrocentric chromosomal fragments (clastogenesis) or by whole

chromosomes(aneugenesis), lagging behind due to mitotic malfunction. Their number increases with increasing chromosomal alterations. The damaged chromosomes, in the form of acentric chromosome fragments, lag behind in anaphase when centric elements move towards the spindle poles. After telophase, the lagging elements are included in the daughter cells in the form of one or several secondary nuclei, which are considerably smaller than the principal nucleus, situated around the main nucleus within the inner half of the cytoplasm and are therefore called the micro nuclei. This takes place in the basal layer of the epithelial tissue, where cells undergo mitosis. This rapid turnover of epithelial tissue brings the cells to the surface, where they exfoliate. [7]

Micronucleus scoring or assay can also be done in human erythrocytes, and lymphocytes. [7]

It has been shown to have a sensitivity of 94%, specificity of 100%, and an accuracy of 95%. [6,7] Micronuclei scoring can be interfered by small dye granules, bacteria and keratohyaline granules. Bacteria can be differentiated by their shape, smaller size and their location either over or in between the squamous cells. Dye granules have a slightly different refractility and color intensity. Keratohyaline granules are numerous and are dispersed throughout the cytoplasm. Misinterpretation of nuclear anomalies like karyorrhexis, karyolysis, condensed chromatin, and binucleates as MN sometimes may occur. [7]

Chemopreventive agents including beta-carotene and other vitamins, have been shown to significantly decrease MN levels in healthy tobacco users, as well as in individuals with precancerous lesions. [7]

DNA specific stains are preferred over Papanicolau stain for evaluating MN, and other nuclear anomalies in exfoliated cells. Many studies have utilized Feulgen-Fast Green as it is DNA specific and enables easy identification of MN in clear transparent cytoplasm [4,16-19]; however method is relatively time consuming and may lead to the underscoring of MN.[16] The assessment of degenerative abnormalities suggestive of apoptosis increases the sensitivity of micronucleus test. [3,4] and therefore can be employed while assessing micronucleus in smears stained with non DNA specific stains.

Other stains include fluorescent dyes, such as diamidino-2-phenylindole (DAPI), acridine orange, Hoechst, and propidium iodide and nonspecific stains like May-Grunwald Giemsa (Giemsa), PAP, hematoxylin and eosin, and Orcien. [6,7]

In our study we used 230 cervical pap smears stained with Papanicolau stain consisting of 63 normal,106 inflammatory smears, 15 ASCUS, 20 LSIL,12HSIL and14 invasive carcinomas. We found that micronucleus scores were higher in invasive carcinomas ,LSIL and HSIL as compared to ASCUS, normal and inflammatory smears(including those with reactive atypia, erosions, polyps and atrophic changes).

The numbers of apoptotic cells were higher in invasive carcinomas, LSIL, HSIL, atrophic inflammatory smears, cervical erosions and polyps as compared to ASCUS, normal and non specific inflammatory lesions.

The mean micronucleus score and mean apoptotic cell counts were higher in ASCUS, LSIL and HSIL groups as compared to inflammatory and normal smears. The smears showing reactive atypia exhibit lower micronucleus scores and apoptotic cells except in cases of atrophic changes, erosions or polyps. Thus micronucleus score and apoptotic cell count helps to distinguish reactive atypia from LSIL and HSIL once the postmenopausal status, erosions and polyps are ruled out.

Micronucleus and apoptotic cells can be counted even in unsatisfactory smears due to low cellularity and those exhibiting micronucleus scores and apoptotic cells in high numbers should be carefully reevaluated to rule out the presence of LSIL, HSIL and invasive neoplasms.

Many authors have reported spuriously higher micronucleus scores while using Papanicolau smears. The accuracy of micronucleus score can be improved in Papanicolau stained smears by combining it with the apoptotic cell count as the LSIL, HSIL and invasive carcinomas exhibit high micronucleus and apoptotic cell counts as compared to benign non-neoplastic conditions as shown in this study.

Thus Bueno C T et al evaluated the association between the frequency of micronuclei (MN) and the cellular changes in 174 Papanicolaou test smears. MN frequencies were significantly higher in the group with pre neoplastic and neoplastic lesions compared to the control group ($p < 0.001$). The mean MN frequencies were 0.95 +/-1.12 (mean +/-SD) in the control group (n = 223), 2.98 +/- 1.20 in individuals with atypical squamous cells of undetermined significance (ASC-US) (n = 50), 4.04 +/- 1.45 in cervical intraepithelial neoplasia (CIN) I (n = 52), 5.97 +/-1.83 in CIN II (n = 30), 7.29 +/- 1.55 in CIN III (n = 17) and 8.64 +/- 1.55 in invasive cancers(n = 25). [8]

Patel BP et al conducted a study on 47Healthy tobacco chewers and 48controls using the peripheral blood

lymphocyte and exfoliated buccal mucosa cells for Chromosomal Aberrations (CA) and micro nucleated cell count (MNC) respectively. MNC was significantly higher (p=0.001) in tobacco chewers than controls. Chromosomal aberration was higher in chewers than controls. However MN test is better indicator for genotoxicity damage than CA. The study concluded that MNC is a better surrogate biomarker to predict genotoxicity than chromosomal aberration for tobacco exposure and DNA damage index in tobacco chewers. [16]

Naderi NJ et al compared micronucleus count in buccal mucosa of smokers to non smokers. Fourteen samples from individuals with a smoking history less than 10 years and 26 samples from individuals with the smoking history of more than 10 years were analysed. The control group consisted of 23 samples from nonsmokers. The mean number of micronucleus of buccal mucosa cells in nonsmokers, smokers with a smoking history less than 10 years and second group smokers with smoking history of more than 10 years was 0.94 ± 0.94, 1.89 ± 0.62 and 2.01 ± 0.93 respectively. The differences between these groups were statistically significant ($P < 0.002$). The mean number of micronuclei in buccal mucosa cells of the nonsmokers was significantly lower than that of the smokers. The mean number of micronucleus of buccal mucosa cells in smokers who smoked more than 10 years was higher than smokers who smoked less than 10 years. [10]

Fareed M et al investigated the frequency of micronucleus in oral mucosa of pan masala chewers and healthy controls and showed that MN frequency was greater in exposed cases (3.56±0.719) as compared to the controls (0.75±0.171). [9]

Ambroise MM et al studied the routine Papanicolaou-stained cervical smears in a total of 132 cases consisting of 42 pre-neoplastic and neoplastic cases and 90 non-neoplastic cases. In each smear, the number of micronucleated cells and binucleated cells were counted under oil immersion. They concluded that the micronucleus count and the binucleated cell count were significantly higher (p value<0.001) in the high-grade squamous intraepithelial lesion (HSIL) and invasive carcinoma patients compared to low–grade squamous intraepithelial lesion (LSIL) and non-neoplastic cases. Expression of HPV 16 E6/E7 inhibits p 53 and RB and facilitates transformation of the binucleated cells which would otherwise undergo apoptotic cell death. Binucleated cells contain double the quantity of chromosomal material. Propagation of these cells can promote genomic instability resulting in development of cervical cancer. Thus binucleated cell count augments the predictive value of MN count. These biomarkers are useful in identifying the borderline cases on cytology. [19]

In a study conducted by Gayathri et al, the mean MN scores ± SD in normal, inflammatory, atypical squamous cells of undetermined significance, LSIL, HSIL and Infiltrative carcinoma cases were 0.84±0.68, 1.06±0.84, 3±0.73, 4.06±1.13, 8.03±1.64 and 10.5±2.01, respectively. MN scores of Infiltrating carcinoma and HSIL were significantly high compared to normal (P<0.000), inflammatory (P<0.000), ASC-US (P<0.000), ASC-H (P<0.000) and LSIL (P<0.000) cases.[20]

Aires GMA et al evaluated distribution of micronucleus and apoptosis among women with normal smears and women with cervical abnormalities, i.e., 12 inflammatory lesions and 10 low- grade and 27 high-grade squamous intraepithelial lesions(HSIL). The frequency of end points of apoptosis was similar in women without cervical abnormalities and women showing HSIL (P > 0.50), and significantly lower in women without cervical abnormalities and in women showing HSIL than in women showing inflammatory lesions or low grade squamous intraepithelial lesions(LSIL)(P < 0.0001). Micronucleus counts were significantly greater in women with HSIL than in the women without cervical abnormalities or inflammatory processes (P < 0.001) or in the women with LSIL(P < 0.005). [3]

Shoji Y et al examined forty six cases of CIN I/I, 75 of CIN III, 16 of microinvasive carcinoma of cervix, and 44 of invasive carcinoma of cervix using formalin fixed and paraffin wax embedded samples. The apoptotic cells were detected using TdT mediated dUTP-biotin nick end labeling (TUNEL) method.

Apoptotic labelling indices were calculated after counting positive nuclei among at least 2000 nuclei. Significant positive correlation with histological malignant grading in CIN and tumour cell invasion into stroma was noted. They concluded that apoptosis in cervical neoplasias may be closely related to tumour progression. [4]

Mergener M et al studied nasal swab samples collected from 40 infants under 12 months of age to evaluate DNA damage related to air pollution in infants.

They recorded 0.13% of cells with micronuclei; 1.20% karyorrhexis; 0.03% pyknosis; 10.85% karyolysis; 1.11% condensed chromatin; 0.54 binucleated cells; and 0.02% nuclear bud. [21]

Conclusions

MN and apoptosis counts helps in mass screening for cervical cancer. The study of micronuclei and apoptosis in Pap smears will increase the sensitivity and specificity of cervical cytology as screening test for cancer.

There was a positive correlation between increased apoptosis and high micronucleus score in our study. These features can be used as biomarkers of cervical intraepithelial lesion and carcinoma in cervical Pap smear even while interpreting Papanicolau stained smears. Also presence of these two markers in high numbers in inadequate cervical smears due to low cellularity indicates the presence of underlying LSIL or HSIL or carcinoma cervix. Hence these patients must undergo repeat Pap test without fail.

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Competing Interest

The authors declare that there is no competing of interest.

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