

Original Article

Association between micronucleus frequency and cervical intraepithelial neoplasia grade in Thinprep cytological test and its significance

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Abstract: A micronucleus is an additional small nucleus formed due to chromosomes or chromosomal fragments fail to be incorporated into the nucleus during cell division. In this study, we assessed the utility of micronucleus counting as a screening tool in cervical precancerous lesions in Thinprep cytological test smears under oil immersion. High risk HPV was also detected by hybrid capture-2 in Thinprep cytological test smears. Our results showed that micronucleus counting was significantly higher in high-grade squamous intraepithelial lesion (HSIL) and invasive carcinoma cases compared to low-grade squamous intraepithelial lesion (LSIL) and non-neoplastic cases. Receiver operating characteristic (ROC) curve analysis revealed that micronucleus counting possessed a high degree of sensitivity and specificity for identifying HSIL and invasive carcinoma. Cut-off of 7.5 for MN counting gave a sensitivity of 89.6% and a specificity of 66.7% ($P = 0.024$ and $AUC = 0.892$) for detecting HSIL and invasive carcinoma lesions. Multiple linear regression analysis showed that only HSIL and invasive cancer lesions not age, duration of marital life and number of pregnancy are significantly associated with MN counting. The positive rate of high risk HPV was distinctly higher in LSIL, HSIL and invasive cancer than that in non-neoplastic categories. In conclusions, MN evaluation may be viewed as an effective biomarker for cervical cancer screening. The combination of MN count with HPV DNA detection and TCT may serve as an effective means to screen precancerous cervical lesions in most developing nations.

Keywords: Micronucleus, cervical intraepithelial neoplasia, Thinprep cytological test

Introduction

Cervical cancer is the most common cancer among women in developing countries and the second most common cancer among women globally. In 2008, it accounted for 9% (529,800) of new cancer cases and 8% (275,100) of all cancer-related deaths among women. More than 85% of cases and deaths occurred in developing countries, including China [1, 2]. Cervical cancer is the only malignant tumor with explicit etiology and has some of the best prospects in terms of prevention and cure. Thinprep cytology test (TCT) is commonly used for the investigation in screening cervical cancer. HPV testing and others like p16 immunostaining would be further applied to estimate

cervical intraepithelial neoplasia (CIN) after TCT screening, which may not develop into invasive cancer. This distinctly improves CIN detection rate and conduces to the reduction of incidence of cervical carcinoma. However, the cost of evaluating and treating CIN is high like HPV testing and p16 immunostaining, so they are seldom used for screening in developing nations. This may be the reason why, in developing countries, cervical cancer goes undetected at higher frequencies than in developed countries [3, 4]. Hence a simple procedure which can be used in conjunction with TCT would be of much use in better detection of CIN in developing countries.

A micronucleus (MN) is an additional small nucleus in the cytoplasm, formed when chro-

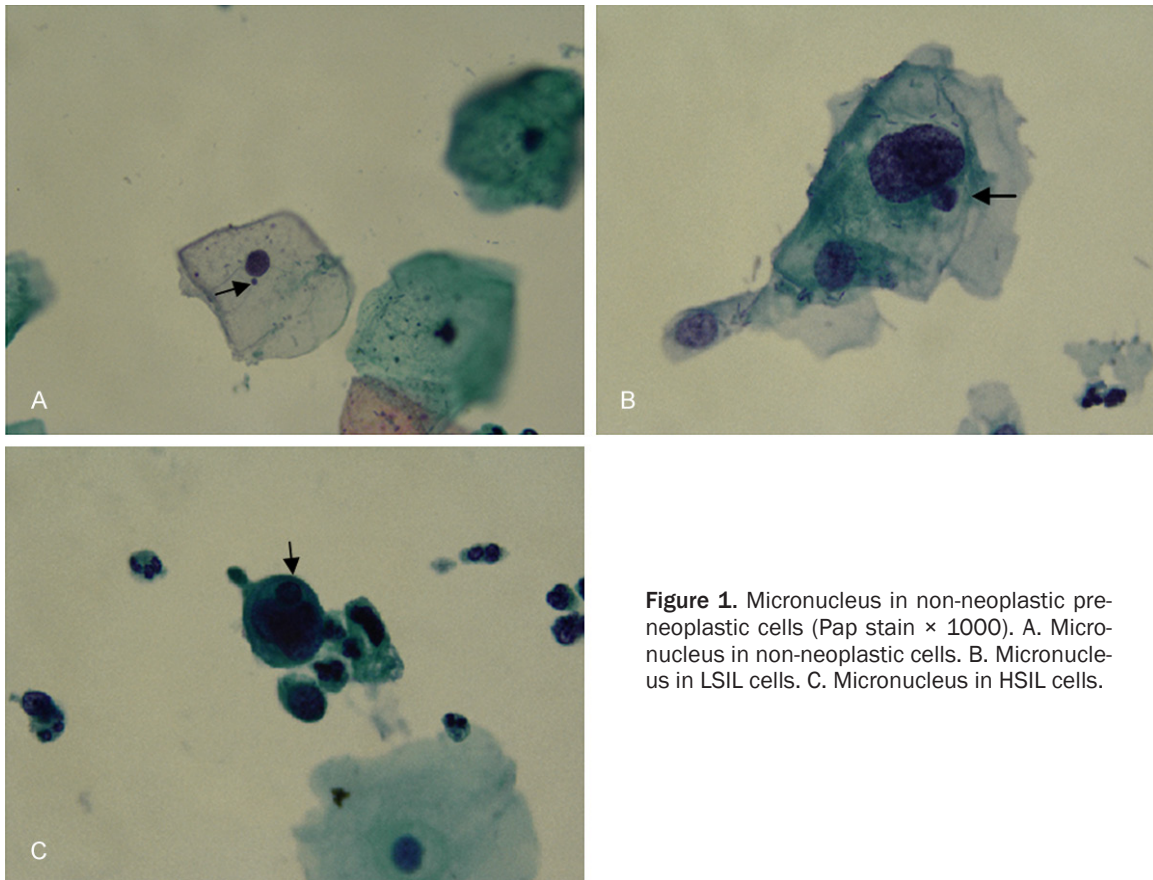


Figure 1. Micronucleus in non-neoplastic pre-neoplastic cells (Pap stain $\times 1000$). A. Micronucleus in non-neoplastic cells. B. Micronucleus in LSIL cells. C. Micronucleus in HSIL cells.

Table 1. MN count in each category

Group	N	Mean rank of MN count	H value	P value
Cervical cancer	27	231.11	176.936	0.000
HSIL	21	219.83		
LSIL	40	171.6		
Normal	47	83.67		
Inflammation	57	93.6		
Atrophy	58	75.64		

Note: using Kruskal-Wallis test.

mosomes or chromosomal fragments fail to be incorporated into the nucleus during cell division. MN test is a well established and widely used technique to evaluate chromosomal abnormalities resulting from exposure to carcinogenic or mutagenic agents [5-7]. Genetic abnormalities in malignant tumors are common events; hence MN also could serve as a potential biomarker for predicting cancer risk. Katarkar A et al. reported that MN frequency can be used with the same effectiveness and greater efficiency in early detection of oral pre-

malignant conditions compared with comet assay of peripheral blood leucocytes [8]. MN frequency in peripheral blood lymphocytes was important for evaluation of prognosis of acute leukemia patients, and it may reflect progression of disease to a certain degree [9].

In this study, MN frequency, HPV DNA and cellular changes in TCT smears were detected in a large cohort of women with different kinds of cervical abnormalities from Xinjiang, China, and the association between them was analyzed.

Materials and methods

Human cervical exfoliated cell smears

250 cases of TCT smears were collected from the Department of Pathology, First Affiliated Hospital of Xinjiang Medical University and Department of Pathology, Hospital of Traditional Chinese Medicine Affiliated to Xinjiang Medical University. Smears were collected from January 2014 to December 2014. 162 cases of non-

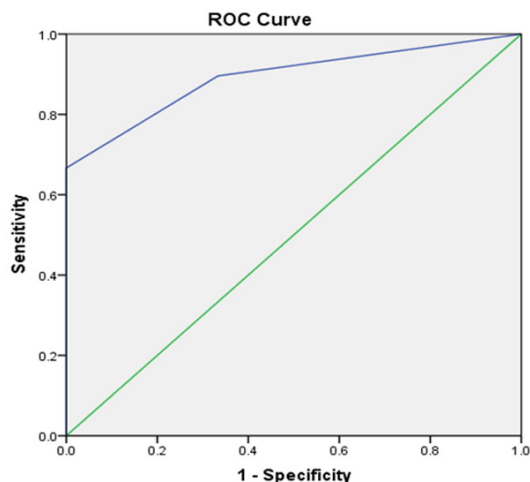


Figure 2. ROC curve for Micronucleus counts to predict HSIL and invasive cervical cancer cases.

Table 2. Test result of area under the curve

Area	Std. Error	Asymptotic Sig.	Asymptotic 95% confidence interval	
			Lower bound	Upper bound
0.892	0.063	0.024	0.768	1.000

neoplasia were obtained comprising 47 normal (mean age 39.09 ± 9.78 years, age range 23~60 years), 57 chronic cervicitis (mean age 40.23 ± 10.02 years, age range 22~72 years), 58 atrophy (mean age 54.5 ± 10.10 years, age range 29~73 years), 40 cases of low grade squamous intraepithelial lesion (LSIL) (mean age 41.13 ± 9.71 years, age range 21~61 years), 21 cases of high grade squamous intraepithelial lesion (HSIL) (mean age 40.95 ± 9.55 years, age range 29~70 years) and 27 cases of cervical carcinoma (mean age 52.74 ± 11.57 years, age range 35~82 years). The smears were stained using standard Pap methodology and classified using the criteria described by The Bethesda System (TBS). Smears of atypical squamous cells of undetermined significance (ASC-US) and atypical squamous cells of undetermined significance not except high lesions (ASC-H) were sorted into relevant diagnosis according to the histological results. All of smears were confirmed by pathological histology. The inclusion criteria were ① a final pathologic diagnosis of CIN and cervical carcinoma; ② no radiotherapy or chemotherapy; and ③ no serious complications.

Cytological preparation

TCT kit (Hologic, USA) was bought. A cytobrush with exfoliated cells collected from uterine cervix was fixed in TCT preserve cytological solution for 15 min. After discarding the cytobrush, the solution bottle was placed into full-automatic slice machine, blended, filtered, and transferred into a glass slide. Then the slide was stained with Papanicolaou staining solution.

MN counting

The TCT smears were analyzed by light microscopy under oil immersion ($\times 1000$) separately and independently by two scorers. For each case, 1000 epithelial cells with well-defined nuclei and cellular borders were counted. Cells showing features of degeneration and apoptosis were not included. Counting was avoided in cell clusters and clumps. A micronucleus was determined according to the following criteria: size less than one-third of the main nucleus, clearly included in the cytoplasm on the same optical plane as the nucleus and distinctly separate from the main nucleus with a similar staining intensity (**Figure 1A-C**). An overall MN count was expressed as the number of micronucleated cells per 1000 epithelial cells [10]. Cervical TCT smear from breast cancer patient using cyclophosphamide was used as a positive control.

Hybrid capture-2 (HC-2) assay

Detection of hybrid capture-2 kit (Digene, USA) was bought. A brush with exfoliated cells collected from uterine cervix was fixed in preserve cytological solution. HC-2 was performed according to the manufacture's instruction. Simply, the process included denaturation, hybridization, capture, amplifying signal and detection. 13 types of high risk HPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68) can be detected at a single time. The criteria of judgement: target DNA (any one in 13 types of high risk HPV) ≥ 500 copy is considered positive.

Statistical analysis

Data were analyzed using SPSS 20.0 statistical software. MN counts were expressed as mean rank. Kruskal-Wallis test was used to compare quantitative variables, since the data did not show normal distribution. The predictive effica-

Table 3. Effect of positivity for HSIL+, age, duration of marital life and number of pregnancies on MN counts

Feature	Unstandardized coefficients		Standardized coefficients	T	Sig.
	B	Std. Error	Beta		
(Constant)	0.606	0.721		0.84	0.402
Groups	8.001	0.222	0.921	35.992	0.000
Age	0.025	0.027	0.087	0.938	0.349
Duration of marital life	-0.025	0.024	-0.088	-1.011	0.313
Times of pregnancies	-0.055	0.151	-0.016	-0.367	0.714

Note: using multiple linear regression.

Table 4. HPV detection in each category

Group n	N	Real case by HPV detection	HPV+	P
Cervical cancer 27	27	27	25	0.000
HSIL 21	21	21	17	
LSIL 40	40	34	28	
Normal 47	47	27	1	
Inflammation 57	57	32	2	
Atrophy 58	58	22	0	

Note: using Fisher exact test.

cy of MN counting was assessed using area under curve (AUC) generated by a receiver operating characteristic (ROC) analysis. The effect of demographic parameters like age, duration of marital life, number of pregnancies on MN counts was analyzed using multiple linear regression. Fisher exact test was used to compare the expression differences of high risk HPV across groups. $P < 0.05$ was considered to be statistically significant.

Ethics statement

The study was approved by the Ethical Committee of the First Affiliated Hospital of Xinjiang Medical University, and the informed consent forms were signed.

Results

MN counting differences are statistically significant across groups

MN counts for the non-neoplastic, preneoplastic and neoplastic categories were showed in **Table 1**. Kruskal-Wallis test yielded significant differences for MN counts among groups ($P = 0.000$). The MN count of HSIL and invasive cancer was significantly higher compared to LSIL

and the normal, atrophic and inflammatory groups, but micronucleus occurrence did not differ among the women showing the normal, inflammatory or atrophic smears (**Table 1**).

Cut-off of 7.5 for MN count provides more than 85% sensitivity and 66% specificity for detecting HSIL or invasive cancer lesions

The ability of MN counts to diagnose HSIL + lesions (HSIL or invasive squamous cell carcinoma) was analyzed using receiver operating characteristic (ROC) curve analysis. ROC curve analysis showed that a cut-off of 8 for MN count gave a sensitivity of 89.6% and a specificity of 66.7% ($P = 0.024$ and $AUC = 0.892$) for detecting HSIL + lesions (**Figure 2; Table 2**). The majority of HSIL +

cases (89.6%) had MN counts ≥ 7.5 . Only 2.5% of the smears in LSIL category had MN counts ≥ 7.5 .

Only HSIL + lesions not age, duration of marital life and number of pregnancy are significantly associated with MN counting

We also analyzed the effect of demographic parameters such as age, duration of marital life, number of pregnancies and positivity for HSIL + on MN counts. Multiple linear regression analysis was performed to determine the relative contribution of these variables. The result revealed that only HSIL + lesions were significantly associated with MN counts ($P = 0.000$), while age ($\beta = 0.087$, $P = 0.349$), duration of marital life ($\beta = -0.088$, $P = 0.313$) and number of pregnancies ($\beta = -0.016$, $P = 0.714$) were not significantly related with MN counts (**Table 3**).

The expression differences of high risk HPV are statistically significant among groups

Detection of high risk HPV for the non-neoplastic, preneoplastic and neoplastic categories were shown in **Table 4**. The positive rate of high risk HPV was distinctly higher in LSIL, HSIL and

invasive cancer than that in the normal, atrophic and inflammatory groups (**Table 4**, $P = 0.000$).

Discussion

Cervical cancer is the consequent result of a long process that has its onset in LSIL and HSIL precancerous lesions. Vaccination against HPV infection, HPV detection and periodical TCT screening are effective measures to prevent cervical cancer, which may reduce the incidence of cervical cancer further. The cost of vaccination against HPV infection and HPV detection is expensive, so they are difficult to be popular in most developing countries. Several studies have indicated an association between micronucleus occurrence and progression of precancerous lesions of cervical cancer. MN frequencies increased with higher grade of cervical lesions, and MN monitoring may be used as an auxiliary measure for monitoring women who are at risk of developing into cervical cancer in routine cervical cytopathological examinations [11-15].

The present study shows that MN count was significantly greater in HSIL + lesions compared to LSIL and control group, the results of ROC analysis showed that MN counts gave a high degree of sensitivity and specificity for identifying HSIL + lesions. Our results indicate that MN evaluation in TCT smears may serve as an easy, useful biomarker in cervical precancerous lesions screening. Our results were similar to other studies. Some reported that MN frequency was significantly higher in the women with HSIL or cervical cancer than in the women with normal cervix or chronic cervicitis or with LSIL [16-18]. MN are small, additional nuclei formed by the exclusion of chromosomal fragments (clastogenesis) or whole chromosomes that are not incorporated into the main nuclei because of mitotic malfunction (aneuploidy) [19], and may be used as markers of genome instability. Chromosomal instability are induced early by deregulated expression of HPV oncogenes E6 and E7 resulting in non-diploid nuclei (aneuploidy). Whereas E6 expression may result in failed cytokinesis, E7 expression may uncouple centrosome duplication from cell division [20, 21]. Consequently, aneuploidy is characteristic for HPV transformed lesions even at cervical precancerous stages [22]. Currently, besides pri-

mary Pap screening, only HPV testing has been assessed as a primary screening marker in larger population based studies [23, 24]. A number of protein biomarkers have been analyzed in serum to detect cervical cancer, among them the SCC antigen [25], VEGF-C [26] and CYFRA 21.1 [27]. The methylation of CDH1 and CDH2 genes has been analyzed in serum samples [28]. None of these markers has shown a clinical utility superior to the analysis of directly sampled exfoliated cells so far.

Several factors such as age, duration of marital life and number of pregnancies are involved in cervical carcinogenesis. We did not observe any significant variation of MN count with the factors. MN counts increased significantly only with HSIL or invasive carcinoma lesions.

Our study also shows that positive expression of high risk HPV is significantly higher in precancerous lesions and cervical cancer compared to non-neoplastic groups. High risk HPV infection is the major factor for cervical carcinoma, therefore HPV DNA detection is widely used clinically as an important means to screen cervical precancerous lesion in advanced nations. However, HPVDNA detection serves only as a qualitative diagnosis tool. Cost of HPV DNA detection is expensive, which is why there is not so many of it in most developing nations. We suggest that the combination of MN count with HPV DNA detection and TCT may serve as an effective means to screen precancerous cervical lesions in developing nations.

On summary, MN counting may be viewed as a simple and convenient test which can be done easily on TCT smears and serves as an effective biomarker for cervical cancer screening in conjunction with HPV DNA detection and TCT cytological analysis in most developing countries. A larger scale study will be needed to further verify our conclusion.

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Disclosure of conflict of interest

None.

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