Assessment of the Association Between Micronuclei and the Degree of Uterine Lesions and Viral Load in Women with Human Papillomavirus

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Abstract. *Infection by human papillomavirus (HPV) is among the main etiologies of cervical cancer. The expression of oncogenic viral proteins enables the onset of the virus, which can trigger the carcinogenic process. One of the main characteristics of this process is the loss of genome stability, including chromosome stability. The micronucleus test is a cytogenetic method for the detection of genetic alterations that change chromosome behavior during cell division resulting in the formation of micronuclei. This method has been applied for the early detection of DNA damage in individuals with a greater likelihood of developing cancer. The aim of the present study was to assess the association between micronucleus expression and the degree of cytological lesions and viral load in patients with HPV. The micronucleus analysis revealed differences in the number micronuclei found in the groups, which ranged from 0.00067 to 0.00133 in the control group and 0.00267 to 0.02433 among patients with HPV. Statistically significant differences ($p<0.05$) were found in the number of micronucleated cervical cells between the patients and healthy women. Moreover, significant associations were found between micronucleus expression and both the degree of uterine lesions ($r^2=0.7237$; $r=0.8507$; $p=0.000002$) and viral load ($r^2=0.7012$; $r=0.8374$; $p=0.000004$). The findings demonstrate the efficacy of micronucleus analysis in monitoring risks to human health.*

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Cervical cancer is the third most common type of cancer among women worldwide, following breast cancer and colon cancer (1-3). Cervical cancer affects approximately six out of 100,000 women and accounts for approximately 275,000 deaths annually in developing countries, which corresponds to 88% of cases worldwide (3-5). Human papillomavirus (HPV) is the main etiological agent of cervical cancer detected in 95 to 100% of cases (1, 6, 7).

Despite its severity, cervical cancer responds favorably to secondary preventive measures when detected in early stages (8). The Papanicolaou test (Pap smear) is the main measure for the prevention of this type of cancer (7) and is capable of detecting pre-invasive lesions in the slow progression of the tumor (1, 5, 9, 10). However, the Papanicolaou test is not completely effective in the diagnosis of lesions as demonstrated by the 20% rate of false-negatives and false-positives (11-15). The sensitivity of the test for high degree pre-invasive lesions and cervical cancer enables a positive diagnosis in no more than 55% of cases (16). Thus, improvements to the Papanicolaou test have been proposed with the suggestion of other cytological markers in the analysis that can contribute toward an increase in its sensitivity (10). Among the markers suggested, the micronucleus test -a cytogenetic method- has been proposed as micronuclei result from numeric and/or structural chromosome alterations (either spontaneous or induced) and failures in the mitotic spindle that lead to the non-

incorporation of either chromosome fragments or whole chromosomes in the main nucleus during cell division (5, 17-19). The presence of micronuclei (in several cell types) has been successfully used to screen populations at risk of cancer and is a sensitive indicator of genetic damage (20).

The evolution of cervical cancer is characterized by lesions that range from low grade to high grade, corresponding to an increase in nuclear atypia and a lack of cell differentiation. These phenotype changes are accompanied by an increase in genetic instability, which is considered a predisposing factor or even a primary event that leads to malignant transformation (21). This instability can be detected through the analysis of the frequency of micronuclei in exfoliated cervical cells (5, 22). A number of studies have demonstrated an association between different degrees of cervical lesions and the frequency of micronuclei (4-8, 23-27). All studies cited have detected a substantial increase in micronuclei in exfoliated cervical cells in patients with cervical cancer.

The aim of the present study was to analyze the association between micronucleus expression and the degree of cytological lesions and viral load in patients with HPV to establish an additional marker of cervical carcinogenesis.

Materials and Methods

Samples were obtained from 20 female patients between 32 and 45 years of age submitted to exams under their informed consent at the ANNALAB Laboratory (Curitiba, Paraná, Brazil) for the determination of high-risk HPV. Fifteen patients with high-risk HPV, based on the results of a hybrid capture method and cytological exam, were selected. These women had not previously undergone treatment, had no history of chronic disease and did not consume alcohol, drugs or cigarettes. Using the same selection criteria, five women negative for HPV made up the control group.

With the use of a hybrid capture kit (HC II; Digene, address, Porto Alegre, Rio Grande do Sul, Brazil), the viral load was determined through the quantification of light emission and expressed as the ratio (sample/positive control) of relative light units. Samples with a ratio of less than 1.0 pg/ml were considered negative. Results equal to or greater than 1.0 pg/ml were considered positive and were divided into two groups: viral load less than 100.0 pg/ml (low viral load) and equal to or greater than 100.0 pg/ml (high viral load). This criterion was based on the method described by Dalstein and collaborators (28). Cytological samples were obtained through the conventional Papanicolaou method and liquid-based cytology (DNACitoq, Digene, Porto Alegre, Rio Grande do Sul, Brazil). Diagnoses were performed using the nomenclature of the Bethesda system (29, 30) and Brazilian National Cancer Institute (31).

The material for the analysis of micronucleus frequency was obtained using the method employed for the Papanicolaou test. Slides containing the cell material were stained with Giemsa, remaining immersed in the stain for five minutes. The slides were then rinsed with distilled water, left to dry at room temperature and analyzed under an optical microscope, with 3,000 cells examined per patient.

The data were log-transformed and the Bartlett's test was used to test the homogeneity of samples. Analysis of variance (ANOVA) was used for the comparison of micronucleus frequencies between groups. Spearman's correlation coefficients were calculated to determine correlations between micronuclei exspression and both degree of uterine lesions and viral load. The level of significance was set to 5% ($p<0.05$).

This study received approval from the Human Research Ethics Committee of Universidade Positivo in the city of Curitiba, Brazil, in compliance with Resolution 003/2002 of the Brazilian National Health Council.

Results

The cytological analyses for the identification of the lesions revealed that six patients tested negative, six patients had atypical squamous cells of undetermined significance (ASCUS) and eight patients had grade I cervical intraepithelial neoplasia (CIN I). The hybrid capture analysis identified five patients with a low viral load (less than 100.0 pg/ml) and five with a high viral load (equal to or greater than 100.0 pg/ml). The micronucleus analysis revealed differences in the number of micronuclei found in the groups, which ranged from 0.00067 to 0.00133 in the control group and 0.00267 to 0.02433 among the patients with HPV (Table I).

ANOVA revealed a significant difference ($p<0.05$) (Figure 1) in the frequency of micronucleated cells between groups. Tukey's unplanned, a posteriori, test also revealed significant differences between groups (Table II).

Spearman's correlation coefficients revealed positive correlations between micronucleus frequency and both viral load ($r^2=0.7012$; $r=0.8374$; $p=0.000004$) (Figure 2) and cytological lesions ($r^2=0.7237$; $r=0.8507$; $p=0.000002$) (Figure 3).

Discussion

A biological marker is an important aspect of the diagnosis, prognosis and risk assessment of a given disease (1). In the present study, the marker analyzed (micronuclei) was capable of differentiating groups at greater and lesser risk based on the degree of genome instability. The findings indicate an increase in genome instability associated with viral load and degree of uterine damage.

A number of studies have demonstrated the importance of evaluating genome instability using the micronucleus test for the categorization of groups at risk of cervical cancer (4-6, 24, 33, 34). In all studies cited, women with HPV had a greater frequency of micronuclei in comparison to those in the control group, which is in agreement with the present findings.

Regarding the correlation between viral load and micronucleus frequency, a significant increase in micronucleated cells was found in women with a high viral load (Table I). These women also had a higher lesion degree (CIN I). According to Avanzi *et al*. (32), repeated cycles of viral infection may increase the number of genetically

Table I. *Results of the cytological analysis, hybrid capture and micronucleus analysis of cervix cells from the patients.*

Patient	Cytological diagnosis	Hybrid capture (pg/ml)	Number of micronucleated cells	Frequency of micronuclei/3,000
1	NEG	0.0	2	0.00067
2	NEG	0.0	5	0.00167
3	NEG	0.0	4	0.00133
4	NEG	0.0	3	0.001
5	NEG	0.0	2	0.00067
6	ASCUS	68.0	12	0.004
7	ASCUS	10.7	8	0.00267
8	ASCUS	37.7	10	0.00333
9	ASCUS	5.0	9	0.003
10	NEG	4.2	9	0.003
11	CIN I	5.0	10	0.00333
12	ASCUS	2.4	8	0.00267
13	ASCUS	4.0	9	0.003
14	CIN I	64.0	20	0.00667
15	CIN I	6.1	11	0.00367
16	CIN I	838.0	42	0.014
17	CIN I	858.0	51	0.017
18	CIN I	885.0	73	0.02433
19	CIN I	1364.0	68	0.22667
20	CIN I	564.0	33	0.011

NEG, Negative; ASCUS, atypical squamous cells of undetermined significance; CIN I, cervical intraepithelial neoplasia grade I; pg, pictograms.

damaged cells in the host and produce cells with an accumulation of chromosome abnormalities. The integration of the viral genome into the epithelial cell genome is a clastogenic event that may increase the number of micronucleated cells, thereby introducing a degree of chromosome instability (4, 27, 35, 36). It is, therefore, plausible that a greater viral load leads to a greater likelihood of genomic instability, as suggested by the present findings.

A number of risk factors have been correlated with the development of CIN, such as infection by high-risk HPVs (for instance, types 16, 18, 31 and 33), which play a crucial role in the development of the disease (6, 21). Subsequent to HPV infection, the increase in genome instability is considered a predisposing factor or even a primary event in malignant transformation (7, 21). These two events were correlated in the present study as most of the patients with CIN in the cytological diagnosis also has a higher viral load and, consequently, greater genome instability, as evidenced by the greater frequency of micronuclei.

The high frequency of micronucleated cells was associated with high-risk HPV. Cortés-Gutiérrez *et al*. (21) also found a direct association between the micronucleus frequency and HPV type. According to the researchers, a lesser frequency of micronuclei among women with low-risk HPV indicates lesser chromosome damage in the early stages than in more advanced stages. In contrast, women with high-risk HPV have a greater frequency of micronucleated cells associated with a less favorable progression of the disease. In the present study, this relationship was evident in Patients 11, 14 and 15 (Table I) who, despite having a low viral load, exhibited genomic instability, as demonstrated by the number of micronuclei in the cells (10, 20 and 11, respectively) and a higher degree of tissue damage (CIN I). These characteristics indicate infection by a more aggressive type of HPV. Thus, more frequent follow-up is necessary in these patients.

Patient 10 (Table I) exhibited a peculiarity as she had no cytological lesions and a low viral load but a high frequency of micronuclei. As genomic instability precedes cytological lesions, as stated by Nersesyan *et al*. (7) and Cortés-Gutiérrez *et al*. (6, 21), the patient in question is at high-risk for the progression of the disease and also requires more frequent follow-up. The characteristics of this patient underscore the importance of micronucleus analysis in combination with the Papanicolau test for the determination of the risk of developing cervical cancer.

The results of the present study add to the evidence that human papillomavirus results in alterations on the genetic level and may compromise cell function, which can trigger the carcinogenic process. The data also demonstrate the efficacy of micronucleus analysis in patients at risk for carcinogenic processes regarding the quantification of genetic damage, which can precede and predispose patients to the malignant process. Thus, despite its methodological simplicity, this test can contribute towards the monitoring of risks to human health.

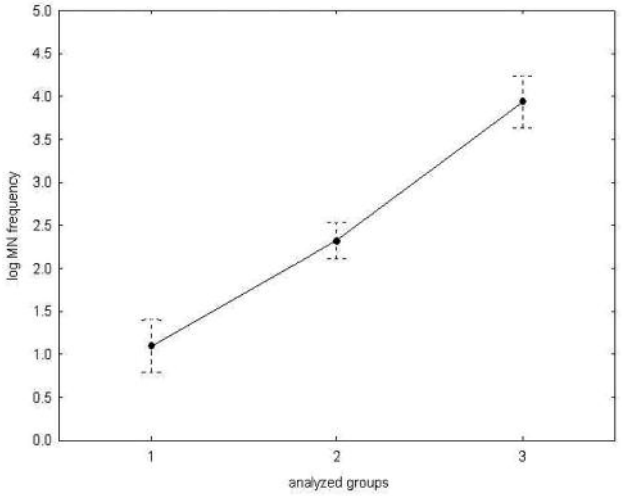

Figure 1. *Result of one-way ANOVA considering the number of micronuclei found in the groups studied. MN, Micronuclei; 1, control group; 2, patients with low viral load; 3, patients with high viral load.*

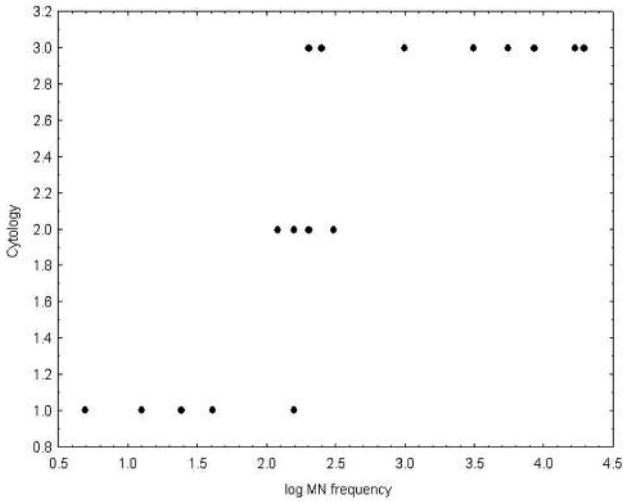

Figure 3. *Result of Spearman's correlation considering frequency of micronuclei and the cytological classification of the lesions in the individuals analyzed. MN, Micronuclei.*

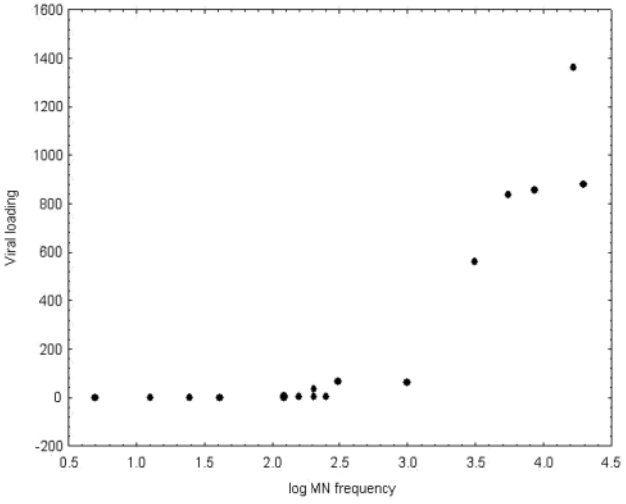

Figure 2. *Result of Spearman's correlation considering frequency of micronuclei and viral load in the individuals analyzed. MN, Micronuclei.*

Table II. *Results of Tukey's test for the comparison of HPV genotoxicity between the groups studied.*

Groups	1	2	3
1			
2	0.000164*		
3	0.000161*	0.000161*	

**p*-Values: 0.05 level of significance.

Conflicts of Interest

The Authors declare that they have no conflicts of interest related to the publication of this manuscript.

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