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Research Article

Association between human papillomavirus (HPV) DNA and micronuclei in normal cervical cytology

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Abstract

The aim of the present study was to investigate the association between HPV-DNA and micronucleus (MN) frequency in women with normal cervical cytology. A total of 158 normal cervical smears were analyzed cytologically. The HPV genome was amplified using the GP5+/bioGP6+ consensus primers. HPV-DNA of high-risk types 16, 18, 31, 33, 39, 45 and 59 were also investigated. Of the 158 samples, 20 (12.7%) and 47 (29.7%) were positive for HPV-DNA and MN, respectively. Evidence for MN was found in 11 out of 20 (55%) HPV-DNA positive samples and in 36 out of 138 (26.1%) HPV-DNA negative ones. MN presence was significantly higher in HPV-DNA positive samples ($p = 0.016$). On the other hand, the absence of MN observed in a considerable number of HPV-DNA negative samples (102) may be of great value in predicting the absence of HPV. The mean age of HPV-DNA positive women (34.2 $\pm$ 12.6) was significantly lower than the mean age of HPV-DNA negative women (43.9 $\pm$ 13.7) ($p = 0.003$). Infection by one or multiple HPV types was found in 11 out of 20 (55.0%) and 9 out of 20 (45.0%) samples, respectively. The evaluation of MN using cervical smears collected for cytology tests could, thus, be used as additional information to monitor a population's exposure to HPV.

Keywords: human papillomavirus, micronucleus, cytology, high-risk HPV, Southern Brazil.

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Introduction

Cervical cancer represents the third most common cancer in women worldwide, with 86% of all deaths occurring in developing countries, where there is lack of access to prevention, early diagnosis and treatment (Jemal *et al.*, 2011). In Brazil, cervical cancer ranks as the second most frequent cancer among women between 15 and 44 years of age (WHO/ICO, 2010). Human papillomavirus (HPV) is considered the major cause of cervical cancer. Most cervical cancers can be detected early through a routine exfoliative cytology test (Papanicolaou test), a screening which has been used for more than 50 years in an effort to decrease the number of deaths from cervical cancer (Stack, 1997).

DNA testing has also been reported as an important tool for detecting HPV (Mayrand *et al.*, 2007) and is being introduced in some countries as an adjunct to the Papanicolaou test or as the primary screening test (Castle *et al.*, 2012). In developing countries, this introduction has been controversial, mainly because cytology-based screening programs suffer from lack of basic healthcare infrastructure (Bradford and Goodman, 2013). In a meta-analysis study, HPV types 16, 18, 31, 33 and 45 were shown to be the most prevalent types associated with cervical carcinomas in Latin America (Ciapponi *et al.*, 2011).

Chromosomes or chromosome segments that fail to be incorporated into nuclei during cell division configure micronuclei (MN). The MN test, readily performed via microscopy, has been successfully used to screen population groups at risk for cancer, representing a sensitive indicator of genetic damage (Samanta and Dey, 2012). The frequency of MN appears to increase in carcinogen-exposed tissue before there are any clinical symptoms (Gayathri *et al.*, 2012). Considering the fact that oncogenic HPV viral proteins may induce cytogenetic changes in exfoliated cells of the uterine cervix, a positive correlation between micronucleated cells and dysplasia in Papanicolaou smears has been reported (Gandhi and Kaur, 2003; Guzmán *et al.*, 2003; Cortés-Gutiérrez *et al.*, 2010; Aires *et al.*, 2011).

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Taking this into account, the aim of the present study was to investigate the association between HPV-DNA and MN in women from Southern Brazil with normal cervical cytology in order to contribute additional information to cervical cytology analysis. Additionally, some of the most prevalent high-risk HPV types were investigated.

Material and Methods

A total of 158 normal cervical smears were analysed cytologically for HPV-DNA and MN comparisons. The smears were collected through the use of a cytobrush in a gynecology clinic at a healthcare medical centre (*Posto de Saúde Modelo*) in Porto Alegre, Rio Grande do Sul, Southern Brazil from March to December 2009. All women who agreed to participate signed an informed consent form. This study was approved by the Ethics Committee of Universidade Luterana do Brasil (number 2008-601H). All participants answered a questionnaire on their clinical history.

Two slides were made, one for the conventional Papanicolaou exam and another for MN analysis. After the slides were prepared, the cytobrush was placed in a tube containing 2 mL TE 1x and stored at -20 °C for later DNA extraction. For the Papanicolaou test, the analyses were based on the Bethesda system (Solomon *et al.*, 2002). Samples with altered cytology results were excluded from the study. The slides for detecting and counting MN were stained with Giemsa, and evaluated four times, twice by each of two observers independently. A total of 1000 cells were counted under a light microscope at a 1000x magnification following the criteria described by Tolbert *et al.* (1992). Both analyses were independently interpreted by two cytologists.

A non-organic method developed in our laboratory was used to extract the DNA from the clinical specimens (Michelon *et al.*, 2011). Briefly, 500 µL of each sample was concentrated by centrifugation. The resulting sediment was resuspended in 50 µL of 1x TE and incubated at 99 °C for 10 min. The DNA was then purified using 5 µL of a glass matrix (Concert Extraction Systems; Life Technologies, Rockville, MD, USA).

The amplification of a 150 bp fragment of the L1 region of the HPV genome was performed using the GP5+/bioGP6+ consensus primers (De Roda Husman *et al.*, 1995). The PCR was carried out in a final volume of 50 µL containing 50 mM KCl, 10 mM Tris-HCl pH 8.5, 2.5 mM $MgCl_2$, 0.1 mM of each deoxynucleoside triphosphate, 50 ng of each primer, 2.5 U of *Taq* DNA polymerase (Invitrogen) and 10 µL of extracted DNA. A negative control (no DNA) was included in each PCR run to ensure that there was no cross contamination. Samples were amplified under the following conditions: initial denaturation step at 95 °C for 5 min, 40 cycles at 95 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min, and a final elongation step at 72 °C for 10 min. DNA of CaSki (HPV-16 positive) cells were used as a positive control in all PCR reactions. The final PCR product (10 µL) was visualized in electrophoresis agarose gel (1.5%) under UV light.

HPV DNA genotyping of high-risk types 16, 18, 31, 33, 39, 45 and 59 was carried out using a microplate colorimetric hybridization assay (MCHA) developed in our laboratory (Barcellos *et al.*, 2011). Denatured PCR amplicons were reverse hybridized to the probes fixed on a microplate. Seven DNA plasmids containing the L1 gene of a high-risk HPV type (HPV16, 18, 31, 33, 39, 45 and 59) were used as positive controls.

The absolute and relative frequencies of the categorical variables and the distribution of the continuous variables were evaluated to define the profile of the population under study. Statistical analysis was performed with SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). The χ^2 test was used to study differences in categorical parameters. Values of $p < 0.05$ were considered statistically significant.

Results

The mean age of the 158 women was 42.7 ± 13.9 years, the mean age of menarche was 11.6 ± 1.7 years and the mean number of children per woman was 1.4 ± 1.2 children. In terms of other baseline characteristics, 107 (65.5%) had a stable marital status, 119 (73%) were Caucasian, 25 (25.2%) smoked, 58 (35.6%) used oral contraceptive, and 78 (47.9%) were post-menopausal.

HPV-DNA was found in 20 out of 158 (12.7%) samples. The mean age of HPV-DNA positive women (34.2 ± 12.6) was significantly lower than the mean age of HPV-DNA negative women (43.9 ± 13.7) ($p = 0.003$).

The positive samples were tested for the presence of HPV 16, 18, 31, 33, 39, 45 and 59. Fourteen of the 20 positive samples (70.0%) were positive for HPV 16, with 7 out of 20 (35.0%) being positive for HPV 31; 4 out of 20 (20.0%) for HPV 33; 2 out of 20 (10.0%) for HPV 39; 3 out of 20 (15.0%) for HPV 45; and 2 out of 20 (10.0%) for HPV 59. No samples were positive for HPV 18. Infection by one and multiple HPV types was found in 11 out of 20 (55.0%) and 9 out of 20 (45.0%) samples, respectively.

The cellular changes consistent with the presence of MN were observed in 47 (29.7%) of 158 slides, of which 11 were HPV-positive and 36 were HPV negative. The presence of MN was significantly associated with HPV-DNA positivity ($p = 0.016$) (Table 1). On the other hand, the absence of MN observed in a considerable number of HPV-DNA negative samples (102) may be of great value in predicting the absence of HPV. Table 1 also shows the frequencies of MN detected in the slides.

Discussion

In developed countries, the variability of performance in cytology-based screening has led to the develop-

Table 1 - Association between micronucleus presence/frequency and HPV-DNA in cytologically normal cells.

	HPV positive (%)	HPV negative (%)	p
MN presence	11 (55.0)	36 (26.1)	0.016
MN/1000 cells			
1	4 (20.0)	22 (15.9)	
2	3 (15.0)	11 (8.0)	
3	2 (10.0)	2 (1.5)	
4	1 (5.0)	1 (0.7)	
5	1 (5.0)	0 (0.0)	
0	9 (45.0)	102 (73.9)	
Total	20	138	

ment of more effective alternatives, such as reliable molecular testing. Several large randomized controlled trials have concluded that HPV testing is more sensitive than cytology for cervical cancer screening (Mayrand *et al.*, 2007; Castle *et al.*, 2012). Despite the importance of HPV testing, its use requires advanced technology and still has a high cost, limiting the possibility of its introduction in developing countries.

Considering the importance of cervical cancer control programs and the limited number of studies on MN scoring in normal cervical cytology (Aires *et al.*, 2011; Gayathri *et al.*, 2012), the present study was intended to evaluate whether MN is a suitable test to complement the results of the Papanicolaou smear in a low-resource setting, analyzing the association between HPV-DNA and MN in normal cervical cytology of women from Southern Brazil. MN scoring on the cytology smear is easy, cheap, reproducible and can be performed on routinely stained Papanicolaou smears as a biomarker of chromosomal damage (Gayathri *et al.*, 2012; Samanta and Dey, 2012).

In the present study, the prevalence of HPV-DNA was 12.7% and was similar to that found in a meta-analysis study, in which the estimated global HPV prevalence in women with normal cytology was 11.7% (Bruni *et al.*, 2010). In South America, Brazil has the highest incidence of cervical cancer, with a prevalence of HPV infection among women with normal cervical cytology of 21.1%, being the northern and northeastern regions with higher mortality than eastern and southern states (Dutra *et al.*, 2012). In another study developed in Rio Grande do Sul state, the prevalence of HPV-DNA found in women with normal cytology was of 32.7% (Coser *et al.*, 2013). It is reasonable to assume that these differences in HPV prevalence can be due to risks factors associated to the characteristics of populations in different countries, sensibility of the study method used to detect DNA, and/or the number of women included in each study (sample size).

The mean age of women with positive HPV-DNA results was 34.2 years. This finding supports previous research, which concludes that HPV prevalence is higher in women younger than 35 years of age, decreasing in women of more advanced age (Sanjosé *et al.*, 2007). Furthermore, a high prevalence of HPV 16 was found (70%). This result corroborates earlier findings that HPV 16 is the most prevalent type of HPV woldwide (Guan *et al.*, 2012).

In the present study, the occurrence of MN was associated with the presence of HPV-DNA in normal cervical cytology, thus corroborating data obtained by other authors (Aires *et al.*, 2011; Gayathri *et al.*, 2012). This finding suggests that the presence of MN, even with no cytological signs of HPV, can be used by the physician as a criterion for repeating the test within a shorter interval of time. On the other hand, the high number of samples with negative results in both tests (MN and HPV-DNA) suggests that these women are at low risk of HPV infection and can be called in for a new round of screening at the normal interval.

Other authors have used the micronucleus test as a biomarker for chromosome instability and malignancy, observing higher frequencies of micronucleated cells in samples with cervical cancer than in controls (Majer *et al.*, 2001; Leal-Garza *et al.*, 2002; Gandhi and Kaur, 2003; Campos *et al.*, 2008; Cortés-Gutiérrez *et al.*, 2010; Gayathri *et al.*, 2012).

Finally, there is a consensus that the most clinically effective and cost-effective methods for reducing the incidence of cervical cancer are those that limit the number of required visits (Hoppenot *et al.*, 2012). Since cervical screening is usually based on cytology alone, the study of MN in Papanicolaou smears can improve the quality of diagnosis and screening. Nonetheless, these results should be confirmed by other researchers.

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