

# Association between Human Papilloma Virus-type Infections with Micronuclei Frequencies

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**Abstract:** To determine the association between Human papillomavirus (HPV)-type infections with the frequency of Micronucleus (MN), a hospital-based, unmatched case-control study was carried out. We evaluated and compared the average number of MN/1,000 cells among three groups of Mexican females. Twenty one women ranging in age from 31–56 years and divided into three groups were studied. Group I comprised seven control women without cervical lesions and with HPV-negative, Group II was composed of seven women with Squamous intraepithelial lesions (SIL) infected with low-risk HPV low-risk, and Group III was made up of seven women with SIL infected with high-risk HPV infection. Analysis of variance (ANOVA) test revealed differences among Groups I ( $5.14 \pm 3.02$ ), II ( $13.43 \pm 3.41$ ), and III ( $25.43 \pm 3.41$ ) ( $F=67.46$ ;  $P=0.0001$ ). We demonstrated an association between HPV type infection and higher MN frequencies. However, a larger controlled study with sufficient follow-up will be required to further evaluate the usefulness of this test in the clinical management of women with HPV infection.

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## Introduction

Cervical cancer represents the second most common malignant neoplasia in women worldwide. In Mexico, cervical cancer is the most common malignancy in females (Secretaria de Salud, Mexico, 1996).

Infection by high-risk HPV was reported to be strongly associated with cervical cancer evolution; however, this genital infection is spontaneously eliminated within several months in the majority of cases (Snijders et al., 2006). Persistent HPV infection is characterized by marked cytological atypia. SIL occur as morphological representations in HPV-related biological and genomic dysfunction (Baseman and Koutsky, 2005). The lesions are classified as low- and high-grade SIL according to degree of atypia and risk of developing cancer by The Bethesda Systems for Reporting Cervical/Vaginal Cytologic Diagnoses (Solomon et al., 2001). Unfortunately, morphological changes are of low specificity for predicting outcome in the early disease phase.

Some of the 80 types of HPV are considered to be involved in development of 95% of pre-neoplastic and neoplastic lesions of the cervix. The most commonly encountered HPV types in lesions of the uterine cervix are types 6, 11, 16, and 18. Types 6 and 11 (low-risk) have been associated with a cervical lesion in dysplasia that evolved favourably. On the other hand, types 16 and 18 (high-risk) have been associated with a cervical lesion with malignant evolution (Herrington, 1994).

HPV infection of replicating immature cells prevents epithelial maturation and differentiation, leading to continued replication and accumulation of genetic abnormalities (Münger et al., 2004; Doorbar, 2005). Chromosomal instability, including aneuploidy, chromosome rearrangement and breakage-fusion-bridge cycles (Duensing and Münger, 2004), and MN (Chakrabarti and Dutta, 1988; Cerqueira et al., 1998; Leal-Garza et al., 2002) have been suggested as a hallmark of malignant tumours.

The MN originates from chromosome fragments or whole chromosomes that are not included in the main daughter nuclei division. Thus, MN provide a measure of both chromosomal breakage and chromosomal loss and it has been shown to be at least as sensitive an indicator of chromosomal as classical metaphase chromosome analysis (Fenech et al., 1999). A significant increase in the number of MN in cervical epithelial cells has been reported previously in women with cervical carcinoma (Chakrabarti and Dutta, 1988; Cerqueira et al., 1998; Leal-Garza et al., 2002). However association between HPV type infection and MN frequency of micronuclei has not yet been established.

In this unmatched case-control study, we determined the association between HPV type infection and MN frequency in three groups of Mexican women (without cervical lesions and with HPV-negative, with SIL infected with low-risk HPV, and with SIL high-risk HPV infected).

## Material and Methods

### *Studied population*

Data analyzed in this research were collected at the Unidad Médica de Alta Especialidad (UMAE) No. 23, Instituto Mexicano del Seguro Social (IMSS) in Monterrey, Nuevo León, Mexico during 2006.

We carried out a hospital unmatched case-control study that was approved by the Ethical Committee of the Centro de Investigación Biomédica del Noreste (CIBIN), IMSS. Twenty one females ranging in age from 31–56 years and divided into the three following groups were studied: Group I, seven women without cervical lesions and who were HPV-negative (control); Group II, seven patients with low- and high-grade SIL and infected with low-risk HPV, and Group III, which was made up of seven women with SIL infected with high-risk HPV.

The International Federation of Gynecology and Obstetrics (FIGO) criterion was used for histological diagnoses (Creasman, 1990).

### *MN Test*

Cytological specimens were collected with a cytobrush from colposcopically abnormal areas (patients) and normal areas (controls). The material collected was submerged into 5 ml of physiological saline (NaCl 0.9%) and centrifuged at 1,200 rpm for 5 min. The pellet was suspended in a 3:1 solution of methanol/acetic acid and centrifuged twice at the same speed for the same amount of time. Finally, the pellet was resuspended in 1 ml of methanol/acetic acid solution and dropped onto clean slides.

The cytological slides were stained by the Feulgen method (Schiff's reagent) (Stich and Rosin, 1983). After this procedure, the slides were washed in distilled water, cleaned in xylene, and mounted in resin (Figure 1).

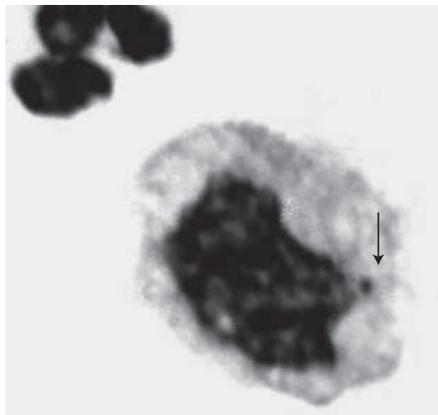


Figure 1 – Micronuclei (arrow) in cytological slides stained by the Feulgen method (Schiff's reagent).

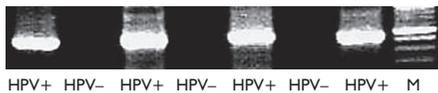


Figure 2 – Molecular detection of HPV in cytological specimens by PCR using L1 (viral region) consensus primers MY09 and MY11. PCR product of 450 bp corresponding to HPV. (M) Molecular weight marker.

For each woman, 2,000 cells from cervical smears were studied by blind analysis under light microscopy with a 100× immersion oil lens following criteria described by Sarto et al. (1987) and Tolbert et al. (1992).

#### *Detection and genotyping of HPV*

DNA was extracted from the cytological specimens as previously described (Bauer et al., 1992). HPV-Polymerase chain reaction (PCR) was performed with L1 (viral region) consensus primers MY09 and MY11, yielding a PCR product of 450 base pairs (bp) (Figure 2). Amplification was carried out for 40 cycles in a Perkin-Elmer/Cetus Thermal Cycler (Perkin-Elmer, Wellesley, MA, USA). Each amplification cycle consisted of 1 min of denaturalization at 94 °C, 1 min of annealing at 43 °C, and 1 min of extension at 72 °C. The amplified product was separated on a 1.5% agarose gel by electrophoresis after staining with ethidium bromide and visualized. A HeLa cell line was used as positive control, and human DNA and water were used as negative controls in each PCR assay. An internal control was employed to indicate the presence and availability of DNA in the sample; this consisted of a region of the human  $\beta$ -globin gene (primers G20 and PO4) that was simultaneously amplified, yielding a PCR product of 260 bp. All 21 specimens were positive for this internal control.

For HPV genotyping, first the amplification product was digested with a combination of Hae III, Pst I, and Rsa restriction endonucleases. Second, the digestion reaction was analyzed by electrophoresis in 3.5% agarose gel. Finally, HPV type was determined according to the banding pattern as visualized using ultraviolet light after ethidium bromide staining. Each sample showed only one HPV type (6, 11, 16, 18, 31, or 33) (Bauer et al., 1992).

#### *Statistical analysis*

Data were entered into a database for statistical analysis that was conducted in two stages. First, data were analyzed by Levene's test for equality of variance. Second, one-way ANOVA with Newman-Keuls test for multiple comparisons at

**Table 1 – Average number and range of micronuclei/1000 cervical epithelial cells in patients with cervical lesions affected by HPV-low risk and HPV-high risk**

Group*	N	No. cells studied	MN ( $\bar{X} \pm SD$ )	MN (range)
I	7	14,000	5.14 $\pm$ 3.02	2–8
II	7	14,000	13.43 $\pm$ 3.41	10–20
III	7	14,000	25.43 $\pm$ 3.41	20–30
Total	21	42,000	–	–

\*Group: I – control women, II – patients with cervical lesions affected by HPV-low risk (types 6 or 11), III – patients with cervical lesions affected by HPV-high risk (types 16, 18, 31 or 33). Levene test = 0.020, P=0.980. ANOVA test: F=67.46, P=0.0001. Newman-Keuls: Group I  $\neq$  Group II  $\neq$  Group III

interpopulation level was utilized to investigate any possible differences among MN averages in the three groups studied. For statistical purposes, SPSS-PC software (v. 10.0) was utilized. A value of  $P < 0.05$  was considered significant.

Sample size was calculated for one-way ANOVA using the *nQuery Advisor Package* (v. 4.0). The test's significance level was 0.01, variance of means was 69.35, common standard deviation (SD) was 3.29, and effect size was 6.43.

## Results

Average age of study participants was 43.04 years (range, 31–55 years) and 45.51 years (range, 34–56 years) for the 14 patients and the seven controls, respectively. Thus, age distribution between patients and controls was similar.

Distribution of number of cells with MN in cervical smear in patients grouped by HPV type is shown in Table 1. ANOVA and Newman-Keuls tests showed increased significance in Groups II ( $13.43 \pm 3.41$ ) and III ( $25.43 \pm 3.41$ ) with respect to Group I or control women ( $5.14 \pm 3.02$ ). Group II was different from Group III.

No significant differences among variances for all comparisons were found when the Levene test was applied.

## Discussion

The number of cells with MN/1,000 cells in the control group was 5.14 according to a previous report (Fenech et al., 1999). Women with HPV infection had higher MN frequencies than those of the control group.

Women infected with high-risk HPV showed the highest number of cells with MN. These findings demonstrated a direct association between frequencies of MN cells with HPV infection type. These women have a higher probability for unfavourable disease evolution according to certain epidemiological data. In addition, MN frequency among women with low-risk HPV indicates that there is a lower chromosomal-damage rate at these earlier disease stages than at more advanced stages.

A significant increase in MN number in cervical epithelial cells has been reported previously in women with cervical carcinoma (Chakrabarti and Dutta, 1988; Cerqueira et al., 1998; Leal-Garza et al., 2002).

HPV-induced genetic instability results in polyploidization, as well as in low-frequency random chromosomal aberrations in squamous cells. This polyploidization appears to be a direct effect of HPV through inhibition of mitotic-apparatus formation in the cell cycle's prometaphase. Centrosome disturbances that occur in the presence of episomal virus genome have been described as a possible mechanism of endoreduplication (Skyldberg et al., 2001). This same mechanism may induce endoreduplication in squamous cells with pre-existing genetic aberrations including aneusomies or structural chromosomal aberrations.

Recently, it has been demonstrated that HPV infection and subsequent HPV E6 and E7 overexpression could induce DNA breakage *in vitro*. Mechanistically,

chromosomal breakage in HPV oncoprotein-expressing cells are likely to involve increased susceptibility to DNA damage or defective DNA damage repair as a consequence of lower p53 or pRB function (Duensing and Münger, 2004). This comprises a clastogenic event that might increase the number of cells with MN by introducing a certain degree of chromosomal instability. This chromosomal instability (Popescu et al., 1990; Sobti et al., 1991; Atkin, 1997) may promote the development of cells with numerical and structural chromosome aberrations, particularly in chromosomes 1, 3, 5, 11, and 17, which has been associated with development of cervical carcinoma (Atkin, 1997).

Induction of chromosomal instability is an emerging theme in viral tumor genesis in humans, and is not only associated with high-risk HPV types (Duensing and Münger, 2004), but also with Hepatitis B virus (Ozkal et al., 2005), Kaposi's sarcoma herpesvirus (KSHV) (Pan et al., 2004), and human T-cell leukemia virus type 1 (HTLV-1) (Majone et al., 2005).

The role of folate and vitamin B12 deficiency as a risk factor for increased MN frequency and cancer risk has been well established (Iarmarcovai et al., 2008). Recent studies suggest that deficiencies of these micronutrients increased risk of developing cervical intraepithelial neoplasia in women exposed to HR-HPV, especially HPV-16 (Piyathilake et al., 2007). However, large cohort studies are required to assess adequately the role of foods and nutrients in cervical HPV carcinogenesis.

## Conclusion

Our results demonstrated a strong association between HPV type infection and MN frequency. MN may comprise an additional criterion for establishing HPV type infection and cervical cancer risk. However, a larger controlled study with sufficient follow-up will be required to evaluate further the usefulness of this test in the clinical management of women with HPV infection.

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