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*Research Article*

# Evaluation of DNA Single and Double Strand Breaks in Women with Cervical Neoplasia Based on Alkaline and Neutral Comet Assay Techniques

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A hospital-based unmatched case-control study was performed in order to determine the relation of DNA single (ssb) and double (dsb) strand breaks in women with and without cervical neoplasia. Cervical epithelial cells of 30 women: 10 with low grade squamous intraepithelial lesions (LG-SIL), 10 with high-grade SIL (HG-SIL), and 10 without cervical lesions were evaluated using alkaline and neutral comet assays. A significant increase in global DNA damage (ssb + dsb) and dsb was observed in patients with HG-SIL (48.90 ± 12.87 and 23.50 ± 13.91), patients with LG-SIL (33.60 ± 14.96 and 11.20 ± 5.71), and controls (21.70 ± 11.87 and 5.30 ± 5.38; resp.). Pearson correlation coefficient reveled a strong relation between the levels ssb and dsb ($r^2 = 0.99$, $P = 0.03$, and $r^2 = 0.94$, $P = 0.16$, resp.) and progression of neoplasia. The increase of dsb damage in patients with HG-SIL was confirmed by DNA breakage detection-FISH (DBD-FISH) on neutral comets. Our results argue in favor of a real genomic instability in women with cervical neoplasia, which was strengthened by our finding of a higher proportion of DNA dsb.

## 1. Introduction

It is generally accepted that genomic instability, whether inherent or induced by external agents, is a primary event leading to neoplastic transformation in the multistep path of carcinogenesis. Genomic instability in women with cervical carcinoma has been demonstrated using chromosomal abnormalities, [1] sister chromatid exchange, [2] micronuclei, [3] single-cell gel electrophoresis assays, also known as the comet assay [4, 5].

Studies relying on the comet assay are of particular interest, because this method is simple and has been used extensively to detect DNA damage at the single-cell level in genotoxic testing and biomonitoring under various circumstances.

The comet assay in combination with fluorescence in situ hybridization is a tool for assessing the distribution of region- or locus-specific DNA damage in the whole genome [6].

Although software-based analysis is the recommended method for the assessment of DNA damage, [7] visual scoring is also acceptable, as significant correlations have been detected between visual scoring and computer-based image analysis [8]. Moreover, visual scoring continues to be the method of choice for laboratories involved in the analysis of large numbers of samples, for example, biomonitoring

studies. The principle of the comet assay is based on alterations found in DNA such as strand breaks resulting in the extension of DNA loops from lysed and salt-extracted nuclei, which, in turn, form a comet-like tail after either alkaline electrophoresis, indicating global DNA damage (ssb + dsb), or neutral electrophoresis, indicating dsb.

The procedure for detecting ssb has been improved by performing a strong alkaline incubation during lysis and/or before and during the electrophoresis step [9]. When the comet assay is performed under neutral conditions, dsbs can be detected. The original comet assay was carried out under neutral conditions but using relatively low doses, so the comet images were dependent on the relaxation of DNA supercoiling by ssb [10]. Since dsb are 25–40 times less frequently induced than ssb, much higher doses and strong lysis are employed for their detection by the neutral comet assay [11, 12].

It is known that ssb can be induced by a number of agents, such as ultraviolet light, reactive oxygen species, and many mutagens, and that they can be easily and rapidly repaired, but do not pose a major threat to genomic integrity. By contrast, dsb represent the most dangerous damage inflicted by mutagens, because they are potent inducers of mutations and chromosomal abnormalities [13]. This type of DNA damage has not been studied comprehensively in women with cervical neoplasia.

The aim of this hospital-based unmatched case-control study was to evaluate the global DNA damage (ssb + dsb) and DNA dsb in cervical epithelial cells in Mexican women with low-grade squamous intraepithelial lesions (LG-SIL) and with high-grade squamous intraepithelial lesions (HG-SIL) using alkaline and neutral comet assays. This information helps build a better picture of the degree and type of DNA damage present in these patients.

## 2. Materials and Methods

*2.1. Study Population.* The data analyzed in this study were collected during 2010 from Unidad Médica de Alta Especialidad (UMAE) No. 23 of the Instituto Mexicano del Seguro Social (IMSS) in Monterrey, Mexico. Written informed consent was obtained from all subjects and approval was given by the local Centro de Investigación Biomédica del Noreste (CIBIN), IMSS Ethical Committee.

The study included 20 women diagnosed with different stages of cervical neoplasia and 10 control women. The average age of the patients was $39 \pm 11$ years (range, 24–59 years), and that of the controls was $43 \pm 10$ years (range, 29–57 years); no significant age difference was found ($P > 0.05$).

All women received a gynecological examination, at which time a Papanicolaou (Pap) smear was obtained using a cytobrush. A biopsy was taken if the examination revealed the presence of a macroscopic cancer lesion. All women who did not have macroscopic cancer lesions were examined using a colposcope and a biopsy was performed if indicated during the examination. Pap smears were read and interpreted by a single pathologist according to the Bethesda system of Pap smear classification [14]. Another pathologist examined the biopsy according to the criteria of the International Federation of Gynecology and Obstetrics (FIGO) [15]. Based on the results of the Pap smears and colposcopy/biopsy, the women were classified into the following categories: 10 had LG-SIL, 10 had HG-SIL, and 10 were controls.

The patients had no previous history of chemotherapy or radiotherapy. None had a clinical history of chronic disease, such as diabetes mellitus, arterial hypertension, or another type of cancer. Women with chronic infection, drug use (including contraceptives), cigarette smoking, antioxidant intake, or radiation exposure (within the preceding month) were excluded from the study.

*2.2. Detection and Genotyping of HPV.* HPV was detected and genotyped using the INNOLiPA HPV Genotyping Kit (Microgen Bioproducts Ltd, Surrey, UK). This kit detect all currently known high-risk HPV genotypes and probable high-risk HPV genotypes (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82) as well as a number of low-risk HPV genotypes (6, 11, 40, 43, 44, 54, 70) and some additional types (69, 71, 74).

*2.3. Slide Preparation.* Cytological specimens were collected from colposcopically abnormal areas (patients) and normal areas (controls) using a cytobrush. The material was submerged in 5 mL of phosphate-buffered saline. The samples were brought to the laboratory under cold conditions and were processed within 1 h of sampling to avoid artifact damage to DNA.

*2.4. Comet Assay.* To perform the comet assay, the cell suspension of cervical epithelial cells was mixed with low melting-point agarose at 37°C, to a final concentration of 0.7%. The mixture (15 $\mu$L) was pipetted onto slides pretreated with 0.5% normal-melting-point agarose, to retain the agarose cell suspension. The drop containing the cells was covered with a glass cover slip (24 mm × 24 mm) and left at 4°C for 5 min.

The cover slips were gently removed and the slides were then ready for processing. The alkaline comet assay was performed using the basic rationale of Singh et al. [16], with modifications. The slides were treated with a lysis solution containing 2 M NaCl, 0.4 M Tris-base, 1% sodium dodecyl sulfate, and 0.05 M EDTA, at room temperature for 30 min.

After protein removal, the resultant nucleoids were washed in TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.3) for 10 min. The slides were treated with fresh alkaline solution (0.03 M NaOH and 1 M NaCl 0.9%) for 2.5 min, to cleave the alkali-labile sites.

The slides were placed horizontally on an electrophoresis tray that was filled with fresh alkaline electrophoresis solution (0.03 M NaOH, pH 13). Electrophoresis was then performed on ice using an electric field of 23 V/cm for 12.5 min. After electrophoresis, the slides were gently removed from the tray and washed with neutralizing buffer (0.4 M Tris-HCl, pH 7.5) for 5 min, and in TBE for 2 min.

To detect dsb, the comet assay was adapted to neutral electrophoresis in TBE buffer at 23 V/cm for 2 min. All steps described previously were carried out in a dark room, to prevent the interference of additional DNA damage.

The slides were dehydrated in sequential baths of 70%, 90%, and 100% ethanol (2 min each) and were then air dried. Finally, the slides were stained with propidium iodide (1 $\mu$g/mL) in Vectashield (Vector Laboratories, Burlingame, CA, USA).

Comets in the alkali assay revealed DNA ssb + dsb and those in the neutral nondenaturing assay exhibited DNA dsb.

Each experiment included two positive controls of sample from one woman without cancer; cells treated with hydrogen peroxide at 20 $\mu$M as DNA ssb control and cells treated with the *AluI* restriction enzyme as DNA dsb control.

Hydrogen peroxide and *AluI* produced pronounced DNA ssb and dsb damage (92% and 84% of comet resp.), which confirmed the accessibility of the cells to the tested chemicals.

The slides were examined at 400x magnification using an inverted fluorescence microscope (IX70; Olympus, Tokyo, Japan) equipped with an excitation filter at 549 nm and a barrier filter at 590 nm and attached to a video camera (Olympus, Tokyo, Japan). The arbitrary units were obtained by one hundred comets scored, and for each comet was assigned a value of 0 to 3 according to its class; 0 representing undamaged cells (comets with no or barely detectable tails) and 1–3 representing increasing relative tail intensities (Figure 1). Summing the scores (0–3) of 100 comets gives an overall score of between 0 and 300 arbitrary units [17]. Apoptotic cells were not considered in the analysis.

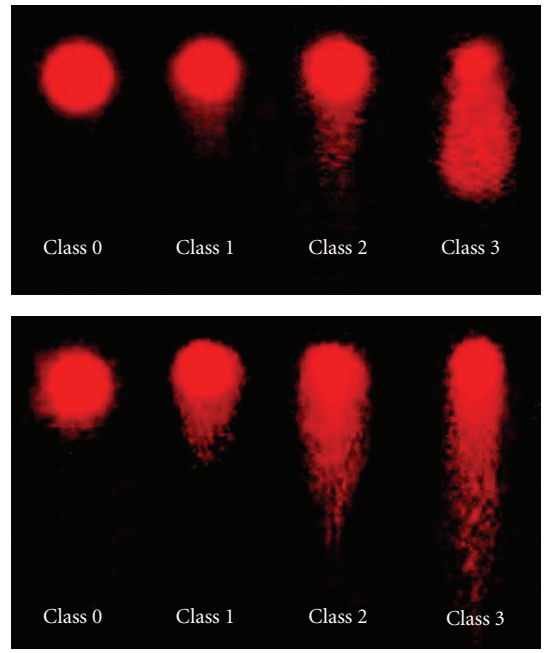


Figure 1: Classification of the comets. Normal nucleus (class 0), halo around the nucleus (class 1), and gradual increase in the length of the comet tail, evolving in parallel with a decrease in the nuclear DNA content (class 2 and class 3). Alkaline comet assay reflecting global DNA damage (ssb + dsb) (upper panel), and neutral comet assay reflecting dsb mainly (lower panel).

*2.5. DBD-FISH on Neutral Comet.* DNA dsb were confirmed by DNA breakage detection-FISH (DBD-FISH) on neutral comets. In this technique, cells are embedded in agarose microgels, lysed, and subjected to electrophoresis under nondenaturing conditions.

Subsequently, the "neutral comets" produced are exposed to a controlled denaturation step that transforms DNA breaks into single-stranded DNA regions, which are detected via hybridization with whole-genome fluorescent probes DNA.

*2.6. Denaturation.* For ssDNA production, protein-depleted slides were incubated in an alkaline unwinding solution (0.03 M NaOH, 1 M NaCl) for 2.5 min at room temperature. After neutralizing with 0.4 M Tris-HCl, pH 7.5, for 5 min, nucleoids were washed in TBE (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH = 8.3) buffer for 2 min. For ssDNA stabilization, slides were dehydrated in sequential 70%, 90%, and 100% ethanol baths for 2 min each, and then air-dried.

*2.7. FISH.* Whole-genome DNA probes were obtained from lymphocyte pellets of women without cancer used a DNA isolation kit for mammalian blood (Roche Diagnostics Corporation, Indianapolis, IN, USA). One microgram from DNA sample was labeled with biotin-14-2′-deoxyuridine 5′-triphosphate (dUTP) employing a commercial nick-translation kit (Roche Diagnostics Corporation, Indianapolis, IN, USA).

The biotin-labeled whole-genome probe was denatured and incubated on slides. After overnight incubation at room temperature, slides were washed twice at room temperature in each of 50% formamide/2 × SSC, pH 7, for 5 min, and then in 2 × SSC, pH 7, for 3 min.

The hybridized DNA probe was detected using 30 min incubation with rhodamine-labeled antidigoxigenin antibody (Roche Diagnostics Corporation, Indianapolis, IN, USA). The slides were counterstained with DAPI (1 $\mu$g/mL) in Vectashield (Vector Laboratories, Burlingame, CA).

The fluorescence intensity and migration length values were calculated using Image J version 1.4.3.67 image analysis software. After background subtraction, 20 different cells for each woman were measured.

The migration length of the DBD-FISH signal is proportional to the number of dsb, whereas its fluorescence intensity depends on the number of ssb (Figure 2) [18].

*2.8. Statistical Analysis.* All analyses were performed using SPSS for Windows 20. First, data were analyzed using Levene's test for variance, and one-way ANOVA with the Student-Newmank-Keuls test for multiple comparisons at the interpopulation level was used to investigate any possible differences between global DNA damage and dsb damage in the three groups of women (LG-SIL, HG-SIL, and control). Second, a Pearson correlation coefficient was using to

Table 1: DNA damage evaluated in alkaline conditions (ssb + dsb) and in neutral conditions (dsb) in women with LG-SIL and HG-SIL and in control women.

| | Mean comet percentage ± SD (Range) | | |
|---|---|---|---|
| | Control | LG-SIL | HG-SIL |
| Global (ssb + dsb) | 21.70 ± 11.89 (8–46) | 33.60 ± 14.96 (9–64) | 48.90 ± 12.87 (34–72)* |
| dsb | 5.30 ± 5.38 (1–16) | 11.20 ± 5.71 (2–20) | 23.50 ± 13.91 (5–49)* |

*ANOVA; Different to control $P < 0.05$.

Table 2: Distribution of percentage of DNA damage evaluated in alkaline comet assay (ssb + dsb) in control, LG-SIL, and HG-SIL women.

| | | Alkaline comet assay (ssb + dsb) | | | | |
|---|---|---|---|---|---|---|
| Group | *N* | Class 0 | Class 1 | Class 2 | Class 3 | Total |
| Control | 10 | 70 (0) | 9 (9) | 9 (18) | 12 (36) | 100 (63) |
| LG-SIL | 10 | 58 (0) | 9 (9) | 10 (20) | 23 (69) | 100 (98) |
| HG-SIL | 10 | 42 (0) | 9 (9) | 17 (34) | 32 (96) | 100 (139) |

The values in parenthesis are "arbitrary units" obtained by the multiplication of the number of cells by each score (0–3). Pearson correlation coefficient ($r^2 = 0.99$, $P = 0.03$).

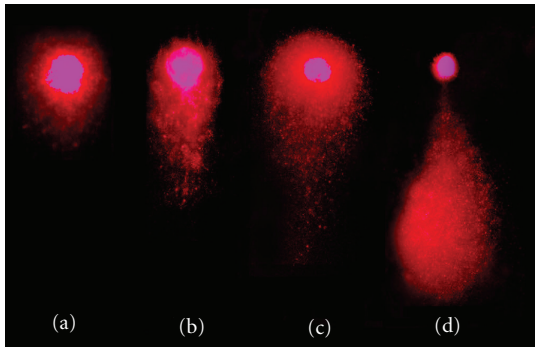


Figure 2: Epithelial cells of the cervix processed using DBD-FISH on neutral comet to determine dsb and ssb simultaneously using whole-genome-labeled (*red*) and DAPI counterstained (*blue*) cells. Cell of patients with HG-SIL (c) and exhibit an increase in the migration length (dsb) and in the fluorescence intensity (ssb) compared with cells from control women ((a) and (b)). Apoptotic cell was concluded to form comets with large fan-like tails and small heads (i.e., so-called hedgehogs) (d).

determine the grade of relation between global DNA damage and dsb damage levels and progression of cervical neoplasia.

The sample size used for ANOVA comparison of means was estimated using the Minitab software (version 15) for DNA ssb damage ($\alpha = 0.05$, $1 - \beta = 0.80$, corrected sum of the squares of means = 371.85, and Sigma = 17.11) and DNA dsb damage ($\alpha = 0.05$, $1 - \beta = 0.80$, = 3.43, corrected sum of the squares of means = 171.17, and Sigma = 11.77). A value of $P < 0.05$ was considered significant for all tests.

## 3. Results

The comet assay allowed the clear visualization of cervical epithelial cells with DNA damage and of cells with no DNA damage. The alkaline denaturing comet assay allowed migration of global DNA damage (ssb + dsb), whereas the neutral nondenaturing method permits the preferential migration of DNA dsb. The morphology, percentage, and length of the tails of comets showed marked differences between the two methods used. Tails were longer with the neutral method, and the percentage of cells showing comet formation was higher in the alkaline assay.

The global DNA damage (ssd + dsb) and DNA dsb in the cervical epithelial cells of LG-SIL, HG-SIL, and controls, as assessed using alkaline and neutral comet assays, are presented in Table 1. ANOVA One-way test revealed a significant increase in global DNA damage and DNA dsb was observed in patients with HG-SIL compared with LG-SIL and controls. The DNA damage observed in controls was considered as normal-level or constitutive damage.

Pearson correlation coefficient revealed a strong relation between the levels of ssb and dsb ($r^2 = 0.99$, $P = 0.03$, and $r^2 = 0.94$, $P = 0.16$ resp.) and progression of neoplasia (Tables 2 and 3).

The woman with HG-SIL showed greater number of DNA dsb. This type of damage was confirmed using DBD-FISH on neutral comets. DBD-FISH signal on neutral comet is proportional to the number of dsb, whereas it depends on the number of ssb (Table 4).

All patients presented a high risk for HPV, with HPV-16, HPV-52, and HPV-51 being the more frequent. Seventy-five percent of the patients (15/20) presented multiple infections. All control women were HPV negative (Table 5).

## 4. Discussion

Genetic instability in women with cervical cancer has been demonstrated previously based on chromosomal abnormalities [1], sister chromatid exchange [2], micronuclei [3], and single-cell gel electrophoresis assays [4, 5].

Udumudi et al., 1998 [4] evaluate the relationship between instability genetic and susceptibitly towards in cervical neoplasia patients with the alkaline comet assay. A significant increased was founded in the mean tail length in peripheral blood leukocytes and cervical epithelial cells of

Table 3: Distribution of percentage of DNA damage evaluated in neutral conditions (dsb) in control, LG-SIL, and HG-SIL women.

| | | Neutral comet assay (dsb) | | | | |
|---|---|---|---|---|---|---|
| Group | *N* | Class 0 | Class 1 | Class 2 | Class 3 | Total |
| Control | 10 | 67 (0) | 28 (28) | 3 (6) | 2 (6) | 100 (40) |
| LG-SIL | 10 | 64 (0) | 25 (25) | 8 (16) | 3 (9) | 100 (50) |
| HG-SIL | 10 | 52 (0) | 25 (25) | 18 (36) | 5 (15) | 100 (76) |

The values in parenthesis are "arbitrary units" obtained by the multiplication of the number of cells by each score (0–3). Pearson correlation coefficient ($r^2 = 0.94$, $P = 0.16$).

Table 4: DNA damage (ssb + dsb) and dsb evaluated by DBD-FISH on neutral comet in women with LG-SIL, HG-SIL and in control women.

| | DBD-FISH on neutral comet | | |
|---|---|---|---|
| | Control (mean ± SD) | LSIL (mean ± SD) | HSIL (mean ± SD) |
| Migration length (dsb)[a] | 6.37 ± 3.11 | 8.41 ± 7.31 | 18.75 ± 6.47* |
| Fluorescence intensity (ssb)[b] | $67^{E3} \pm 42^{E3}$ | $256^{E3} \pm 172^{E3}$ | $731^{E3} \pm 275^{E3}$* |

[a]Migration length is defined as the distance between the center of the comet head and the end of the tail ($\mu$m). [b]Average pixel grey. *ANOVA test; Different to control $P < 0.05$.

patients with dysplasia and cervical cancer respect to control group.

Recently we studied the association between the progressive stages of neoplasia cervical and DNA damage by alkaline comet assay using visual scorning. An increase of high DNA damage in cervical epithelial cells was found according to neoplasia development [5].

The present study confirmed these observations, as it revealed significantly higher level of DNA damage compared with control. In addition, we demonstrated a similar elevation in DNA dsb in women with cervical neoplasia than was confirmed by DBD-FISH.

DBD-FISH had a much higher resolution, showing a hybridization signal at the end of the tail that was not detected by DAPI staining (comet). Thus, DBD-FISH on neutral comets allows the easy and simultaneous estimation of both DNA break types in the same nucleus. In fact, the present study was performed, analyzing the overall genome, using a whole genome probe. However, many different specific probes could be hybridized, with the possibility of simultaneous analysis of ssb and dsb induction [18].

DNA ssb are considered an indicator of early damage [19]. Moreover, DNA ssb are intermediate processing products of DNA damage, which, if left unrepaired, may develop into mutagenic and lethal dsb [20]. By contrast, DNA dsb are considered more biologically relevant lesions [19]. DNA dsb are a more disruptive form of strand break, as, if left unrepaired, they can lead to either cell death or loss of genetic information, or, if the repair process is compromised, they may cause neoplastic progression [21].

It is well established that inaccurate repair or lack of repair of dsb can lead to mutations or to larger-scale genomic instability via the generation of dicentric or acentric chromosomal fragments. Furthermore, the loss and/or amplification of chromosomal material that is characteristic of many cancer cells is explained most easily as having arisen via inappropriate dsb repair events. Defects in cellular responses to dsb may be a frequent initiating event of carcinogenesis [22]. In view of these facts, our results confirmed other observations of a higher number of deletions, breaks, and gaps in these patients [23].

One previous study [24] suggests that inflammation may be involved in directing the course of disease progression by accumulating higher levels of DNA lesions. The P-1, PL-1, PL-2 and L-1 (P, polarity; L, lipophilicity; PL, adducts that are neither very polar nor lipophilic; number, descending order of polarities) adducts were elevated 3- to 13-fold in inflammation compared with normal cervix, and were also higher in dysplasia and cancer.

We found that all patients with cervical neoplasia presented a high-risk HPV. Perhaps, the association of HPV in DNA breaks in patients with neoplasia, with and without HPV infection was not possible determinate. However in one previous study we demonstrated this association evaluated by micronuclei test [25].

In this context, the possible mechanisms that could explain the progressive increase in genetic instability in these patients are related directly to HPV-16 infection [26]. The HPV-16 E7 oncoprotein induces centrosome abnormalities, thereby disrupting mitotic fidelity and increasing the risk for chromosome missegregation and aneuploidy. In addition, expression of the high-risk HPV

E7 oncoprotein stimulates DNA replication stress, which is a potential source of DNA breakage and structural chromosomal instability [27].

An increase in the number of dsb, mediated by Ku70 depletion, is associated with HPV-16 episomal loss in cervical keratinocytes and with a new integration event. Normal levels of host DNA repair proteins, including Ku70, may protect against such events by preventing the generation of host dsb and linearized virus. Interestingly, the HPV-16 E7 protein may play a direct role in inducing integration by interference with the nonhomologous end joining (NHEJ) pathway. Expression of HPV-16 E7 in the HPV-negative

Table 5: HPV Genotyping in cytologic specimens of women with LSIL and HSIL.

| | HPV GENOTYPING | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Diagnosis | 16 | 18 | 31 | 33 | 39 | 44 | 51 | 52 | 54 | 58 | 68 | 70 | 82 |
| LSIL | X | | X | X | | X | | X | X | | | | |
| | X | | X | | | | X | X | X | | | | X |
| | | | | | | | X | | | | | | X |
| | | | | | | | X | X | | | | | |
| | | X | | | X | | | | | | | | |
| | | | | | | X | | X | | X | | | |
| | | | X | X | | X | X | X | X | | | | |
| | X | | X | X | | X | | X | X | | | | |
| | X | | | | | | | | | | | | |
| | | | | | X | | | | | | | | |
| Subtotal | 4 | 1 | 4 | 3 | 2 | 4 | 4 | 6 | 4 | 1 | — | — | 2 |
| HSIL | | X | | | X | | X | X | | X | | | |
| | X | | X | | | | X | X | X | | | | X |
| | | | X | | | X | | X | X | | | | |
| | X | X | | | X | X | | | X | | | | |
| | X | | | | X | | | | | | X | X | |
| | | | | | | | X | | | | | | X |
| | X | | | | X | | X | | | | | | |
| | X | | | | | | | | | | | | |
| | X | | | | | | | | | | | | |
| | | | | | | | X | | | | | | |
| Subtotal | 6 | 2 | 2 | — | 4 | 2 | 5 | 3 | 3 | 1 | 1 | 1 | 2 |
| Total | 10 | 3 | 6 | 3 | 6 | 6 | 9 | 9 | 7 | 2 | 1 | 1 | 4 |

HPV high risk: 16, 18, 31, 33, 39, 51, 52, 58, 82.
HPV low risk: 44, 54, 68, 70.

cervical keratinocyte cell line C33A resulted in the upregulation of the Ku70-binding protein [28], which may interfere with normal NHEJ and increase the frequency of dsb.

Moktar et al., 2009 reported that cigarette smoke condensate-mediated DNA strand breaks are highly persistent, and suggest that persistence of cigarette smoke-associated DNA damage in the presence of HPV infection may lead to increased mutations in cervical cells and ultimately higher cancer risk [29], however, in our study the status of smoking was not ascertained.

From a clinical perspective, the presence of DNA dsb, as assessed using the comet assay in neutral conditions, may differentiate patients with clinically significant cervical lesions from those with insignificant lesions, thus discriminating lesions with a high risk of progression from those with a low risk of progression.

In conclusion, our results argue in favor of the presence of a real genomic instability in women with cervical neoplasia, which was strengthened by our finding of a higher proportion of dsb. This information should provide additional data to determine whether the comet assay is a useful early diagnostic and prognostic biomarker for cervical cancer.

## Conflict of Interests

The authors declare no conflict of interests.

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