Published in final edited form as:

Exp Mol Pathol. 2012 December ; 93(3): 422–427. doi:10.1016/j.yexmp.2012.10.016.

Vaginal cells of smokers are more resistant to human papillomavirus infection than that of non-smokers

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Abstract

To evaluate effect of HPV and smoking on DNA double-strand breaks in vaginal samples, vaginal specimens collected from participants (n=76) were classified based on HPV and smoking status and DNA double-strand breaks measured using comet assay. Mean tail length (31.2 $\pm$ 18.7 μ m) and tail moment (2.4 $\pm$ 2.8 arbitrary units) for HPV-positive patients were lower ($p < 0.001$) compared with HPV-negative patients (61.7 $\pm$ 22.6 μ m; 8.7 $\pm$ 4.9). Never-smokers were found to have higher level ($p < 0.001$) of double-strand breaks (57.7 $\pm$ 24.5 μ m, 7.5 $\pm$ 5.5 AU) compared with ever smokers (35.3 $\pm$ 21.9 μ m; 3.4 $\pm$ 3.7 AU). Among HPV infected patients, never-smokers have more double-strand breaks compared to smokers ($p < 0.001$) which correlated with age ($p < 0.001$). Highly differentiated vaginal epithelium may be resistant to DNA damage associated with HPV infection and smoking, which may be attributed to adoptive survival mechanisms of vaginal epithelium.

Keywords

cigarette smoking; comet assay; DNA double-strand breaks; HPV infection; vaginal abnormalities

Introduction

Vaginal tumors account for an incidence of approximately 1–2% of lower genital tract malignancies (1–2). The etiology of vaginal cancer remains mainly equivocal. Nevertheless, there are a few population-based case-control studies for the etiology of this rare abnormality (Brinton et al., 1990; Daling et al., 2002; Daling et al., 1992; Madsen et al., 2008). These studies reveal that the majority of vaginal cancers have common risk factors with cervical cancers; the most reported shared risk factor among women with cervical and

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Disclosures

None of the authors have a conflict of interest.

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vaginal tumors is infection with cancer-associated human papillomavirus (HPV) types. High-risk HPV prevalence is comparable in cervical and vaginal samples (Castle, 2004), which, in turn, signifies that both tissues can retain cancer-associated HPV infection. However, cervical abnormalities are far more common compared with vaginal tumors (Sillman et al., 1997). The possible justification for the infrequent occurrence of vaginal cancer, despite infection with cancer-associated HPV, may be due to the different transformation potential of the virus in cervical and vaginal tissues (Sugase and Matsukura, 1997).

Other epidemiological and etiological variables associated with cervical cancer have also been reported to be pertinent to the etiology of vaginal tumors, such as cigarette smoking (Madsen et al., 2008). Although conflicting data exist on the role of smoking in the development and progression of HPV lesions in vaginal cells (Ho et al., 1998; Madsen et al., 2008; Sellors et al., 2003), the exact mechanism by which cigarette smoking exerts its cytotoxic effects is not fully understood.

Cigarette smoke is a heterogeneous mixture of approximately 5,000 chemical compounds, of which several dozen are carcinogens, co-carcinogens, mutagens and tumor promoters (Hecht, 2003), including polycyclic aromatic hydrocarbons (PAHs), N-nitrosamines, inorganic and organic compounds (Hecht, 1999; Thun et al., 2002). It has been reported that cigarette smoke-induced genotoxicity results in induction of DNA damage, such as double-strand breaks (DSBs) (DeMarini, 2004). Single-strand breaks are considered an indicator of early damage (Calini et al., 2002) and they are intermediate processing products of DNA damage, which, if left unrepaired, may develop into mutagenic and lethal double-strand breaks (Caldecott, 2007). DSBs are a more disruptive form of strand break since they can either lead to cell death or genetic instability, if left unrepaired, or they may cause neoplastic progression if the repair process is compromised (Chu, 1997; Nocentini, 1999).

Previous studies with human HPV-transfected Ect1/E6 E7 cervical cells suggested that cigarette smoke-mediated DNA DSBs are highly persistent, which may lead to increased mutation and ultimately higher cancer risk (Moktar et al., 2009). Hence, the effect of cigarette smoking on human vaginal cells warrants an investigation. The aim of this study was to investigate whether cigarette smoking, given the infection with high risk HPV types, leads to detectable DNA damage in vaginal cells. In order to examine this, we employed single cell gel electrophoresis (SCGE), also known as the comet assay. That is a sensitive technique to detect DNA strand breaks at the single cell level (Singh, 1998; Singh, 2000).

Materials and Methods

Patients

The protocol, informed consent and questionnaire for this study were approved by the Institutional Review Board. Patients (n = 100) who visited the Gynecologic Oncology Clinic at James Graham Brown Cancer Center and University of Louisville Ob/Gyn Colposcopy Clinic during 2007 through 2009 (Table 1) were indiscriminately approached to assess their willingness to be a part of our study. Upon further assessment of their eligibility, we excluded the potential participants only if they satisfied the exclusion criteria in order to avoid selection bias. Patients answered an NIH-approved questionnaire about their medical history and smoking habits. The patients' high-risk HPV status was obtained from hospital records and/or a detailed questionnaire. Women who were pregnant and/or receiving radiation/chemotherapy in the last year prior to the time of their visit were excluded from the study. Thus, 76 subjects who satisfied the exclusion criteria were included. The study was conducted in a blinded fashion.

Materials

Keratinocyte serum-free medium (K-SFM), bovine pituitary extract (BPE), epithelial growth factor (EGF), penicillin/streptomycin, trypsin/EDTA solution and low-melting agarose were from Invitrogen (Carlsbad, CA, USA); sodium N-lauroyl sarcosine, DMSO, Tris and spermine were from Sigma Aldrich (St. Louis, MO, USA); proteinase K was from Roche (Switzerland); EDTA was from Mallinckrodt Chemicals (Hazelwood, MO, USA).

Collection of human specimens

A DacronTM cervical swab was used to obtain vaginal material from the lateral vaginal wall. Swabs were immediately placed in a 5-ml solution consisting of K-SFM pre supplemented with 0.3% BPE, 0.1 ng/ml EGF, 0.4 mM CaCl₂, 1% penicillin and streptomycin at 4°C for up to 2h.

HPV status

The assessment was made based on either the HPV testing or the Pap results done at the time of the visit. In case HPV testing was not done, the Pap results of low- grade squamous intraepithelial lesion (LGSIL) or high squamous intraepithelial lesion (HGSIL) were classified as HPV positive. The final decision on the HPV status of each participant was confirmed by the Gynecologic Oncologist.

Smoking status

Information on the smoking history of the patients was obtained from the questionnaires. Patients were characterized to two broad categories: those who currently or formerly smoked (ever smoker) and those who did not smoke (never smoker). Thus, there were 49 ever smokers (28 current and 21 ex-smokers) and 27 never smokers.

Comet assay

DNA DSBs were detected by neutral comet assay, as described previously (Moktar et al., 2009; Singh, 1998; Singh, 2000). Briefly, vaginal samples were centrifuged, washed with PBS and processed immediately. 100,000 cells per slide were embedded in 1% low-melting agarose on frosted microscopic glass slides. The cells were subsequently lysed (1.25 M NaCl, 0.1% sodium N-lauroyl sarcosine, 50 mM Na-EDTA, 100 mM Tris-HCl, pH 10), digested with proteinase K (1.25 M NaCl, 5 mM EDTA, 5 mM Tris-HCl, 0.5 mg/ml proteinase K, pH 10) and the DNA was precipitated (50% ethanol, 1 mg/ml spermine, 20 mM Tris-HCl, pH 7.4). DNA was unwound using a neutralizing buffer (500 mM Na-EDTA, 0.2% DMSO, 500 mM NaCl, 100 mM Tris-HCl, pH 9.0) and then electrophoresed in the same buffer (26 V; 250 mA at 4°C). After staining the cells with ethidium bromide, comets were visualized using a light microscope attached to a digital camera with 510 excitation and 590 emission filters. The images were analyzed using the software Comet Assay IV (Perspective Instruments, Haverhill, UK). A total of 50 cells per slide and 2 slides per sample were scored for tail length (TL) and tail moment (TM) as described before (Moktar et al., 2009). Tail length is defined as the distance between the center of the comet head and the end of the tail. Tail moment is often considered a more significant indicator of DNA damage since they refer both to the distance of DNA migration and the amount of DNA in the tail (Olive et al., 1990). The higher the values of tail length and tail moment, as computed by the software, the higher the level of DNA damage.

Statistical analysis

The analysis was conducted to examine the influence of age, HPV infection, smoking and disease status as individual variables and multi-variables on the extent of DNA double-strand breaks using the generalized linear regression model (McCullagh and Nelder, 1989).

The mean, standard deviation (SD), median, minimum and maximum along with 95% confidence intervals for TL and TM were computed within each level of the categorical variables. The p-values were computed using the Wilcoxon rank-sum test between HPV positive and negative subjects, ever and never smokers, and between old (>36 years) and young (<36 years) patients for TL and TM. We further performed the subset analyses on smokers and non-smokers based on their HPV status. Age was grouped using a median cut-off and non-parametric test was used. Furthermore, we performed the multi-variable analysis to estimate the simultaneous effects of the HPV and smoking covariates on TL and TM. We have observed very strong association between HPV status and disease status ($p < 0.001$). Therefore, age, HPV infection and smoking status were analyzed using PROC GLM (McCullagh and Nelder, 1989) in SAS system (v9), where age was considered as both a categorical and continuous variable.

Results

The influence of HPV infection on induction of DNA double-strand breaks

We investigated the potential role of cancer-associated HPV types in inducing DNA DSBs. The percentage of women enrolled in this study with high-risk HPV DNA was higher ($n = 46$; 60.5%) compared with women who were reported to be negative for the virus ($n = 30$; 39.5%). Two most common types of DNA strand break measurements were recorded: tail length and tail moment. The mean tail length for those who were infected with HPV was significantly ($p < 0.001$) lower ($31.2 \pm 18.7 \mu\text{m}$) compared with women who were HPV negative ($61.7 \pm 22.6 \mu\text{m}$) (Fig. 1, Table 2). These data indicate that those with HPV infection showed a lower degree of DNA damage compared to those who did not have HPV DNA. Similar results were found for tail moment (HPV positive, $2.4 \pm 2.8 \text{ AU}$; HPV negative, $8.7 \pm 4.9 \text{ AU}$) demonstrating the significant ($p < 0.001$) inverse association between HPV infection and extent of double-strand DNA breaks in the studied population.

DNA double-strand breaks induced by cigarette smoking

We examined whether DNA strand breaks correlated with the smoking status of the study population. The study group was ($n = 76$) comprised of the population who never smoked ($n = 27$; 35.5%) and ever smokers (either currently or formerly) ($n = 49$; 64.5%). The clinical and general characteristics of the study population are presented in Table 1. The mean tail length for never smokers was $57.7 \pm 24.5 \mu\text{m}$. The ever smokers group had a mean tail length of $35.3 \mu\text{m} \pm 21.9$. We found a significant inverse association between smoking status and tail length ($p < 0.001$) indicating that those who never smoked have, on average, a higher degree of DNA DSBs compared with individuals who had reported as smokers (Fig 1, Table 2). Similarly, mean tail moment was $7.5 \pm 5.5 \text{ AU}$ among never smokers compared with $3.4 \pm 3.7 \text{ AU}$ for those who smoked. This finding signifies that those who never smoked showed a higher extent of DNA strand breaks ($p < 0.001$).

The effect of age on DNA double-strand breaks

In order to understand the effect of age on DNA damage, analysis was carried out taking into account the age as both a continuous and categorical variable (Table 2). Forty patients (52.6%) were less than or equal to the median age of 36 and 36 patients (47.4%) were above the median age. There were significantly higher DNA DSBs in older women compared with younger women: mean TL of 61.2 ± 22.5 versus $27.1 \pm 13.9 \mu\text{m}$ ($p < 0.001$) and mean TM of $8.3 \pm 5.0 \text{ AU}$ versus $1.8 \pm 1.6 \text{ AU}$ ($p < 0.001$).

The interaction between HPV infection and smoking status

Subjects with HPV-positive and -negative status were further categorized based on their smoking status as summarized in Table 3. Ever-smoker and HPV-positive women ($n = 32$) had significantly lower mean TL compared to never-smoker and HPV-positive individuals ($n = 14$) ($23.9 \pm 11.3 \mu\text{m}$ versus $48.0 \pm 21.8 \mu\text{m}$; $p < 0.001$) (Fig. 2). This inverse association was also observed in mean TM (Ever smoker and HPV positive, 1.4 ± 1.0 AU versus never smoker and HPV positive, 4.7 ± 4.1 AU) (Fig. 2). A non-significant association with TL ($p = 0.258$) and TM ($p = 0.079$) was observed among smokers who were negative for HPV infection (Fig. 2).

The interaction among HPV infection, cigarette smoking and age

We examined the influence of HPV infection, smoking status and age on induction of DNA DSBs in study population (Table 4). Age as either a categorical (≤ 36 and >36) or continuous variable showed a strong association when using the multivariable generalized linear regression model. Significant association was observed between TL and HPV for age as categorical ($p = 0.019$) and as continuous ($p = 0.034$) variable. This finding indicates that women with HPV infection had a lower mean TL ($31.2 \pm 18.7 \mu\text{m}$) compared to HPV negative individuals ($61.7 \pm 22.6 \mu\text{m}$) regardless of their age. Similarly, TM and HPV status conferred a significant association ($p = 0.002$) for age as both categorical and continuous variable, indicating a lower TM (2.4 ± 2.8 AU) in women with HPV infection compared with those who were HPV negative (8.7 ± 4.9 AU). The smoking status was significantly correlated with TL ($57.7 \pm 24.5 \mu\text{m}$ vs. $35.3 \pm 21.9 \mu\text{m}$) and TM (7.5 ± 5.5 AU vs. 3.4 ± 3.7 AU) for age as categorical ($p < 0.001$) and continuous variable ($p = 0.003$). These findings suggest the negative correlation between smoking status and DNA DSBs regardless of age.

Comment

We examined the interplay of cigarette smoking and high-risk HPV infection on the DNA integrity of vaginal cells. Based on our experimental findings, we confirmed that the levels of DNA double-strand breaks are inversely correlated with both smoking and HPV status. This observation suggests that the vaginal epithelium may have a different transformational ability by HPV infection.

We collected the vaginal specimens from the lateral vaginal wall to minimize the possible contamination by cervical and metaplastic epithelial cells. This site-specific specimen collection was further validated by microscopic evaluation of the mature squamous cells encompassing the vaginal epithelium. However, we cannot rule out the possibility of sloughed cervical cells in those vaginal samples. Nonetheless, the great majority of the cells were mature squamous types and the homogeneous nature of the collected samples was cytologically confirmed.

One of the intriguing findings that emerged from our study was that HPV infection showed an inverse relation with the extent of DNA strand breaks in the studied population. Considering that a link between high-risk HPV and Vaginal Intraepithelial Neoplasia has been reported (Hampl et al., 2006), the induction of DNA damage by the virus is a logical hypothesis. Moreover, even though the natural histories of cervical and vaginal lesions appear similar (Dodge et al., 2001), neoplastic and invasive vaginal abnormalities are far less common than both cervical intraepithelial neoplasia and invasive cervical carcinoma (Sillman et al., 1997). This obvious discrepancy may be explained by considering two facts. One is that the vagina lacks a squamo-columnar junction, known as transformation zone (Woodruff, 1981). The importance of the transformation zone is evident by the fact that most of the dysplasia caused by persistent HPV infection frequently arises, for unknown reasons, in areas where two different types of epithelium merge such as that occurring in the

cervix, anus and oro-pharynx (Parkin and Bray, 2006). It has also been reported that the transformation zone is more sensitive to HPV infection (Castle, 2004) since the squamous metaplastic cells - constituents of the transformation zone - are needed to support the carcinogenic alteration by high risk HPV (Castle, 2004). Secondly, the discrepancy may also be explained by the variable manifestations of HPV infection in the vagina compared to that in the cervix, even though the virus infects vagina and cervix with the same frequency (Castle et al., 2006). HPV replication in lytic infection is more efficient and results in producing progeny virions, which eventually destroy the infected vaginal cells. In contrast, HPV replicates less efficiently in the cervix and tends to remain within the cells as latent infection, a phenomenon involved in persistence of the infection (Sugase and Matsukura, 1997).

Even though persistent high-risk HPV infections account for a great majority of cervical cancers (Schiffman et al., 2005; zur Hausen, 2000), cell-mediated immunity plays a significant role in clearance of the infection within 1–2 years of exposure (Stanley, 2006). There are, however, no data reported on role of HPV persistence on DNA damage particularly in vaginal walls. Based on our experimental data indicating decreased level of DSBs in HPV-positive individuals, we deduce that vaginal cells may be more resistant to the DNA damaging effect of HPV infection. This speculation was partly derived from the fact that HPV has the ability to hinder host defenses by several means, such as the lack of viral-induced cell death in keratinocytes and downregulation of interferon genes' expression (Stanley, 2006). As part of the HPV infectious cycle, which depends on the differentiation status of the target cell, the virus infects primitive keratinocytes; the expression of viral proteins and viral assembly occur at much higher level in the superficial squamous cells that are terminally differentiated (Middleton et al., 2003). These differentiated keratinocytes that mainly cover the vaginal wall are intended for death and desquamation. Therefore, HPV replication, assembly and particle release do not trigger any cytolysis, and therefore an immune response due to lack of inflammatory reaction (Stanley, 2006). A limitation of the study is that the HPV status was based on the detection of the collective presence of carcinogenic HPV types instead of the individual HPV genotypes. Stratification of the patients based on specific HPV genotypes may provide further insights into the complex interrelation of smoking and progression of HPV infection in vaginal carcinogenesis. The HPV testing used for this study, however, employed a combination of DNA probes to test for high-risk viral types, including HPV 16, which is the most prevalent high-risk HPV strongly associated with vaginal carcinogenicity (Daling et al., 2002; Ikenberg et al., 1990; Ostrow et al., 1988).

Based on significant increase in DNA DSBs in HPV-negative individuals, we infer that, in our study population, the extent of DNA damage is independent of HPV infection. However, DNA strand breaks were the only measured endpoint used in this study. Investigation of other forms of HPV-associated damage, including chromosomal instabilities (Duensing and Munger, 2004) is warranted. Other pre-disposing factors, such as history of cervical and/or vaginal abnormalities, chronic irritation (Merino, 1991) and cigarette smoking (Daling et al., 1992) may have an impact on DNA integrity of vaginal cells.

We have clearly shown that the extent of DNA DSBs increases as a function of age. Our finding is congruent with the studies of Singh *et al.* and Morris *et al.* who demonstrated an increase in DNA strand breaks with age in human sperm (Morris et al., 2002; Singh et al., 2003). Additionally, age-related differences have been reported among individuals diagnosed with cervical cancers compared to those with vaginal cancers (Hellman et al., 2004). Carcinoma of vagina mainly occurs in elderly women (> 60 years) (Al-Kurdi and Monaghan, 1981; Eddy et al., 1991; Hellman et al., 2004; Stock et al., 1995). This finding signifies the age-associated alterations in DNA structure, such as double-strand breaks

(Singh et al., 2003) that may, in turn, attribute to genetic instability and ultimately vaginal carcinogenesis. The extent of double-strand DNA breaks increases with age of the population examined, which implies that older women are at higher risk for accumulation of mutation and genetic instability. Thus, the relevance of our findings to public health in identifying high-risk population is significant.

Our analysis demonstrated a significant inverse correlation between DNA strand breaks and smoking habit. Data on smoking was obtained by self-reporting smoking history. However, the smoking history was obtained prior to medical examination, which minimizes the chance of reporting inaccurate smoking habit (underestimation or denial) among the participated women in the study. Biochemical measures such as cotinine, thiocyanate and carbon monoxide are also considered to be subjective (Patrick et al., 1994). Self-reporting smoking history, when compared with biochemical validation, has been reported to have sensitivity and specificity of above 85% (Patrick et al., 1994).

Few studies, however, have reported a positive association between cigarette smoking and vaginal abnormalities (Daling et al., 2002; Daling et al., 1992; Levi et al., 1998; Sherman et al., 2008). These studies were based on an epidemiological association that appears to link cigarette smoking with vaginal cancer. In contrast, in the current study, DNA strand breaks, as a biological endpoint, was measured to provide insights into the mechanistic effect of the cigarette smoking on the etiology of vaginal cancer. Our finding of a negative association of smoking with vaginal abnormalities was in agreement with the two other studies, where a non-significant epidemiological relationship was found (Brinton et al., 1990; Hellman et al., 2004). Furthermore, oxidative DNA damage caused by treatment with cigarette smoke condensate were repaired in HPV-transfected, HPV-positive and HPV-negative human cervical cancer cells with equal efficiency, which may be attributed to p53-dependent and – independent DNA repair pathways (Moktar, *et al.*, unpublished data). We infer from this and other studies, that cigarette smoke exposure can alter the mechanisms of gene expression or their functions, which may, in turn, have a variable effect on DNA integrity. Equally important, we cannot overlook the confounding cumulative effects of polymorphisms in DNA repair genes, the heterogeneous nature of cigarette smoke constituents, and exposure to other environmental factors in evaluating the influence of smoking on HPV-mediated vaginal tumorigenesis.

Our data derived from the *ex-vivo* study clearly showed an inverse relationship between the interaction of cigarette smoking and HPV infection in the induction of DNA strand breaks regardless of age. These findings are congruent with the results of Sellors *et al.*, who also reported that smoking was not a risk factor for HPV infection or persistence (Sellors et al., 2003). On the contrary, in several studies elevated levels of tobacco related carcinogens such as benzo[a]pyrene and nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) were identified in cervical mucosa of cigarette smokers compared to non-smokers (Hellberg et al., 1988; Melikian et al., 1999; Prokopczyk et al., 1997). It is worth noting that the systemic effect of tobacco smoke on vaginal epithelium may not manifest itself by DNA DSBs, the only endpoint investigated in this study. Importantly, the scarcity of literature on the biological effects of cigarette smoke on vaginal epithelium provide us with a challenging and unique prospect to caution ourselves in making a generalized statement from a public health perspective.

In conclusion, we found that HPV infection, cigarette smoking and the interaction between the two have a significant inverse relation with DNA DSBs detected in vaginal epithelium of the population studied. Even though the overall conclusion is restricted by the small sample size, the highly differentiated nature of vaginal squamous cells and lack of persistence of HPV infection may partly explain the negative association between the investigated

parameters. From an etiological point of view, further studies with broader scope and larger sample size are required to elucidate the impact of cigarette smoking as a cofactor in vaginal carcinogenesis.

Acknowledgments

This work was supported from Agnes Brown Duggan Endowment and in part from the NIEHS grant T32-ES011564. Ramesh Gupta is the Agnes Brown Duggan Chair in Oncological Research. The authors thank Betty Royce, a physician assistant at Gynecologic Oncology Clinic at James Graham Brown Cancer Center, and Terry Campbell, a registered nurse at University of Louisville Ob/Gyn Colposcopy Clinic, for their assistance in patient recruitment. The authors thank Dr. Wendy Spencer for reading the manuscript.

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Highlights

- Literature on biological effects of cigarette smoke on vaginal cells is scarce. This work contributes to the limited body of knowledge in understanding the interplay of cigarette smoking and HPV infection in an unprecedented ex vivo study.
- This study suggests that both cigarette smoking and HPV infection have a significant inverse correlation and age has significant positive correlation with DNA double-strand breaks detected in vaginal epithelium of the population investigated.
- The latter finding implies that older women are at higher risk for accumulation of mutation and genetic instability and corroborates the known vaginal cancer risk in elderly women.
- The relevance of these findings to public health in identifying high-risk population is significant.

Figure 1. Representative comet assay images. Panel (A) depicts intact nuclei in a HPV positive individual. Panel (B) shows DNA double-strand breaks in a HPV negative participant. Panels (C) and (D) represent double-strand breaks in DNA of never and ever smokers, respectively.

Figure 2.

Scattered plot presentation of the DSBs in the studied population. Panels represent the distribution of (A) tail length and (B) tail moment, respectively in HPV-positive and –negative individuals. Panels (C) and (D) depict the distribution of tail length and moment among ever- and never-smokers.

Correlation between HPV infections and smoking status presented by scattered plot in the studied population. Panel (E) represents the distribution of tail length (E1) and tail moment (E2) in HPV positive women. Panel (F) depicts the distribution of tail length (F1) and tail moment (F2) in HPV negative individuals, with respect to smoking status.

Table 1

Clinical and General Characteristics of the Studied Population

Characteristics	Numbers (%)
Total	76 (100)
Age	
Mean $\pm$ SD	40.6 $\pm$ 17.1
Median (Min-Max)	36.0 (18.0–80.0)
Race	
White	50
African American	15
Unknown	11
HPV Infection	
Positive	46 (61)
Negative	30 (39)
Smoking Status	
Never	27 (36)
Ever	49 (64)
Pap/Pathology Status	
Normal	30 (39)
LGSIL	16 (21)
HGSIL	30 (39)

Table 2

Comet Assay Measured in the Vaginal Cells of the Subjects

	Numbers (%)	Mean TL $\pm$ SD	95% CI	P Values	Mean TM $\pm$ SD	95% CI	P Values
HPV Infection							
Positive	46 (61)	31.2 $\pm$ 18.7	25.2 – 37.2	<0.001	2.4 $\pm$ 2.8	1.3 – 3.5	<0.001
Negative	30 (39)	61.7 $\pm$ 22.6	54.3 – 69.1		8.7 $\pm$ 4.9	7.3 – 10.0	
Smoking Status							
Never	27 (36)	57.7 $\pm$ 24.5	48.9 – 66.5	<0.001	7.5 $\pm$ 5.5	5.8 – 9.3	0.001
Ever	49 (64)	35.3 $\pm$ 21.9	28.8 – 41.8		3.4 $\pm$ 3.7	2.1 – 4.7	
Age							
36 (%)	40 (52.6)	27.1 $\pm$ 13.9	22.8 – 31.4	<0.001	1.8 $\pm$ 1.6	1.3 – 2.3	<0.001
>36 (%)	36 (47.4)	61.2 $\pm$ 22.5	53.9 – 68.6		8.3 $\pm$ 5.0	6.6 – 9.9	
Age							
Continuous				<0.001			<0.001
Est. $\pm$ SE	1.07 $\pm$ 0.12				0.20 $\pm$ 0.02		

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Table 3
Comet Assay Measured in the Vaginal Cells of the Subjects Stratified for HPV and Smoking Status

	Numbers (%)	Mean TL ± SD	P Value	Mean TM ± SD	P Value
HPV Positive					
Never	14 (18)	36.9 ± 21.9	<0.001	4.7 ± 4.1	<0.001
Ever	32 (42)	23.9 ± 11.3		1.4 ± 1.0	
HPV Negative					
Never	13 (17)	68.1 ± 23.6	0.258	10.7 ± 5.4	0.079
Ever	17 (22)	56.8 ± 21.1		7.2 ± 4.0	

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Table 4

Generalized Linear Regression Model on Age, Smoking and HPV Status

Dependent	Intercept		HPV Positive		Ever Smoking		
	Est.	P Value	Est.	P Value	Est.	P Value	
Age as Categorical	TL	59.56	<0.001	-13.015	0.019	-15.318	<0.001
	TM	7.2	<0.001	-3.419	0.002	-2.852	<0.001
Age as Continuous	TL	16.93	0.0155	-11.312	0.034	-12.582	0.003
	TM	0.61	0.644	-3.298	0.002	-2.468	0.003