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Expression of human papillomavirus E6 and E7 oncoprotein mRNA in women with low-grade squamous intraepithelial lesions or less

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ABSTRACT. We verified the prevalence of human papillomavirus (HPV) E6/E7 protein mRNA expression in patients with low-grade squamous intraepithelial lesions (LSILs) and negative cervicovaginal cytology. To investigate the relationship between mRNA expression and viral infection type, we assessed genotyping in single infections. Samples from 825 women were submitted to the E6/E7 survey. We noticed a larger percentage of E6/E7 mRNA expression in the atypical squamous cells of undetermined significance (ASC-US) and LSIL cytologies. Negative results of mRNA expression were in accordance with negative cytologies. In positive cases, the infection by a single HPV type was most common, with type 16 being most prevalent. The expression of mRNA was most prevalent in ASC-US and LSIL cytologies, compared with the negative cytology. The infection by a HPV type was more frequent in cases of positive expression, with HPV

type 16 being found most frequently. Patients with LSIL cytologies had a higher percentage of multiple infections.

Key words: E6/E7 mRNA; Human papillomavirus; Negative cytology; ASC-US cytology; LSIL cytology

INTRODUCTION

Cervical cancer is the second most common type of neoplasia in women throughout the world and the third cause of mortality by cancer in women, exceeded only by breast and colon tumors. In developing countries, it is one of the most frequent types of carcinoma and can account for over 25% of all female cancers. In Brazil, this cancer ranks third in frequency of the most common types of cancers in women (WHO, 2009; INCA, 2014).

It is believed that the human papillomavirus (HPV) is the cause of pre-malignant and malignant lesions in the cervix, and is recognized by researchers and epidemiologists as the agent of one of the most common sexually transmissible diseases, in both men and women. Such a relationship was first demonstrated by Harald zurHausen, in the early 1980s, work for which he won the Nobel Prize in Physiology or Medicine in 2008 (zur Hausen, 2002; Narisawa-Saito and Kiyono, 2007).

Almost half of women of reproductive age and nearly 80% of teenagers and young adults below 30 are infected by HPV at some point. Most of the infections caused by HPV are of benign evolution and 80-90% is temporary, being eliminated spontaneously by the host organism within 10-18 months; persistence is liable to occur in only 20% of cases, leading to the onset of early lesions and cervical cancer (Kjaer et al., 2002; Plummer et al., 2007; Smith et al., 2007; Winer et al., 2011; Panatto et al., 2012).

Some co-factors can modify risk, including: smoking, multiple sexual partners, multiparity, early sex initiation, the use of oral contraceptives for five years or more, co-infection by such other infectious agents as the human immunodeficiency virus (HIV), *Chlamydia trachomatis*, herpes simplex virus type 2 (HSV-2) and acquired immunosuppression following organ transplantation (Bouvard et al., 2009).

A crucial aspect of understanding this cancer has been the elucidation of the natural history of infections caused by HPV. This has led to the conclusion that the persistence of high-oncogenic risk genotypes is the main risk factor for the development of high-grade intraepithelial neoplasia in cervical cancer. Other factors determine the viral load progression per cell unit and the integration of virus DNA into the cell (Castellsagué, 2008; Handisurya et al., 2009; Muñoz et al., 2009).

Infection by high-oncogenic risk HPV is a necessary but is not a sufficient condition for the progression of the invasive disease. The persistence of infection for an extended period of time, changes infected cells, leading to cervical intraepithelial neoplasia and the loss of viral transcriptional regulation, with mutations, instability and chromosomal reconnections, that are indispensable factors for cervical cancer (zur Hausen, 2002; Lizano et al., 2009; Moody and Laimins, 2010).

Oncogenes E6 and E7 are important for the coordination of transcription and viral replication, and are also essential for malignant transformation (Passos et al., 2008; Villa and Sichero, 2008; Paralta-Zaragoza et al., 2012).

Once the disease-causing agent and the importance of the immunological mechanisms involved had been characterized, molecular biology techniques were developed to aid the

screening, diagnosis, and treatment of cervical cancer.

A key step in current screening methods is to identity HPV-positive women, with higher risk of developing high-grade lesions and the subsequent invasive cancer. In this respect, the detection of oncogenes E6 and E7 allows easier distinction between the temporary the HPV-infection and active infection that has the potential to evolve into cervical cancer (Martin and O'Leary, 2011).

E6/E7 transcriptions indicate the expression of those oncogenes that start the process of cervical carcinogenesis. In other words, the quantification of E6 and E7 mRNA expression can be used to evaluate the transforming capacity of the present HPV, implying the immortal proliferation and mutation of the host squamous epithelial cells.

The overexpression of the E6/E7 oncogenes can be used as marker for the transition from productive to abortive infection, which eventually promotes cell transformation. That marker might be used to reduce the number of women referred to a more careful monitoring and follow-up regime, raising the specificity of assessment in women with atypical squamous cells of undetermined significance (ASC-US) and low-grade squamous intraepithelial lesions (LSILs) (Varnai et al., 2008; Keegan et al., 2009; Brown and Trimble, 2012; Tornesello et al., 2013).

This method has shown better positive predictive and prognostic results for the stratification of risk and the ability to indicate the progression of high-risk intraepithelial lesions and invasive cancer (Lie et al., 2005; Molden et al., 2006; Varnai et al., 2008; Jeantet et al., 2009).

The first objective of this study was to assess the expression of E6/E7 oncogene mRNA of high-oncogenic risk HPV from cervicovaginal cytopathological examinations of women, with results within the limits of normality, ASC-US and LSIL.

The second objective was to investigate the relationship between the E6 and E7 oncoproteins mRNA expression of high-risk HPV and viral infections of single or multiple types of HPV, as well as with the genotyping variation in single infections.

MATERIAL AND METHODS

The medical records of 825 women were examined, for the years to 2009 and 2010, and the mRNA E6/E7 oncoprotein examinations and cervicovaginal oncotic cytology data were collected in a private laboratory, Salomão & Zoppi Diagnósticos Laboratory. That laboratory is considered a standard for the diagnosis of HPV-induced lesions. The patients referred for the oncoprotein examinations presented a history of HPV infection, with the real clinical diagnosis of the case being unknown.

The retrospective observational study comprised a data survey by means of the SILAB computer-based system for mRNA E6/E7 data collection, through the biomolecular test for E6/E7 oncogene mRNA, using NucliSENSEasyQ® HPV technology, and a DNA-HPV survey by means of real-time polymerase chain reaction (PCR).

The criteria for inclusion were: women with cytological findings within the limits of normality (negative), for ASC-US and LSIL.

Women with cytological abnormalities exceeding low-grade lesions were not included in the study.

The study was approved by the Ethics in Research Committee of HSP-UNIFESP and the Ethics Committee of Salomão & Zoppi Diagnósticos Laboratory. All patients signed a Deed of Free and Informed Consent and were given the standard information provided by the laboratory.

RESULTS

We analyzed the results of 825 examinations of women aged 16 to 73 years, submitted to the E6/E7 oncogene mRNA test, for high-oncogenic risk associated with cervicovaginal cytology in a private laboratory.

The results of the cytopathological examinations were: negative (within the limits of normality) in 478 (57.9%) cases, ASC-US in 258 (31.3%) cases and LISL in 89 (10.8%) cases. In respect of E6/E7 oncogene mRNA expression of high-risk HPV, 203 cases (24.6%) were positive and there was no expression in 622 cases (75.4%).

The prevalence of high-oncogenic risk HPV E6/E7 oncogene mRNA expression in the three cytological types is presented in Table 1.

Table 1. Prevalence of high-oncogenic risk HPV E6/E7 oncogene mRNA expression in patients with negative, ASC-US and LSIL cytologies.

Cytology	E6/E7 mRNA		
	Negative	Positive	Total
Negative	413 (86.4%)	65 (13.6%)	478 (100.0%)
ASC-US	158 (61.2%)	100 (38.8%)	258 (100.0%)
LSIL	51 (57.3%)	38 (42.7%)	89 (100.0%)
Total	622 (75.4%)	203 (24.6%)	825 (100.0%)

HPV = human papillomavirus; ASC-US = atypical squamous cells of undetermined significance; LSIL = low-grade intraepithelial lesion; E6/E7 mRNA = expression of E6/E7 oncogene mRNA. Pearson chi-square, $P < 0.001$. ASC-US *vs* LSIL. $P = 0.513$.

The results showed that there was an association between E6/E7 oncogene mRNA expression and cytology. There was a higher percentage of positive E6/E7 mRNA expression in the ASC-US and LSIL cytologies than in the negative cytology ($P < 0.001$). No statistically significant difference was observed between the ASC-US and LSIL cytologies ($P = 0.513$).

In the cases of positive E6/E7 oncogene mRNA expression, we investigated the prevalence of single or multiple infections of HPV genotypes in the three cytological forms presented.

In 5 of the 203 positive cases of E6/E7 mRNA expression, HPV genotyping could not be performed owing to technical problems, so only 198 cases were recorded (Table 2).

Table 2. Investigation of the presence of single or multiple infections in patients with positive E6/E7 oncogene mRNA expression and negative, ASC-US or LSIL cytologies.

Cytology	HPV genotype		
	Single	Multiple	Total
Negative	57 (89.1%)	7 (10.9%)	64 (100%)
ASC-US	87 (87.9%)	12 (12.1%)	99 (100%)
LSIL	24 (68.6%)	11 (31.4%)	35 (100%)
Total	168 (84.8%)	30 (15.2%)	198 (100%)

ASC-US = atypical squamous cells with undetermined significance; LSIL = low-grade intraepithelial lesion; HPV = human papillomavirus. Pearson chi-square, $P < 0.001$; multiple LSIL infection *vs* negative or ASCUS. $P = 0.012$.

For the positive cases of E6/E7 mRNA expression, infection by a single type of HPV was more prevalent than infection by multiple types.

The type of HPV infection was associated with cytology, there was a higher percentage of multiple infection in the LSIL (31.4%) cytology, compared with the negative and ASC-US cytologies.

There was a higher percentage of single infection in the negative and ASC-US cytologies, compared with the LSIL cytologies.

HPV genotyping in positive cases of E6/E7 mRNA expression for patients with a single infection was analyzed.

HPV types 16, 18, 31, 33 and 45 occurred with similar frequency in the three cytology types: there was a higher frequency of HPV genotype 16, followed by genotype 33, in the altered cytologies, but without statistical significance (Table 3).

Table 3. HPV genotyping in patients with positive E6/E7 oncogene mRNA expression single infections and negative, ASC-U.

	HPV types					
Cytology	16	18	31	33	45	Total
Negative	20 (35.1%)	9 (15.8%)	5 (8.8%)	8 (14.0%)	15 (26.3%)	57 (100%)
ASC-US	35 (40.2%)	9 (10.3%)	13 (15%)	19 (21.8%)	11 (12.6%)	87 (100%)
LSIL	10 (41.6%)	2 (8.3%)	3 (12.5%)	7 (29.2%)	2 (8.3%)	24 (100%)
Total	65 (38.7%)	20 (12.0%)	21 (12.5%)	34 (20.2%)	28 (16.6%)	168 (100%)

S or LSIL cytologies. ASC-US = atypical squamous cells with undetermined significance; LSIL = low-grade intraepithelial lesion; HPV = human papillomavirus. Pearson chi-square, P = 0.289.

DISCUSSION

In Brazil, the screening program for cervical cancer is based on an oncotic cytology examination using the Papanicolaou technique. The program is started at age 25 and is run at annual intervals; assuming the results lie within the limits of normality for two consecutive years, it is recommended that the screening procedure be carried out every 3 years.

Sankaranayanan et al. (2008) commented that the oncotic cytology examination is questionable because its accuracy varies from 31 to 78%, thereby leaving a large percentage of women at the margin of diagnosis and with a higher risk of progression to pre-neoplasic lesions.

The molecular detection of HPV provides evidence of the infection and Rijkaart et al. (2012) demonstrated the risk of cervical intraepithelial neoplasia grade 2 or higher (≥CIN2) in 55% of women with positive expression of E6/E7 mRNA, HPV DNA with high positive oncogenic risk and normal oncotic cytologies, compared with 20% in those with negative test results. The authors concluded that the test could aid in the selection of women with high-oncogenic risk HPV for prompt referral or a colposcopic examination.

Our study analyzed retrospectively 825 women, to assess the prevalence of E6/E7 mRNA expression in patients with a history of HPV infection. The negative cytology finding was observed in 478 (57.9%) cases, ASCUS cytology was found in 258 (31.3%) cases and LSIL was found in 89 (10,8%) cases. E6/E7 mRNA expression was found in 24.6% of the total, with 13.6% in women with negative cytologies, 38.8% in those with ASC-US cytology and 42.7% in patients with LSIL.

The prevalence statistics were a little below those from a study by Perez Castro et al. (2013); a prospective analysis revealed the corresponding results from their analysis to be 37.8, 55.7 and 77.5%. A follow-up investigation revealed that nearly 26% of the women with ASC-US or LSIL cytologies in that study had moderate/severe intraepithelial neoplasia.

The authors therefore concluded that the E6/E7 mRNA test could be used for a screening of patients with negative and ASC-US cytologies, which would be positive for HPV 16 and/or 18, detected by a DNA test, owing to the test's high sensibility and specificity for the detection of moderate/severe intraepithelial neoplasia.

Our study did not perform a follow-up of the assessed women, but a prevalence of approximately 40% of E6/E7 mRNA expression was noticed in the ASC-US and LSIL cytologies, which qualifies it as a factor for stratification of patients under risk, and therefore thus deserving of stricter surveillance.

A significant association between the ASC-US and LSIL cytologies and the positive expression of E6/E7 mRNA was also noticed, in comparison with negative cytologies. This demonstrates that cellular alterations, even to minimal extent, can indicate the oncogenic activity of the virus, identifying the cases deserving of more care.

Molden et al. (2005 and 2006) demonstrated that women with ASC-US and LSIL cytologies had almost 70 times more chance of developing CIN 2 within two years of follow-up if the results of the test were positive in comparison with negative results. In this way, the researchers indicated that the E6/E7 mRNA test for HPV types 16, 18, 31, 33 and 45 is a promising oncotic cytology technique for identifying the risk of progression in patients with ASC-US and LSIL alterations.

Other authors, such as Sørbye et al. (2010, 2011 and 2013) have also suggested that the E6/E7 mRNA test could improve early diagnosis and lead to a better prediction in patients with lower cytological alterations, negative biopsies, and intraepithelial grade 1 tumors.

Our study, however, excluded women with cytological examinations results compatible with high-grade neoplasia, failed to assess the sensitivity or specificity of the test, and did not compare diagnostic methods, since these were not its objectives.

When the type of infection was analyzed in respect of the number of HPV types involved in positive cases of oncoproteins expression, a higher prevalence was seen for infections by a single HPV type, particularly in negative and ASC-US cytologies, compared with the LSIL cytology.

A higher prevalence by multiple viral types (31,4%) was found in patients with LSIL. 12.1% of cases had ASC-US and 10.9% had a negative cytology. Such findings agree with the results from studies by Gargiulo et al. (2007), Brismar-Wendel et al. (2009), Carozzi et al. (2010), and Correnti et al. (2011).

When the HPV type prevalence of single-type infections with expression of E6/E7 mRNA was assessed, our study demonstrated that type 16 was the most common, while there was no statistically significant difference in the other types. Such a predominance of HPV 16 with positive expression of oncogenes confirms its more aggressive behavior in respect of oncogenesis, a finding that is corroborated by most of the relevant publications.

Several meta-analyses demonstrate that HPV 16 is the most common viral type in low- and high-grade infections. Types 16 and 18 are associated with a higher probability of progression to malignancy, even in patients with small cytological alterations (ASC-US and LSIL), who exhibit E6/E7 mRNA expression (Clifford et al., 2005; Brismar-Wendel et al., 2009; Lizano et al., 2009; Muñoz et al., 2009; Sjoeborg et al., 2010; Correnti et al., 2011).

Also in respect of HPV types, our study found that HPV 33 was the second most common type in ASC-US and LSIL cytologies, in women with a single infection expressing E6/E7 mRNA and type 45 was the second most common in negative cytologies. This agrees with the results reported by Keegan et al. (2009) and Sørbye et al. (2011).

No great prevalence of HPV 18 was noticed in any of the three cytologies. It is known that this type of HPV is more closely related to glandular alterations and adenocarcinoma, which are difficult to diagnose through oncotic cytology alone, and alterations in glandular cells were not investigated in this study.

According to the findings by Khan et al. (2005), early positive infections by HPV types 16 and 18 can be misleading and deserve follow-up, owing to the higher chance of persistence

by these viral types. The authors found a cumulative risk of 17.3 and 13.6%, respectively, of the development of intraepithelial neoplasia grade 3 or higher (≥CIN3), even with a negative cytology, over 10 years, compared with patients infected with other high-oncogenic risk HPV types and also by other non-oncogenic viral types.

Our study allows us to infer the stratification of patients with positive expression of E6/E7 mRNA and ASC-US and LSIL cytologies are under risk of progression. As such, they deserve a strict follow-up program, for the possible detection of early lesions, since the persistence and integration of high-risk viruses are factors of risk for the development of cervical cancer.

The use of E6/E7 oncogenes of high-oncogenic risk HPV as markers of persistent and productive infection by HPV in patients with smaller cytological alterations is an improvement on existing markers. E6/E7 oncogenes can serve as better markers for the assessment and monitoring of HPV DNA-positive patients and could perhaps be used to predict the risk of high-grade intraepithelial neoplasia and cervical cancer.

However, with the current information from official publications, it is not possible to confirm such an assertion, because studies assessing the cost-benefit ratio and real clinical relevance of such tests still need to be performed, so as to verify their applicability (Salimović-Bešić et al., 2013).

Other studies are required to assess this methodology, as well as studies that comprise a larger number of patients and analyze population screening to determine whether the E6/E7 mRNA tests would be useful and feasible in daily clinical practice.

In view of the achieved results, we conclude that: high-oncogenic risk HPV E6/E7oncogene mRNA expression was more prevalent in the ASC-US and LSIL cytologies, compared with the negative cytology. Infection by a single HPV type was more prevalent in cases of positive expression of E6/E7 oncogene mRNA.

In such cases, a higher percentage of multiple infections in LSIL cytologies was observed; a higher number of single infections were evidenced, particularly by HPV 16, in the negative and ASC-US cytologies, followed by type 33 in all the cytopathological variations studied.

Conflicts of interest

The authors declare no conflict of interest.

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