

# Clinical characteristics of women diagnosed with carcinoma who tested positive for cervical and anal high-risk human papillomavirus DNA and E6 RNA

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Received: 25 August 2014 / Accepted: 3 February 2015  
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**Abstract** High-risk human papillomavirus (hrHPV) is an essential cause of cervical carcinoma and is also strongly related to anal cancer development. The hrHPV E6 oncoprotein plays a major role in carcinogenesis. We aimed to evaluate the frequency of hrHPV DNA and E6 oncoprotein in the anuses of women with cervical carcinoma. We analyzed 117 women with cervical cancer and 103 controls for hrHPV and the E6 oncogene. Pos-

itive test results for a cervical carcinoma included 66.7 % with hrHPV-16 and 7.7 % with hrHPV-18. One case tested positive for both HPV variants (0.9 %). The samples from the anal canal were positive for HPV-16 in 59.8 % of the cases. Simultaneous presence of HPV in the cervix and anal canal was found in 53.8 % of the cases. Regarding expression of E6 RNA, positivity for HPV-16 in the anal canal was found in 21.2 % of the cases, positivity for HPV-16 in the cervix was found in 75.0 %, and positivity for HPV-18 in the cervix was found in 1.9 %. E6 expression in both the cervix and anal canal was found in 19.2 % of the cases. In the controls, 1 % tested positive for HPV-16 and 0 % for HPV-18. Anal samples from the controls showed a hrHPV frequency of 4.9 % (only HPV16). The presence of hrHPV in the anal canal of women with cervical cancer was detected at a high frequency. We also detected E6 RNA expression in the anal canal of women with cervical cancer, suggesting that these women are at risk for anal hrHPV infection.

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**Keywords** HPV · Cervical cancer · Anus · HPV E6 · HPV DNA · HPV RNA

## Background

Persistent infection with high-risk human papillomavirus (hrHPV) is strongly associated with the development of gynecological and non-gynecological cancers [1, 2]. Cervical cancer has been studied extensively, and it has been established that hrHPV infection is a significant cause of cervical carcinogenesis [3]. Current HPV testing, vaccinations, and cytology examination are expected to enhance the quality of cervical cancer prevention programs [4]. Well-conducted screening programs are recognized as efficient instruments

for preventing cervical cancer, but the efficacy of these initiatives varies based on the public health systems in place in different countries. Socioeconomic factors, including developed medical and social infrastructures, accessible laboratory facilities, and professional human resources, play a significant role in the effectiveness of screening efforts. Unfavorable conditions in poor and developing countries make screening challenging, and HPV vaccination may represent an alternative approach to effectively reduce the burden of HPV-related cancer [5]. Concomitant hrHPV infections in the uterine cervix and anal canal are associated with different risk factors that can increase the risk of HPV-induced cancer development; importantly, high incidences of anal hrHPV infection have been associated with cervical lesions [6]. Although anal hrHPV infection is a source for hrHPV cervical re-infection, there are notable biases [7]. Due to the numerous discrepancies and differences in variables assessed in previous studies, it is difficult to determine the true cause of anal and cervical concomitant HPV infection. In males, this association is remarkably high in men who have sex with men and persistent in patients infected with the human immunodeficiency virus. The likely auto-contamination and differences in HPV assays also interfere with establishing the cause of simultaneous HPV infection of the cervix and anus [7].

We investigated the prevalence of HPV in the anal canal in a small group of women with grade 3 biopsy-proven cervical intraepithelial neoplasia (CIN3). We compared the HPV frequency, using Hybrid Capture2™ technology, among 40 women with CIN3 and 40 women with normal results from colposcopic examinations and Pap tests. Of the women with CIN3, 39 (97.5 %) tested positive for HPV in the uterine cervix, and 14 (35 %) tested positive in the anal canal; interestingly, four women in the control group (10 %) tested positive for HPV in both the cervix and the anus. Despite the small sample size, the prevalence of HPV in the anal canal was significantly greater in women with CIN3 than in controls [8].

## Objectives

In this context, the current study sought to evaluate the frequency of hrHPV DNA and E6 oncoprotein expression in a larger, biopsy-proven group of women with carcinomas of the cervix and determine whether there was any correlation with the presence or absence of hrHPV in anal samples.

## Materials and methods

A cross-sectional study was conducted in women with biopsy-proven carcinoma of the cervix who were outpatients at the gynecology department, and the control group comprised

women recruited from the Prevention Department of Foundation Pius XII (Cancer Hospital of Barretos—SP) between February 2010 and February 2012. Eligible subjects were females aged 18 years or older. All participants provided informed consent, and the ethics committee approved the study.

We analyzed the following two groups of women: the cases, which comprised 117 women with cervical tumors, and the controls, which comprised 103 women without gynecological abnormalities or colposcopic or cytological alterations. We collected biopsies from the cervix and brushed cytological samples from the cervical and anal canals from the cases to test for hrHPV DNA and expression of the HPV E6 oncoprotein. We also obtained brushed samples from the cervical and anal canals of the controls but did not perform biopsies. All brushed materials were stored at  $-20$  and  $-80$  °C for the subsequent extraction of DNA and RNA, respectively. Both groups underwent blood sampling to test for the presence of HIV-1 and HIV-2.

### Cervical sample collection

With the women in a gynecological position, and after obtaining direct visualization of the cervix, a cytobrush device was introduced (Rover, Netherlands) 1.0 to 1.5 cm into the endocervical canal (when visible) until the largest bristles touched the ectocervical region. The brush was then turned clockwise five times. When the endocervical canal was not accessible, samples were collected from the surface of the tumor. The samples were immediately placed in a preservative solution.

### Anal canal sample collection

To collect material from the anal canal, the women remained in the same position, the examiner's gloves were changed, and a different brush was introduced into the anal canal. This brush was placed 3.0 cm anterior to the year-cutaneous line, to ensure the dentate line had been reached. The brush was turned clockwise five times, and the samples were immediately placed in a preservative solution.

The cell samples were subsequently placed into a tube containing Tris-EDTA buffer (TE, 10 mM Tris-HCl and 1 mM EDTA, pH 7.4) and centrifuged at 1000 rpm for 5 min. The cell pellet was resuspended in 500  $\mu$ L TE and divided into two aliquots of 250  $\mu$ L placed in 1.5-mL DNase/RNase-free microfuge tubes (Axygen, Union City, CA, USA). One aliquot was stored at  $-80$  °C and the other at  $-20$  °C to preserve the samples for RNA and DNA extraction, respectively.

### DNA extraction

DNA was extracted from fresh samples and from samples preserved in an in-house buffered methanol-based

solution material using a QIAamp DNA Mini Kit and QIAamp DNA Micro Kit (Qiagen, Biotecnologia do Brasil Ltda), respectively, according to the manufacturer's instructions. The DNA elution volume at the end of the extraction process was 200  $\mu$ L.

#### E7 HPV-16/HPV-18 type-specific real-time PCR

The samples were submitted to a type-specific TaqMan-based real-time qPCR assay targeting HPV16/HPV18 E7. The qPCR assays were performed in an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). All samples and controls were run in duplicate. The HPV16 E7 qPCR assays included the following oligonucleotide primers: forward (5'GATGAAATAGATGGTCCAGC3') and reverse (5'GCTTTGTACGCACAACCGAAGC3') primers, [9] and a probe (5'FAM-CAAGCAGAACC GGACAG-MGB-NFQ). In a final reaction volume of 25  $\mu$ L, each qPCR reaction contained 1X TaqMan master mix (Applied Biosystems, Foster City, CA), 400 nM each of the forward and reverse primers, 200 nM of fluorogenic TaqMan probe, and 5  $\mu$ L of extracted DNA. The amplification conditions consisted of 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 1 min, and 60 °C for 1 min. Each qPCR run included the following controls: (i) SiHa cell line DNA (harboring 1–2 copies/cell of HPV16) and (ii) water as a negative control. The HPV18-E7 qPCR assays included the following oligonucleotide primers: forward (5'AAGAAAACGATGAAATAGATGGA3') and reverse (5'GGCTTCCACCTTACAAACA3') primers, [9] and a probe (5'VIC-AATCATCAACATTTACCAGCC-MGBNFQ3'). In a final reaction volume of 25  $\mu$ L, each qPCR contained 1X TaqMan master mix (Applied Biosystems, Foster City, CA), 400 nM each of the forward and reverse primers, 400 nM fluorogenic TaqMan probe, and 5  $\mu$ L extracted DNA. The amplification conditions consisted of 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 50 °C for 1 min, and 60 °C for 1 min. The qPCR was performed using an ABI 7300 machine, and each run included the following controls: (i) HeLa cell line DNA (harboring 20 copies/cell of HPV18) and (ii) water as a negative control.

#### General standards for HPV identification

Real-time qPCR was standardized to detect HPV copies per milliliter of plasma. To determine the analytical sensitivity, six replicates for each point of the standard curve were subjected to the standard assay. For construction of the standard curve, DNA extracted from Caski and HeLa cells was used. To construct the standard curve for HPV-16, DNA extracted

from Caski cells was used at a concentration of 50 ng. Initially, 10  $\mu$ L of DNA extracted from Caski cells were added to 1 mL of negative plasma. Given that one diploid cell has 6.6 pg of DNA, and cells in the Caski cell line have 600 copies of HPV-16 per cell, we know that  $4.5 \times 10^7$  copies of HPV-16 were added in 1 mL of negative plasma. Subsequently, serial dilutions were made up to a concentration of  $4.5 \times 10^1$  HPV-16 copies. To construct the standard curve for detection of HPV-18 reactions, the same procedure was adopted. Initially, 10  $\mu$ L of the DNA extracted from HeLa cells at a concentration of 30 ng/ $\mu$ L were added to 1 mL of negative plasma. Given that one diploid cell has 6.6 pg of DNA and cells in the HeLa cell line have 20 copies of HPV-18 per cell, we know that  $9.1 \times 10^5$  copies of HPV-18 were added in 1 mL of negative plasma. Subsequently, serial dilutions were made up to a concentration of  $9.1 \times 10^1$  HPV-18 copies. To ascertain the analytical specificity of the PCR reaction using standard real-time methodology, we subjected the DNA of the tumor samples that tested positive for different types of HPV (16, 18, 31, 33, 35, 45, 58, 39, 73, 68, 6, 11, 42, 44, and 51) to specific reactions for HPV-16 and HPV-18. The alignment of the primers and probes of the E7 region of HPV 16 sequences was carried out for different types of HPV, obtained from GenbankGenBank, to confirm the presence of mismatches, and all had at least one or more mismatches. We also performed a second test to evaluate real-time qPCR specificity using 20 plasma samples from healthy pregnant women in whom detection of the presence of viral DNA was not expected. All 20 samples tested negative for both HPV 16 and HPV 18.

#### Human $\beta$ -globin PCR

$\beta$ -globin PCR was performed as previously described in the literature [10].

#### RNA analysis

RNA assays were performed on the samples preserved with a buffered methanol-based solution prepared in our lab. RNA extraction was performed using the RNeasy Mini Kit (Qiagen, JP) according to the manufacturer's protocol. The samples were initially found to have low levels of RNA; therefore, a TRIzol extraction was performed to obtain higher levels of RNA. However, the RNA quantity remained low and we opted to extract RNA from all samples with TRIzol. QuantiTec Whole Transcriptome Kits (Qiagen, JP) were used to increase the levels of RNA for conversion into cDNA. After this process, real-time PCR was performed using plates for the device, the E6 primers, and Platinum SYBR Green (Invitrogen).

## Ethics

All women enrolled in this study signed an informed consent form. The study protocol was approved by the Barretos Cancer Hospital Ethics Committee.

## Statistical analysis

Data were analyzed using SPSS for Windows® version 20.0 (Inc., Chicago, IL, USA). A Student's *t* test was used to compare the means of quantitative variables between the two groups. To analyze the association between groups and qualitative variables in the study, we used a Fisher's exact test. To evaluate the association between related groups (more than one measurement per patient), we used the McNemar test. We adopted the significance level of 5 % for all analyses.

## Results

A total of 117 women with cervical tumors and 103 women without cervical lesions were enrolled in this study. The detailed characteristics of the control and case groups

are listed in Table 1. Anal intercourse, tobacco consumption, and alcohol consumption were not significantly different between the groups. However, the control and case groups showed significant differences in skin color ( $<0.001$ ), education level ( $<0.001$ ), and marital status ( $<0.003$ ). In addition, the mean age was higher in the cases than the controls (Table 2). The age at first sexual intercourse, age at menarche, and number of sexual partners were not significantly different between the two groups. However, the number of births was greater in the cases ( $<0.001$ ).

Table 3 shows the significant differences in the frequency of hrHPV detection in the control and case groups, according to cervical and anal regions. The rates of positive hrHPV tests in the anal canal were significantly different between groups ( $<0.001$ ); HPV16 was significantly more prevalent in the anal canal samples of women diagnosed with cervical cancer when compared to controls ( $<0.001$ ). Two cases of HPV18 were detected in the anal canals of women with cervical cancer ( $p=0.500$ ).

Regarding the uterine cervix tests, hrHPV was detected in only 1 % of the women in the control group, and this rate was significantly higher in the cases (75.3 %;  $p<0.001$ ). HPV16 was more prevalent in women with cervical cancer ( $<0.001$ ),

**Table 1** The number and percentage of patients included in the study, according to social and cultural variables

Variable	Category	Control group		Case group		<i>p</i> value <sup>a</sup>
		<i>N</i>	(%)	<i>N</i>	(%)	
Skin pigmentation	White	37	(35.9)	77	(66.4)	<0.001
	Non-White	66	(64.1)	39	(33.6)	
Education level	Illiterate	1	(1.0)	15	(12.9)	<0.001
	Basic	46	(44.7)	73	(62.6)	
	High school	43	(41.7)	22	(19.0)	
	University	13	(12.6)	6	(5.2)	
Marital status	Single	23	(22.3)	12	(10.4)	0.003
	Married/stable union	70	(68.0)	72	(62.6)	
	Divorced	6	(5.8)	15	(13.0)	
	Widowed	4	(3.9)	16	(13.9)	
Oral contraceptive use (current)	No	85	(82.5)	109	(94.7)	0.005
	Yes	18	(17.5)	6	(5.3)	
Tobacco (current)	No	88	(85.4)	97	(84.3)	0.852
	Yes	15	(14.6)	18	(15.7)	
Alcoholism (current)	No	96	(93.2)	108	(95.6)	0.558
	Yes	7	(6.8)	5	(4.4)	
Anal intercourse	No	97	(94.2)	102	(89.5)	0.229
	Yes	6	(5.8)	12	(10.5)	
Histology	(Squamous) In situ	–	–	3	(2.6)	NA
	Invasive, SCC	–	–	97	(82.9)	
	Invasive, AdenoCA	–	–	17	(14.5)	

NA not applicable

<sup>a</sup> Fisher exact test

**Table 2** The mean values (and standard deviations) of variables related to the women's age, reproductive history, and age at the first sexual intercourse according to the presence or absence of cervical cancer

Variable		Control group		Case group		<i>p</i> value <sup>a</sup>
Age (years)	<i>N</i>	103		117		<0.001
	Mean (sd)	44.8	(11.2)	53.1	(15.8)	
Age at first sexual intercourse (age)	<i>N</i>	101		114		0.162
	Mean (sd)	18.6	(4.7)	17.8	(3.8)	
Menarche (years)	<i>N</i>	101		115		0.489
	Mean (sd)	13.1	(1.6)	12.9	(1.8)	
Number of sexual partners	<i>N</i>	101		114		0.113
	Mean (sd)	1.9	(2.3)	2.4	(2.7)	
Number of births	<i>N</i>	102		115		<0.001
	Mean (sd)	2.5	(1.9)	4.1	(2.8)	

*sd* standard deviation

<sup>a</sup> Student's *t* test

with positive test results in almost 67 % of cases. HPV18 tests were positive in nine women in the case group (7.7 %), whereas only one case of HPV16 was found in women from the control group. Simultaneous HPV16 and HPV18 infection was found in the anal canal and cervix in one woman in the case group. Finally, positive hrHPV tests were found in both

the uterine cervix and anal canal samples of 63 (53.8 %) women with cervical carcinoma but no positive results were found in the control samples (<0.001).

E6 RNA analysis for HPV16 and HPV18 was performed exclusively in samples from women with cervical cancer, and material for analysis was available in 52

**Table 3** The number and percentage of women according to HPV status, the expression of E6 oncoprotein, and the presence or absence of a cervical tumor

Variable	Category	Control group		Case group		<i>p</i> value <sup>a</sup>
		<i>N</i>	(%)	<i>N</i>	(%)	
HPV—anal canal	Negative	98	(95.1)	46	(39.3)	<0.001
	HPV16	5	(4.9)	69	(59.8)	
	HPV18	0	(0.0)	1	(0.9)	
	HPV16+HPV18	0	(0.0)	1	(0.9)	
HPV—uterine cervix	Negative	101	(99.0)	29	(24.8)	<0.001
	HPV16	1	(1.0)	78	(66.7)	
	HPV18	0	(0.0)	9	(7.7)	
	HPV16+HPV18	0	(0.0)	1	(0.9)	
HPV simultaneously in the uterine cervix and anal canal	No	103	(100.0)	54	(46.2)	<0.001
	Yes	0	(0.0)	63	(53.8)	
E6 (HPV16)—canal anal	Negative	—	—	41	(78.8)	NA
	Positive	—	—	11	(21.2)	
E6 (HPV18)—canal anal	Negative	—	—	52	(100.0)	NA
	Positive	—	—	0	(0.0)	
E6 (HPV16)—cervix	Negative	—	—	13	(25.0)	NA
	Positive	—	—	39	(75.0)	
E6 (HPV18)—cervix	Negative	—	—	51	(98.1)	NA
	Positive	—	—	1	(1.9)	
E6 in cervix and anal canal simultaneously	No	—	—	42	(80.8)	NA
	Yes	—	—	10	(19.2)	

NA not applicable

<sup>a</sup> Fisher exact test



of these cases (adequate RNA quality and concentration). Analysis of the anal canal samples resulted in identification of 11 positive cases (21.2 %) of HPV16 E6 RNA and no positive cases of HPV18 E6 RNA. Analysis of the uterine cervix samples indicated a positive result for HPV16 E6 RNA in 39 (75 %) cases and a positive result for HPV18 E6 RNA in one (1.9 %) case. Simultaneous detection of hrHPV RNA E6 in both the cervical and anal samples was observed in 10 cases (19.2 %).

Table 4 shows that women without cervical cancer demonstrated similar rates of HPV in the uterine cervix and anal canal ( $p=0.219$ ). However, women with cervical lesions showed a higher frequency of hrHPV DNA in both the anal and cervical regions compared to the negative cases ( $p=0.005$ ). The hrHPV E6 RNA was expressed in the cervix and anal canal of the women with cervical tumors and was more frequent in the cervix ( $p<0.001$ ).

## Discussion

We found that a normal anal canal region may act as a reservoir for hrHPV infection in women with cervical carcinoma, as 54 % of women with HPV-induced cervical tumors demonstrated concomitant positive DNA-HPV tests. In contrast, a very low frequency of hrHPV was found in the anal canals of women without cervical lesions, which is consistent with previously published reports on women with cervical high-grade lesions [8]. Importantly, HPV infection was shown to be more common in HIV-infected women than uninfected women [11], although all women tested negative for HIV in our study. Positive tests for hrHPV were significantly associated with cervical cancer, which is consistent with the alleged reservoir role of the anal canal for hrHPV infection. Anal investigation of hrHPV in patients with cervical cancer has been adjudicated because of the augmented risk of HPV-induced lesion

recurrence. As mentioned previously, the anal canal may serve as a reservoir for subclinical HPV in cases of cervical carcinoma; consequently, it is justifiable to test for anal hrHPV in patients with cervical tumors [6, 7]. HPV autoinoculation from the cervix to the anus or vice-versa is common; however, the duration of this reservoir of HPV is largely unknown, mainly because the clearance of anal HPV infection is frequent [7].

The assessment of concomitant anal and cervical HPV infection using DNA assays is relatively common, although few studies have included RNA-targeted tests. The detection of HPV RNA is believed to be a prognostic parameter due to its potential value as a marker for lesion progression [12]. Moreover, a positive test for RNA is thought to be related to the progression of HPV-induced disease, involving oncoprotein production and the activation of a molecular cascade of cellular alterations. However, RNA-based tests are typically less sensitive than DNA tests for the identification of HPV [12]. This was confirmed in our study, as the RNA investigation detected an almost threefold lower hrHPV frequency compared to the DNA assay ( $<0.001$ ). There may be a number of reasons for this significant difference, although the most likely reasons include reduced integrity of RNA and the technical difficulties involved in standardizing an RNA assay in fresh and fixed materials [13]. Different results have been reported regarding the performance of commercially available molecular assays kits for HPV DNA and RNA, although these generally provide superior specificity for RNA and higher sensitivity for DNA [14–16].

Of note, the high frequency of concomitant HPV16 infection in both the anal canal and uterine cervix corroborates data from previous studies [12, 17]. For example, heterosexual women with cervical HPV infection were shown to be at risk of concomitant, same-type anal hrHPV infection [17], and another recent report documented the exclusive association of low-risk HPV6 and HPV11 in anal high-grade lesions [18]. However, our study was limited because we only evaluated HPV16 and HPV18, which have been recognized

**Table 4** The number and percentage of women with and without cervical lesions distributed according to hrHPV DNA and E6 hrHPV RNA in the anal and cervical regions

Group	Variable	Category	Anal canal		Uterine cervix		<i>p</i> value <sup>a</sup>
			<i>N</i>	(%)	<i>N</i>	(%)	
Without cervical cancer	HPV (16 or 18)	Negative	97	(95.1)	101	(99.0)	0.219
		Positive	5	(4.9)	1	(1.0)	
With cervical cancer	HPV (16 or 18)	Negative	46	(39.3)	29	(24.8)	0.005
		Positive	71	(60.7)	88	(75.2)	
	E6	Negative	41	(78.8)	12	(23.1)	<0.001
		Positive	11	(21.2)	40	(76.9)	

<sup>a</sup> McNemar test

as the most prevalent forms of HPV in the cervical and anal regions [19].

The HPV vaccine has great potential for preventing lower genital tract cancer and precursor lesions. The effect of this vaccine on the prevention of anal cancer and anal HPV infection has also been explored, as well as reducing the rates of anal high-grade intraepithelial neoplasia among the group of men who have sex with men [20]. The identification of the specific types of HPV involved in anal infection and the risk factors for anal HPV infection and dysplasia may provide insight into the potential efficacy of the HPV vaccine in preventing anal intraepithelial lesions and anal cancer in high-risk groups of women [20]. Further studies should evaluate the potential usefulness of the HPV vaccine in preventing not only cervical cancer and precursor lesions but also all HPV-related lesions of the anogenital tract.

**Funding** We acknowledge the Research Support Fund of the State of São Paulo (FAPESP), process number 2010/16795–4, for the financial support grants. FAPESP had no other type of influence on this manuscript beyond the financial support.

**Conflicts of interest** None

**Ethical approval** The study was approved by the Ethic Committee number 310/2010, signed in 04 May 2010.

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