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Original Article

p16 protein expression in identification anal intraepithelial lesions related to human papillomavirus infection in women

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Abstract: Our objective was to assess p16 protein expression in anal lesions related HPV infection in patients out of main risk group for anal cancer. We studied immunohistochemical expression of p16 in 65 immunocompetent women with different degree of lesions in anal canal. Cell collection and biopsies were performed. HPV DNA was identified by PCR using GP5+/6+ and MY09/11 primers and biopsy fragments were subjected to routine hematoxylin-eosin staining for the histological assessment and immunohistochemistry reaction using anti-p16 antibody. The results histopathological were grouped as: I-Negative: Ia-52.31% (34/65) and Ib-benign changes of a proliferative nature 41.54% (27/65), II-Neoplasia 6.15% (4/65). The mean age of patients was 36.83 years. There was HPV positivity in 78.46% (51/65), more frequently observed in young women, who had between 2-5 sexual partner and reported anal intercourse, but was not statistically significant. Positivity for p16 was frequently associated with group II than groups Ia and Ib, statistically significant (P = 0.0002) and continuous pattern was predominant. 75% of II group were positive for HPV and p16. The majority of cases Ia and Ib positive HPV were not p16 staining (P = 0.0001). We conclude that p16 have expression associated neoplasia, but the low number of high-grade lesion impede evaluate about specificity of the marker. The HPV infection is not necessarily indicative of several clinical lesion, thus additional tools such as immunohistochemistry and histological diagnosis to assess the degree of lesion combined molecular biology offer a more accurate diagnosis.

Keywords: Anal Intraepithelial Neoplasia (AIN), immunohistochemistry, epithelial markers, viral infection, molecular biology

Introduction

The anal cancer is a rare malignancy that accounts for 2.5% of all gastrointestinal cancers. In 2015 was estimated 7,270 new cases encompassing anus, anal canal and anorectum cancer [1]. The development of Anal Intraepithelial Neoplasia (AIN) have as most common risk factor the human papillomavirus (HPV) infectionbeside history of anal intercourse and smoking [2-4]. The main risk groups for anal cancer are human immunodeficiency virus (HIV)-positive persons and men who have sex with men (MSM) which shown an increased risk for anal cancer [5]. Squamous cell carcinoma, the most prevalent histological type of anal cancer, arises in areas of high grade of AIN, as consequence of chronic HPV infection and appears to be related to a high load of virus in infected individuals [6].

More than 150 HPV genotypes are known and approximately 40 are related to anogenital infections [7]. Currently, it is known that HPV can also be found in cancer of the penis, vagina, vulva, anus, perianal region, head and neck. HPV in the anogenital region may give rise to genital warts and lead to the development of lesions with a possible progression to cancer [8, 9]. The natural history of a HPV infection

includes the following: transmission of the virus; the development of a persistent infection and interaction with the immune system, which plays a crucial role in the progression from a lesion to cancer. HPV infection tends to cause cancer in the area referred to as the "transformation zone". The anal region, as well as the cervix and tonsils are examples of areas prone to carcinogenesis by HPV [10, 11].

The infection for HPV occurs in the basal layer of the squamous epithelium cell through a microtrauma, the viral show tropism by epithelium. Malignant transformation in infected cells depends on the integration of the oncogenic HPV E6 and E7 genes in the host genome [12]. HPV has late structural genes (L1 and L2) that are involved in the structure of capsid and early functional genes (E1-E7) which are associated to viral replication processes [13]. The viral proteins (E6 and E7) inactivate the tumor suppressor proteins p53 and retinoblastoma (pRb), both of which are critical to cell cycle regulation. This promote deregulation of cell cycle and abnormal expression of cellular proliferation markers, for example p16 [14].

A negative feedback process resulting from this interaction activates the expression of p16 inhibitory proteins of the cyclin-dependent kinase complexes [15]. p16 regulates the transition from the G1 to the S phase of the cell cycle [16] and is accumulated in the nuclei and cytoplasm of cells infected by HPV. Biomarkers for HPV infection, such as p16, have been shown to correlate with a histological grade of AIN [17, 18]. However, these researches were realized in mixed groups (men and women), where the majority were men and HIV status was not considered. The aim of the present study was to assess p16 protein expression in anal lesions related HPV infection in patients out of main risk group for anal cancer, characterized by immunocompetent women.

Materials and methods

Characterization and sample collection

The present study was conducted with 65 women voluntary HIV-uninfected, aged between 18 and 60 years, previous HPV-related anogenital disease (cervix, vagina, vulva, or anal) or complaint pain or itch anal. All the patients were submitted to high-resolution anoscopy (HRA) in Service Pelvis of the Cancer Hospital of Pernambuco.

Exclusion criteria: Patients without lesion in anal canal, out of the age range (18-60 years), men and immunodeficiency and who was under treatment were considered as exclusion criteria. Patients with reagent result for HIV also were excluded.

Cells were collected from the anal canal (from the transition zone to the rectum) with gynecological brush before use acetic acid with subsequently biopsy after preview the lesion marked by acetic acid 5%. Immunoassay ELISA (Enzyme Linked Immuno Sorbent Assay) was performed to detection HIV-1 and HIV-2.

HPV analysis

DNA was extracted from anal cells using DNA IQ™ Casework Pro kit for Maxwell® 16, following manufacturer's instructions. PCR gene β-globin was done in order to show that samples contained adequate DNA and were free of substances inhibitory to PCR. DNA amplification was performed using specific primers (5'-CAACTTCATCCACGTTCACC-3'/5'-GAAGAGCCAAGGACAGGTAC-3') yielding a 268 bp amplicon. Ultrapure water was used as negative control and DNA extracted from human blood was used as positive control.

The presence of HPV in biological samples was analyzed by PCR using consensus primers for the region of the HPV L1 gene: GP5+/6+ 5'-TTTGTTACTGTGGTAGATACTAC-3'/5'-GAAAAATAAACTGTAAATCATATTC-3') [19] and MY09/11 5'-CGTCCMARRGGAWACTGAT-3'/5'-GCMCAGGGWCATAAYAATGG-3') [20] that yield a 150 bp and 450 bp amplicon, respectively. PCR was prepared with GoTaqGreen of Master Mix (Promega®) using 1 μL DNA (~30 ng DNA). Ultrapure water was used as negative control and pBR322. HPV16 plasmid was used as positive control. Amplification conditions were as follows: (i) 94°C for 3 minutes, (ii) 34 cycles at 95°C for 1 minute, annealing for 1 min, 72°C for 1 minute, (iii) final extension at 72°C for 10 min. MY09/11 primers annealed at 55°C, whereas GP5+/6+ annealed at 45°C. Amplicons were observed on 1% agarose gel electrophoresis stained with ethidium bromide.

Histology and immunohistochemistry

Tissue fragments from the biopsies were fixed in 10% buffered formalin and embedded in paraffin. Histological slices were obtained (4 μm thickness) and stained with hematoxylin-eosin

Table 1. Patients profile and HPV infection

PARAMETER	n	Percentage	HPV (n)		*P* (x^2)
			Positive	Negative	
Age range					*02322*
18-30 years	23	35.38%	20	3	
31-45 years	25	38.46%	20	5	
46-60 years	17	26.16%	11	6	
Total	65	100%	51	14	
Lifetime Sexual Partner					*0.6302*
1	14	21.54%	10	4	
2-5	35	53.85%	29	6	
More than 5	16	24.61%	12	4	
Total	65	100%			
Anal intercourse					*0.1967* *0.2207**
Yes	42	64.61%	35	7	
No	23	35.39%	16	7	
Total	65	100%			
Condom in anal intercourse					*0.6508* *1.0000**
Yes	1	2.38%	1	0	
No	41	97.61%	34	7	
Total	42	100%			

*Fisher's exact test.

(HE) or used in the immunohistochemical test. Two pathologists (N.T.P.F and R.J.V.M) evaluated the immunohistochemical reactions and histological diagnosis without knowledge of the original histologic diagnoses.

Immunohistochemical staining of tissue sections was done using the CINtec® Histology kit to detect tissue of $p16^{INK4a}$ (Biogen). Cervical carcinoma samples were used as positive control. The histological sections were mounted on silanized slides, deparaffinized and incubated in antigen retrieval solution (1:10) previously heated in a steamer at 95°C for 10 minutes. After cooling to room temperature (RT), the slides were washed with wash buffer (1:10), dried and 50 µl of reagent was added in order to block endogenous peroxidase in tissues and then incubated for 5 minutes at RT. After rinsing in wash buffer, tissue sections were incubated for 30 minutes at RT with 50 µL of the primary monoclonal antibody anti-p16 (clone E6H4). Tissue sections were washed twice in wash buffer and incubated for 30 minutes at RT with visualization reagent (polymer reagent conjugated with horseradish peroxidase). After being washed in wash buffer, peroxidase activity was detected by incubating tissue sections for 10 minutes at RT with DAB Chromogen (3.3'-diaminobenzidine chromogen solution). Tissue sections were counterstained with Harris's hematoxylin.

Histological and immunohistochemical assessment

In our study was proposed a classification to histopathological patterns in accordance diagnoses obtained, thereby the data were grouped and classified as follows: I-*Negative:* Ia-*without significant changes (Normal), benign reactive or inflammatory changes* and Ib- *benign changes of proliferative nature* (including condyloma, polyps and hyperplasia) and II-Neoplasia *(Anal Intraepithelial Neoplasia grade I (low-grade)*, *II* and *III (high-grade).*

The p16 positivity was defined by the presence of nuclear and/or cytoplasmatic staining. The classification criteria were based on the thickness of the anal epithelium marked and defined as follows: one third of inferior epithelium (basal layer), between one third and two thirds of the epithelium, or three thirds, corresponding to the entire thickness of the epithelium. The staining pattern was considered as focal or continuous.

Ethical approval

The Brazilian ethical committee for medical and health research in Federal University of Pernambuco accepted this study under identification CEP/CCS/UFPE 280/10.

Statistical study

The statistical analysis was calculated using Chi-square and Fisher's exact test to comparison between patients profile and HPV positivity, histological diagnosis and p16 staining. The comparisons between Histological diagnosis HPV positive and p16 staining were performed with three groups (Ia, Ib and II) through Qui-square. *P* values < 0.05 were considered statistically significant. The GraphPad Prism 5.01 was used for statistical analysis.

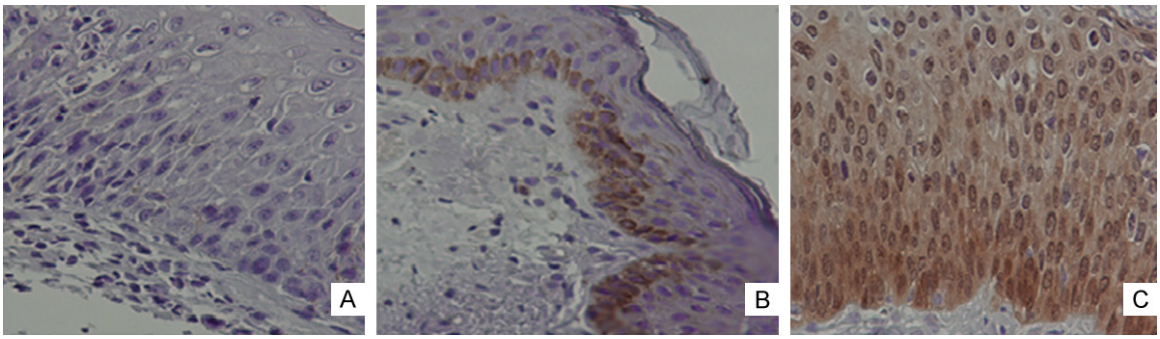

Figure 1. A: Anal epithelium negative (I)(*without significant changes*) with absence of p16 markings (40×). B: Histologic diagnosis II group AIN I, p16 markings in 1/3 of the thickness of the anal epithelium (40×). C: Histologic diagnosis II group AIN III, p16 markings in all thickness of the anal epithelium (40×).

Table 2. p16 protein expression in identification of the degree of anal intraepithelial lesion

Histological diagnosis (n = 65)	p16 staining								
	Negative	Positive	P	1/3	3/3	P	Focal	Continuous	P
			0.0002			*0.4594*			*0.4594*
Ia: Normal, benign reactive or inflammatory changes (n = 34)	28	03		02	01		01	02	
Ib: Proliferative changes (n = 27)	23	01		01	-		-	01	
II: Neoplasia (n = 4)	01	03		01	02		-	03	
Total	52	07		04	03		01	06	

Results

Patients profile

The characteristics of patients are summarized in **Table 1**. The mean age of patients included in this study was 36.83 years (range 20-59 years). Concerning sexual activity, 53.85% (35/65) of the women had between two and five lifetime sexual partners and 64.41% (42/65) reported a history of anal intercourse. A total of 97.61% (41/42) did not use condoms during anal intercourse.

Histology and immunohistochemistry

According to classification previously described, our results to histological diagnosis were: Ia-52.31% (34/65); Ib-*41.54% (27/65)* and II-6.15% (4/65).

Nuclear and/or cytoplasmatic staining were observed in 75% (3/4) in group II (neoplasia) with staining restricted to the basal layer (AIN I) (**Figure 1B**) or staining the entire thickness of the anal epithelium (AIN III) (**Figure 1C**). In 80% (52/65) of the cases evaluated there was not staining for p16, including 01 case of AIN I, 28 cases classified as Ia and 23 cases as Ib (**Table 2**). p16 staining indeterminate were found in 06 cases.

Positivity for p16 (presence or absent staining) was frequently associated with cases of neoplasia (II) than negative cases (Ia and Ib). The difference was statistically significant (P = 0.0002). Concerning thickness of the anal epithelium stained and staining pattern no showed statistical difference (P = 0.4594). Continuous pattern was predominantly observed in p16 staining, where all cases group II showed continuous staining. Histological diagnosis Ia (02 cases) and Ib (01 case) showed staining restricted to the basal layer of the epithelium. However, staining pattern in three thirds of the epithelium was observed in one case classified as Ia (inflammation), though focal pattern (**Table 2**).

Molecular analysis

Concerning molecular analysis to identify HPV was observed a frequency of 78.46% (51/65) being considered as positive results when at least one set of primers was amplified. The viral L1 region amplification analyzed by GP5+/6+ set presented a positivity rate of 67.69%

Table 3. Comparison between histological diagnosis HPV positive results and p16. Positive results for PCR were considered when at least one set of primers was positive

Histological diagnosis HPV positive	p16 staining		P
	Positive	Negative	*<0.0001*
Ia: Normal, benign reactive or inflammatory changes (n = 24)	02	21	
Ib: Proliferative changes (n = 23)	-	20	
II: Neoplasia (n = 04)	03	01	
Total	05	42	

*Ia: 01 case indeterminate; Ib: 03 cases indeterminate.

(44/65) while for MY09/11 set 43.07% (28/65) was achieved.

Although there was not statistically significant difference between HPV positive and the following profile parameters of the patients as age range (P = 0.2322), lifetime sexual partner (P = 0.6302), anal intercourse (P = 0.1967) and use condom in anal intercourse (P = 0.6508). There was a higher frequency, in this study, HPV positivity in young women (18-30 years), who had between 2-5 lifetime sexual partner and reported anal intercourse (**Table 1**).

The comparison between histological results and identification of HPV revealed 100% positivity HPV in lesions with a histological diagnosis of neoplasia, independent of the degree. In patients diagnosed as Ia 70.58% (24/34) and Ib 85.18% (23/27) also exhibited positivity for HPV. This results were not statistically significant (P = 0.2157).

Regarding to histological diagnosis HPV positive and 16 staining, 75% cases classified as II group were positively concordant for both. The majority of cases classified as Ia and Ib positive HPV were not p16 staining, only two cases were positive, so that statistical data showed significance between three groups (P < 0.0001), despite the low number of cases in group II (**Table 3**).

Discussion

In this study the most patients who reported having anal intercourse did not use condoms, which increases the probability of HPV infection and reveals a lack of knowledge about the risk of infection in the anal region. The anal canal, as well as the entire anogenital tract, can be a part of HPV circuit or may even serve asreservoir for the virus due to the presence of squamous epithelium. The lifetime number of sexual partners is a significant risk factor for HPV even after adjustment for recent sexual behavior [21]. This can be verified in this study, where women who had multiple partners showed higher HPV positivity.

HPV infection is a risk factor for the development of squamous premalignant and malignant lesions in the anal area. The interaction of viral oncoproteins, E6 and E7, with cell cycle regulatory proteins, p53 and pRb, and the relationship between pRb and the transcription factor E2F, can lead to the activation of cyclin-dependent kinases (CDK4 and CDK6) which, in turn, leads to the expression of associated protein kinases, such as p16 [22, 23]. p16 expression is up-regulated in the transformation caused by infection with oncogenic HPV, and showed itself asa sensitive marker for squamous intraepithelial lesions of the female genital tract [24, 25].

So far, the majority of studies focus on marking with p16 in cervical samples [26, 27] In cases of grade II and III Cervical Intraepithelial Neoplasia (CIN), a greater p16 expression occurs while positivity in cases of CIN I, although uncommon, appears to have a risk of progression to malignancy [28]. According to Lu et al. [29], there is a similarity in markup with p16 in both anal and cervical samples and showed strong and diffuse nuclear staining (with some cytoplasmatic staining) in all cases of anorectal squamous cell carcinoma.

Our findings are in agreement with Kreuter et al. [30], when demonstrated that protein expression in normal anal mucosa is only null or positive in a number of isolated cells and restricted to the basal layer, which may suggest that the lesion has a higher potential progression. Thus p16 protein identification through immunohistochemistry is an excellent addition-

al tool in the diagnosis of high grade anal dysplasia.

Pirog et al. [31], stated that p16 is a highly sensitive and specific marker for high grade AIN, in relation to low-grade lesions, and thus can aid in the classification of neoplasia related to HPV. 100% sensitivity and 100% specificity to diagnose high-grade AIN was observed by Kreuter et al [30]. Our results showed higher positivity for p16 in neoplasia cases but due the low number of neoplasia cases cannot reach a conclusion about the specificity of the marker.

Large cases severe lesions were not prevalent in our results, however this was due to type of population studied, composed of immunocompetent women, that represent a group has lower-risk to development of higher degree lesion. Although anal cancer is a rare disease in the general population, it was demonstrated that the incidence of anal cancer is higher in certain high risk groups, such as MSM (Men who have sex with men) and immune suppressed individuals, including those with HIV [32]. Studies comparing p16 staining with the degree of anal lesions were predominantly composed of HIV-positive patients [30, 33].

Regarding the molecular analysis of HPV in our study, the index of positive samples for the virus was higher (78.46%), considering lower degree lesions and low number cases classified as neoplasia. The type sample contributes to a viral detection more efficient. Fresh anal cells result in higher HPV positivity and broader spectrum of HPV genotypes compared to tissue biopsies, once collected cells represent the whole anal canal [34].

Cimino-Mathews et al [35] showed in your study that all (100%) cases of anal and rectal small cell carcinomas were positive for p16, and 100% of anal and 82% of rectal small cell carcinomas were positive for high-risk HPV, demonstrating that high-risk-type HPV can be detected using in situ hybridization in the majority of anorectal small cell carcinomas.

The prognosis of anal squamous cell carcinoma is substantially affected by the disease stage. The early detection and treatment of precursor lesions entails benefits to the patient [30]. The presence of p16 overexpression detected by immunohistochemical staining is a simple technique that can be used as a prognostic marker [16]. Bean et al. [17], revealed how routine p16 staining can assist in confirming a histological diagnosis or in detecting intraepithelial lesions that appear as negative using the histological method. This demonstrates the importance of combining the histological method with other methods to assist in the results confirmation.

We observed higher positivity for HPV and p16 in group II, but ingroup Ib HPV positive none showed p16 staining. However, only viral presence in anal regionis not necessarily indicative of clinical lesion with higher severity, is important to analyze the viral type involved in infection. Thereby, benign lesions p16 and HPV positive require a monitoring by lesion progression risk.

Histological exam for AIN diagnosis is sometimes difficult and the utilization of additional tools, such as immunohistochemistry and molecular test, PCR, is important to aid the diagnostic accuracy for AIN. The p16 expression is clearly associated with degree lesion. However, considering the results of the present study need more cases of neoplasia for reach a conclusion about the expression this protein in high AIN. There is a need to disseminate about infections in the anal region, similar to that which occurs related to the cervical region, that due similarity of the epitheliums, the anal epithelium is target of HPV infection, as well as the implementation of periodic screening sessions in healthcare systems.

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