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Original Article

Claudin-1 expression in HIV/HPV co-infected anal intra-epithelial neoplasia

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Abstract: Background: Claudin-1 expression is poorly characterized in anal intraepithelial neoplasia (AIN) from HIV-positive individuals. Aim: To investigate claudin-1 *in situ* expression in HPV-positive AIN lesions from HIV-positive individuals compared with non-dysplastic anal tissues. Materials and Methods: By means of immunofluorescence microscopy, claudin-1 was analyzed in 46 anal tissues: 18 AIN1; 14 AIN2/3 and 14 non-dysplastic specimens. Results: A higher proportion of cells with overexpression of claudin-1 were found in HIV-positive AIN lesions compared with controls. Overexpression of claudin-1 was most evident in AIN 2/3 lesions. HPV16 was detected in 21.8% anal specimens and HPV18 in 3.1%. A great diversity of 18 low-risk HPV types were detected, 40% of AIN specimens had multiple HPV types. Conclusions: Our data show that there is an over-expression of claudin-1 in HIV-positive AIN lesions compared with non-dysplastic anal biopsies. A high prevalence of HPV16 and diversity of HPV types was detected. The overexpression of claudin-1 in HIV-positive AIN specimens may play a role on the development of anal cancer, however further studies should be performed to better understand the role of claudin-1 in AIN and anal cancer development in the HIV-positive individuals.

Keywords: HPV, HIV-1, claudin-1, anal dysplasia, men who have sex with men, women

Introduction

Although claudins-1 and -2 are frequently expressed in a similar pattern in a variety of cancers, some differences do exist. The claudin family consists of 24 different trans-membrane proteins with individual expression profiles that vary among different tissues resulting in a unique, characteristic pattern for each claudin [1]. Recently a report highlighted the role of claudins in tumorigenesis [2], claudin-1 overexpression was associated with increased invasiveness of oral carcinoma cells [3] and can regulate breast cancer cell motility and proliferation, affecting miRNA dynamics in human basal-like breast cancer cells [4]. Overexpression of claudin-1 was also shown to induce the generation of tumor lymphatic vessels, increasing metastasis in the lymph node [5], and suggesting the potential for these proteins to serve as novel molecular targets in cancer treatment strategies [1]. Progression of high-grade lesions to invasive cancer can take many years, but can occur more rapidly in HIV-positive individuals [6]. Previous study demonstrated that Infections with human immunodeficiency virus (HIV) and human papillomavirus (HPV)-associated tight junction (TJ) dysfunction can also contribute to a reduction in the barrier function of oral and anal mucosal epithelium. There is evidence that the envelope of HIV-1 gp120 or Tat (trans-activator of viral replication) can cause changes on the mucosal epithelial barrier [7].

The precise details of the process in the context of the natural progression of HPV-associated carcinogenesis in the HIV infection are not well clarified. Previously we described a local differential expression of several immune cytokines and classes of inflammatory cells in cervical HIV-1/HPV co-infection. An immune shift of cytokine profile from type 1 (interleukin 2 and interferon gamma) to type 2 (interleukin 4 and

Table 1. Clinical and socio-behavioural data from study patients

Variables	HIV Status						Total (N = 46)
	Diagnosis-N (%)						
	HIV-negative			HIV-positive			
	Non-dysplastic (N = 5)	Low-grade AIN (N = 1)	High-grade AIN (N = 2)	Non-dysplastic (N = 9)	Low-grade AIN (N = 17)	High-grade AIN (N = 12)	
Behavioural variables							
Age (Years)							
Mean (SD)	40.1 (6.7)	-	-	41.9 (7.5)	43.8 (9.1)	45.6 (8.8)	43.3 (8.6)
Median (IQR)	40.1 (33.4-46.8)	-	-	42.2 (39.9-47.1)	41.6 (37.3-52.7)	42.8 (40.4-50.2)	41.6 (37.3-50.1)
Sex at birth-Male	5 (100.0)	1 (100.0)	2 (100.0)	5 (55.6)	7 (41.2)	1 (8.3)	21 (45.7)
Current smoking	-	-	-	1 (11.1)	3 (17.6)	1 (8.3)	5 (13.2)
Lifetime illicit drug use	1 (33.3)	-	-	-	1 (5.9)	-	2 (5.0)
No. of sexual partners in the past 6 months (only for women)							
0	-	-	-	-	1 (10.0)	4 (40.0)	5 (20.8)
1	-	-	-	4 (100.0)	9 (90.0)	6 (60.0)	19 (79.2)
No. of sexual partners in the past 12 months (only for men)							
Mean (SD)	7.7 (3.2)	-	-	6.8 (3.6)	5 (7.7)	-	5.5 (5.6)
Median (IQR)	9.0 (4.0-10.0)	-	-	5.5 (4.5-9.0)	2 (1-5)	-	4.0 (1.5-7.5)
No. of sexual male partners in the past 12 months (only for men)							
Mean (SD)	7.7 (3.2)	-	-	3.8 (2.6)	3.1 (3.6)	-	3.9 (3.5)
Median (IQR)	9.0 (4.0-10.0)	-	-	4.5 (2.0-5.5)	2.0 (0.0-5.0)	-	4.0 (0.5-5.5)
Variables related to HPV							
History of anal sex	2 (66.7)	1 (100.0)	-	1 (12.5)	5 (29.4)	2 (16.7)	11 (26.8)
Cervical lesion history	-	-	-	3 (75.0)	8 (80.0)	10 (90.9)	21 (84.0)
Anal lesion by HPV history	-	-	-	3 (33.3)	5 (31.3)	3 (27.3)	11 (27.5)
Treatment for HPV history	-	-	-	3 (33.3)	6 (37.5)	3 (27.3)	12 (30.0)
Variables related to HIV							
Time since HIV diagnostic (months)							
Mean (SD)	-	-	-	132.7 (79.6)	110.1 (79.6)	106.8 (76.7)	114.4 (77.3)
Median (IQR)	-	-	-	165.9 (43-184.7)	113.7 (39.5-147.7)	110.6 (42.9-165.6)	115.8 (40.0-180.1)
Nadir CD4*							
Mean (SD)	-	-	-	220.3 (163.2)	199.1 (158.6)	153.6 (179.3)	189.7 (164.0)
Median (IQR)	-	-	-	197.0 (140.0-226.0)	161.0 (73.0-286.0)	63.0 (29.0-208.5)	166.5 (57.0-280.0)
< 50	-	-	-	-	2 (11.8)	5 (41.7)	7 (18.4)
50-199	-	-	-	5 (55.6)	7 (41.2)	4 (33.3)	16 (42.1)
200-349	-	-	-	3 (33.3)	6 (35.3)	1 (8.3)	10 (26.3)
≥ 350	-	-	-	1 (11.1)	2 (11.8)	2 (16.7)	5 (13.2)
Current CD4+*							

Mean (SD)	-	-	-	626.1 (107.5)	601.7 (277.2)	339.1 (159.5)	517.2 (243.7)
Median (IQR)	-	-	-	605.0 (577.0-704.0)	571.0 (369.0-904.0)	393.0 (182.0-468.0)	476.0 (369.0-688.0)
< 50	-	-	-	-	-	-	-
50-199	-	-	-	-	1 (6.7)	4 (33.3)	5 (14.3)
200-349	-	-	-	-	2 (13.3)	1 (8.3)	3 (8.6)
≥ 350	-	-	-	8 (100.0)	12 (80.0)	7 (58.3)	27 (77.1)
HIV RNA viral load (6 months) undetectable	-	-	-	7 (77.8)	9 (60.0)	7 (58.3)	23 (63.9)
cART exposure	-	-	-	8 (88.9)	13 (76.5)	10 (83.3)	31 (81.6)

interleukin 10) cytokine and lower number of CD68 cells could be associated to the risk of transmission of HIV-1 and increased development of cervical cancer in these women [8].

Prospective studies are needed to better elucidate the mechanisms mediated by cellular immune responses in HIV-1/HPV co-infection, as well as factors that may contribute to increased severity and progression of the disease. To date, there have been few studies to demonstrate the prognostic implications of *in situ* claudin-1 expression in anal tissue. To our knowledge this is the first study to analyze claudin-1 expression in HIV-infected anal tissues. We hypothesized that HIV/HPV co-infection may be associated with claudin-1 overexpression which in turn could facilitate the development of anal intraepithelial lesions. The present study aimed to analyze claudin-1 *in situ* expression in anal specimens from HIV/HPV co-infected individuals in comparison to non-dysplastic anal tissues.

Materials and methods

Study design, population, tissue samples and data collection

This was a cross-sectional study that included 46 anal biopsy specimens. Forty-two biopsies were collected with consent at the Evandro Chagas National Institute of Infectious Diseases of the Oswaldo Cruz Foundation (INI-Fiocruz in Rio de Janeiro Brazil) at baseline. One patient underwent additional follow-up biopsies at 1 and 5 months from baseline. Additionally, four normal squamous epithelium/non-dysplastic biopsies from HIV-negative man were collected at the UCSF clinics and were used as controls. Tissue donors included HIV-infected women (25/46; 54%), HIV-infected men (13/46; 28%) and men without HIV infection (8/46; 17%). Biopsies were evaluated by two expert pathologists and classified as low-grade anal intraepithelial neoplasia (AIN 1), high-grade anal intraepithelial neoplasia (AIN 2/3) or no evidence of AIN (that were used as control).

Socio-demographic information along with behavioral and clinical variables were extracted from the database on the cohort studies at INI-FIOCRUZ-RJ [9]. The following socio-demographic variables were selected: age at the time of the biopsy and sex. The behavioral variables were: smoking, illicit drug use, history of anal sex, number of sexual partners in the last 6 months for women, and in the last 12 months for men and number of sexual male partners in the last 12 months for men. Variables related to HPV were: history of anal lesions, history of cervical lesions and history of treatment for HPV-associated lesions. The variables related to HIV were: time since HIV was diagnosed; CD4+ nadir, defined as the lowest CD4+ value; CD4+ level at the time of the biopsy (current); HIV-1 viral load (detectable at > 49 copies/IU); use of high activity antiretroviral therapy (HAART) defined as two or more analog reverse transcriptase inhibitors and one nucleoside reverse transcriptase inhibitor, a non-nucleoside analog or at least one protease inhibitor; and duration of treatment with HAART.

The study received approval prior to recruiting participants from the institutional ethical review board at the Evandro Chagas National Institute of Infectious Diseases (INI) of the Oswaldo Cruz Foundation (Fiocruz) in Rio de Janeiro, Brazil (under the protocol CAE 0044.0.009.000-09 and from UCSF CHR approval #: H8597-30664-03 for the HIV negative non-dysplastic donors). Written informed consent for participation in the study was obtained from all participants in strict compliance with the ethical guidelines involving human subjects in Brazil as required by Resolution No. 466/2012 of the National Health Council.

Immunofluorescence microscopy and image analysis

The biopsies were stored in liquid nitrogen and embedded in Tissue Tek®. Using a cryostat, 3 sections of 5 μm thickness were cut and fixed in 5% paraformaldehyde (PFA) for 10 minutes. Tissue sections were immunostained as previously described [7]. The primary antibody was a rabbit anti-claudin-1 (Zymed, Lab Inc-Daly City, CA) detected by anti-rabbit secondary antibodies (Jackson Immuno Research-Baltimore Pike-USA) labeled with either fluorescein isothiocyanate (FITC), tetramethyl rhodamine isothiocyanate (TRITC), or cyanine 5 (Cy5). Specificity of each antibody was confirmed by negative staining with the corresponding primary isotype control antibody. Cell nuclei were counter-

Table 2. Semi-quantification of claudin-1 expression across AIN compared with non-dysplastic tissues from HIV+ and HIV-subjects

Histopathology diagnosis	Claudin-positive stained cells/Percent of cells					
	0-25%	26-50%	51-75%	76-100%	Total N (%)	*P* value*
HIV-: Normal	2 (40.0)	2 (40.0)	-	1 (20.0)	5 (100.0)	0.002
HIV-: Low-grade AIN	-	-	-	1 (100.0)	1 (100.0)	
HIV-: High Grade AIN	-	2 (100.0)	-	-	2 (100.0)	
HIV+: Normal	2 (28.6)	2 (28.6)	-	3 (42.9)	7 (100.0)	
HIV+: Low Grade AIN	-	2 (12.5)	2 (12.5)	12 (75.0)	16 (100.0)	
HIV+: High Grade AIN	-	-	-	10 (100.0)	10 (100.0)	
Total	4 (9.8)	8 (19.5)	2 (4.9)	27 (65.9)	41 (100.0)	

*Fisher's exact test.

stained with TO-PRO-3 iodide (ThermoFisher Inc- Waltham, MA-USA).

Tissue sections were analyzed at 40× using a Leica SP 5 laser confocal microscope. Immune reactive cells were quantified in all epithelium field and categorized as the percent of cells expressing claudin-1 (0-25%, 26-50%, 51-75% and 76-100% of cells) Non-dysplastic tissue from HIV-negative subjects were used as a control to define the percentage of normal claudin-1 patterns.

HPV DNA extraction and HPV genotyping of the tissues

Biopsies were stored in liquid nitrogen prior to embedding in Tissue Tek®. Using a cryostat, 3 sections of 5 μm thickness were cut for DNA testing. DNA was isolated using the Isolation Kit Cells & Tissue Genomic Prep Mini Spin (GE Healthcare, Buckinghamshire, United Kingdom) according to the manufacturer's protocol. PCR was performed as previously described [10]. In brief, HPV DNA typing was performed using MY09/MY11 consensus HPV-L1 primers as well as primers for amplification of the human β-globin gene, as a control. PCR products from positive samples were typed by dot-blot hybridization using 39 individual type-specific probes, including oncogenic HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) and non-oncogenic types (6/11, 26/69, 30, 32/42, 34, 53, 54, 57/2/27, 61, 62, 67, 68, 70, 71, 72, 73, 81, 82, 83, 84, 85, 76/87, 90/106, 97, 102/108, as well as 2 separate mixtures, mix1 contains 7/13/40/43/44/55/74/91, and mix2 contains 3/10/28/29/77/78/94 plus all those HPV types that hybridized only with the consensus probe.

Statistical analysis

Two separate analyses were performed based on HIV status and histopathological diagnosis. One comparison was between subjects without dysplasia from HIV-infected and HIV-uninfected individuals, to analyze differences correlated with HIV infection. The second comparison was done for HIV-infected individuals with different grades of histopathologic severity (no dysplasia, AIN1 and AIN2/3), to analyze differences correlated with disease grade in HIV-infected subjects. Categorical variables of sex, smoking, illicit drug use, history anal sex and HPV variables were analyzed by Chi-square test and Fisher's exact test. Continuous variables of age, number of partners, HIV variables, and histopathologic scoring of tissue samples were analyzed by the non-parametric Kruskal-Wallis test. Statistical analysis was performed with SPSS 15.0 software.

Results

Study population

Clinical and socio-demographic data from the patients analyzed on this study are depicted on **Table 1**. There were no statistically significant differences regarding the variables analyzed between HIV-negative and HIV-positive patients. Forty-six patients were included in this study, forty-five contributed with one anal biopsy at baseline and one patient contributed with two anal tissue samples at 1-month (AIN2/3) and 5-months (AIN1) from baseline, 54.3% (25/46) were from HIV-infected women, 28.2% (13/46) from HIV-infected men, and 17.3% (8/46) from men without HIV infection. A total of 38 HIV-infected and 8 HIV-uninfected subjects were included. The mean age of HIV-

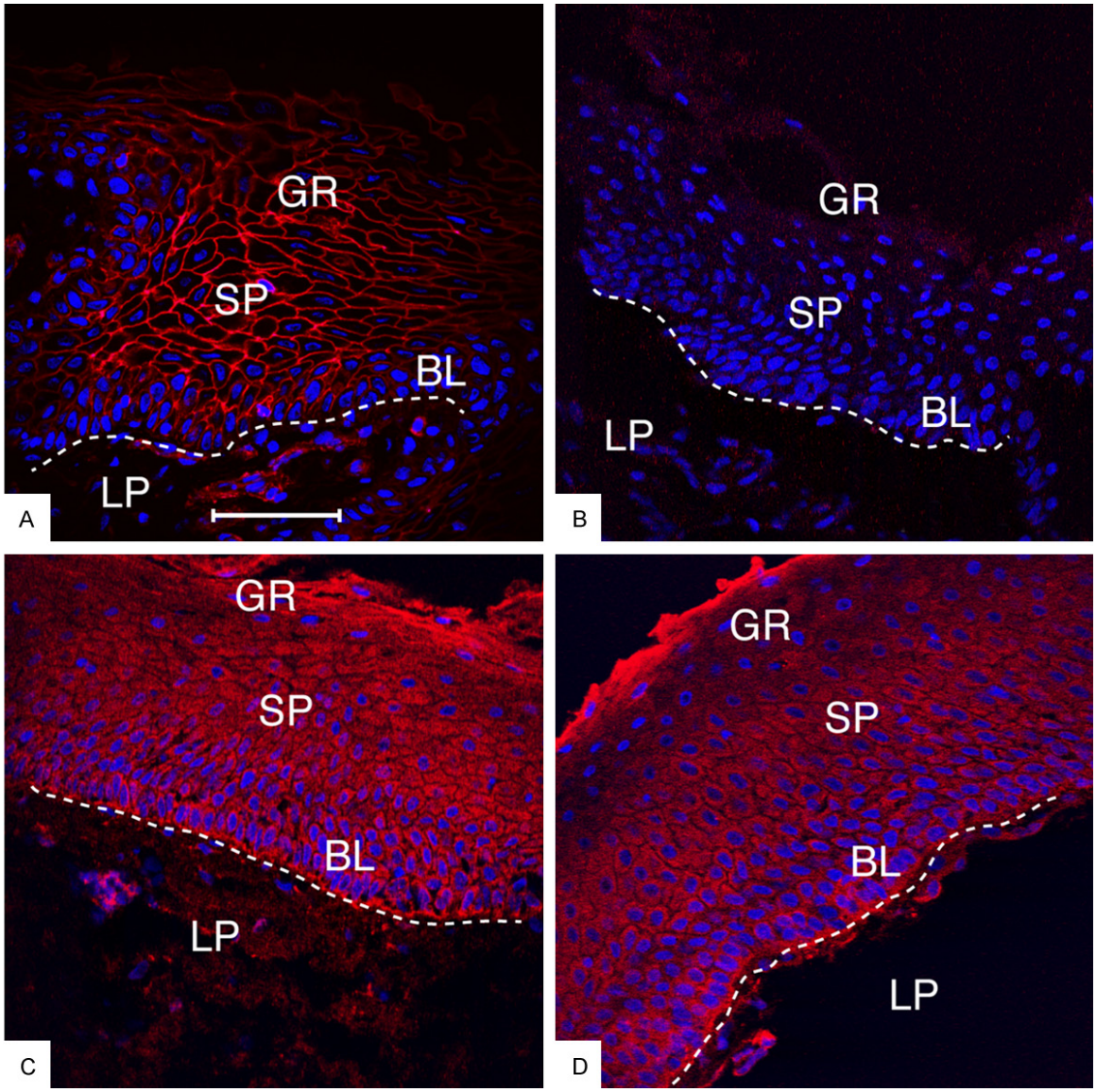

Figure 1. Claudin-1 expression in anal epithelium. (A) Shows HIV-negative non-dysplastic (control), normal pattern of claudin-1, exclusively on the cell membrane compared with high-grade (AIN 2/3) anal specimen (C and D) in HIV-1 infected individuals. (B) IgG, negative control. (A) high level of expression claudin-1 in the spinosum/intermediate layers of two AIN-2/3 tissues was observed, showing membrane and cytoplasmic pattern (C and D). Color combined images of claudin-1 staining (red) and nuclei (blue). GR, granulosum; SP, spinosum; BL, basal; LP, lamina propria. Scale, 50 μm.

infected patients was 43.9 years old (8.5 SD), HIV-uninfected patients (control) were 37.1 years old (8.1 SD). Among the HIV-infected subjects, 81.6% (31/38) had previous cART exposure, CD4+ count ≥ 350 (77.1%), < 50 (0%) and CD4+ > 50-349 (22.9%). Regarding the nadir CD4+ count, data showed that most patients had 50-199 (42%) and only 13.2% had ≥ 350. HIV RNA viral load was undetectable (for 6 months) in 64%. Only 5 (13.2%) subjects were current smokers and 2 (5%) had used illicit drugs, 19 (76%) of women had a sex partner in the past 6 months and the average number of sex partners in the past 12 months for men was 5.5; 21 women (84%) had a history of a cervical lesion. The average number of months since HIV diagnosis was 114. HIV-infected patients with high grade AIN (AIN2/3), had lower nadir and current CD4+ compared with those with low-grade AIN (AIN1).

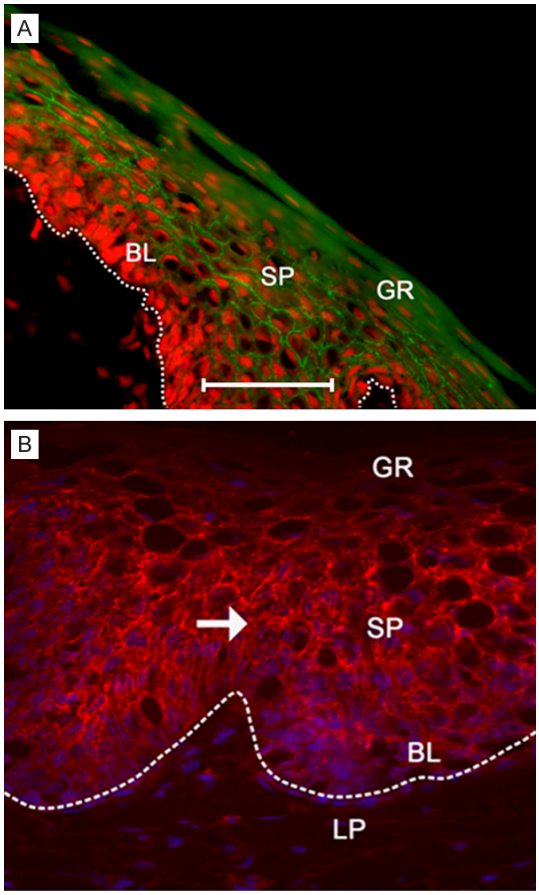

Figure 2. Claudin-1 expression in normal and low-grade anal epithelium. (A) Shows claudin-1 membrane expression in normal anal epithelium from HIV-negative individual. (B) Shows claudin-1 expression in low-grade anal epithelium from HIV-1 infected individual, which has mostly membrane localization (B). Color combined images of claudin-1 staining (Green in panel A; Red in panel B) and nuclei (blue). GR, granulosum; SP, spinosum; BL, basal; LP, lamina propria. Scale, 50 μm.

Claudin-1 expression

We analyzed claudin-1 expression and its location within the epithelial layers and across the histopathology diagnosis in both HIV-positive and HIV-negative anal specimen. We quantified claudin-1 expression among HIV-infected AIN I and AIN II/III compared with control/non-dysplastic anal tissues from both HIV-infected and HIV-uninfected patients, claudin expression increased with the AIN severity as shown in **Table 2**. Claudin-1 expression in controls (normal/non-dysplastic anal tissue) and AIN from HIV-infected subjects is shown in **Figures 1** and **2**. Claudin-1 expression in normal anal epithelia was mainly within the parabasal and spinosum layers of epithelium and exclusively in the cell membranes with a ring shape. In contrast, claudin-1 expression in dysplastic anal tissues was detected within the all layers of epithelium. Claudin-1 localization in low-grade anal tissue was mostly in the membrane, however, in the high-grade epithelia claudin-1 was detected in the cell membranes as well as in the cytoplasm. The expression of claudin-1 in the dysplastic epithelial tissues was higher than in normal anal epithelia.

HPV genotyping

Of the 46 anal specimens, fourteen were not evaluated due to insufficient tissue for extracting DNA. Of the remaining 32 specimens, seven (21.87%) had no detectable HPV DNA and 78.12% (25/32) of the anal biopsies had HPV DNA that by sequencing revealed 15 high-risk HPV types (16, 18, 26, 30, 31, 33, 35, 39, 51, 58, 59, 68, 69, 82, 97). A great diversity of 18 low-risk HPV types were detected in the anal specimens. The most common types detected were HPV 6 (10/32; 31.2%), HPV11 (10/32; 31.2%) and the high risk HPV16 (7/32; 21.8%). Only one case of HPV18 (3.1%) was detected. A high prevalence of multiple HPV infection (40.6%) was found in the anal biopsies (**Table 3**).

We next evaluated the HPV type according to the histopathology diagnosis. Data showed that 50% of normal squamous epithelium were negative for HPV and 25% had both low-risk and high-risk HPV types. Of the high-grade AIN specimens, 50% were infected by both high-risk and low-risk HPV types. Of AIN1, 41.7% specimens had a low-risk HPV type and 8.3% had a high-risk HPV type detected. **Table 3**, shows the oncogenic and non-oncogenic HPV types found on this study in both HIV-positive and HIV-negative patients.

Discussion

To date, there have been few studies to demonstrate the prognostic implications of *in situ* claudin-1 expression in anal specimens. In our study, increased claudin-1 expression was highly correlated (P = 0.008) with AIN development. Other groups have also suggested that increased claudin-1 expression is related to cervical intraepithelial lesions (CIN) and HPV positive tonsillar cancers [11, 12]. The mecha-

Table 3. Prevalence of HPV across the anal biopsies

Anal HR HPV genotypes	Histopathology diagnosis-N (%)						Total (N = 32)
	HIV-			HIV+			
	No dysplasia (N = 3)	Low Grade AIN (N = 0)	High Grade AIN (N = 2)	No dysplasia (N = 5)	Low Grade AIN (N = 12)	High Grade AIN (N = 10)	
Both LR and HR HPV	-	-	100.0	40.0	33.3	50.0	40.6
LR HPV only	-	-	-	20.0	50.0	30.0	31.3
HR HPV only	33.3	-	-	-	-	10.0	6.3
No HPV infection	66.7	-	-	40.0	16.7	10.0	21.9

*p (Fisher's exact test) = 0.236.

nisms underlying the up-regulation of claudins in cancer are still unclear. Since anal carcinomas commonly evolve from AIN 2/3 lesions, our data would suggest that the invasive cancer may maintain the high claudin-1 activity as seen in tonsillar HPV+ cancers [12]; further data are required from anal cancers to address this point.

Thus, taken together, our results when added to the published literature suggests that HIV-1 infection per se may increase claudin-1 expression in the epithelia, especially towards the surface, which in turn could facilitate HPV infection and, thus, AIN lesions. It is well documented that HIV-1 infected people indeed have a much higher increase of HPV related intraepithelial lesions and cancers in the genital tract and anus and a greater HPV prevalence in homosexual and bisexual individuals (men and women) that are HIV positive to those that are HIV negative [13, 14]. Moreover, co-infection by HPV-HIV exhibits polarization of Th2 cytokine profile, both locally and at the systemic level, which can facilitate neoplastic transformation [8]. In the present study, of the 38 HIV-positive patients, over fifty percent have cervical lesion history, suggesting that the cervical lesion history could be a risk factor for anal lesion (AIN) in women [6]. HIV-associated cell junction dysregulation seems to have an important role in increasing the risk of HPV infection, together with others immune factors which down regulate the immune response and contribute to increase the risk of subsequent development of HPV-associated neoplasia [7]. Although several clinical studies have shown that HIV-1 is associated with increased permeability of the intestinal tract, there are very few studies on the mechanisms underlying HIV-related deregulation of tight junctions in the skin. A recent study showed that exposure to HIV-1 can directly breaking the integrity of the epithelial barrier of the mucosa, which enables translocation of viruses and bacteria [15]. This suggests that HIV-1 infection per se may be partly responsible for the increased claudin-1 expression noted in this study in the AIN lesions.

Persistence of HPV DNA is one of the main risk factors for the malignant transformation in the anal and cervical epithelium. The host immune response is crucial to eliminate HPV infection, especially the immune response mediated by cells that is an essential factor to infection control. In this study, we found a high prevalence for multiple HPV infection in the AINII/III anal specimens (58.3 %). Our data are in agreement with a previous study that also detected a high prevalence of multiple HPV infection in the anal canal of HIV positive men who have sex with men [16].

The use of highly active antiretroviral therapy (HAART) has reduced the prevalence of several opportunistic infections in AIDS patients [17]. However, despite the immune reconstruction, this therapy seems to have a limited impact on the development of HPV-associated lesions. In this study, 81.57% of HIV infected individuals had previous CART exposure and 31.57% had high grade anal lesion.

The present study has some important limitations, one of them is the small sample size to analyze the different AIN grade. However, this is a molecular study and nevertheless, we showed important findings about the claudin-1 pattern expression in HIV infected subjects. Further investigations with large sample size are needed to determine whether claudin-1 is correlated with the development of AIN lesions in the HIV infected patients. It is also important to accompany the progression of AIN to determine the

relationship between changes in the claudin-1 pattern with cancer.

In conclusion, our data show that an HIV-positive AIN has a higher percentage of cells with elevated claudin-1 expression compared to controls. This, in turn, may contribute to a reduction in the barrier function epithelium that can increase the risk of AIN.

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Disclosure of conflict of interest

None.

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