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Secretory leukocyte protease inhibitor expression and high-risk HPV infection in anal lesions of HIV positive patients

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Abstract

Objective—The aim of the current study was to evaluate secretory leukocyte protease inhibitor (SLPI) expression in anal biopsies from HIV-positive (HIV^+) individuals, and compare that to anal intraepithelial neoplasia (AIN) diagnoses and human papillomavirus (HPV) status.

Design—This is a cross-sectional study of a cohort of 54 HIV^+ (31 males and 23 females) from an AIDS clinic in Rio de Janeiro, Brazil.

Methods—The study material consisted of anorectal tissue biopsies obtained from HIV^+ subjects, which were used to construct tissue microarray paraffin blocks for immunohistochemical analysis of SLPI expression. Biopsies were evaluated by an expert pathologist and classified as low-grade anal intraepithelial neoplasia (AIN1), high-grade anal intraepithelial neoplasia (AIN2/3), or normal squamous epithelium. Additionally, DNA from the biopsies was extracted and analyzed for the presence of low- or high-risk HPV DNA.

Results—Histologically normal squamous epithelium from the anorectal region showed strong positive SLPI staining in 17/20 (85%) samples. In comparison, 9/17 (53%) dysplastic squamous epithelial samples from AIN1 patients showed strong SLPI staining, and only 5/17 (29%) samples from AIN2-3 patients exhibited strong SPLI staining, which both were significantly fewer than

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those from normal tissue (p=0.005). Furthermore, there was a significantly higher proportion of samples in which oncogenic high-risk HPV genotypes were detected in low SLPI expressing tissues than that in tissues with high SLPI expression (p=0.040).

Conclusion—Taken together these results suggest that low SLPI expression is associated with high-risk HPV infections in the development of AIN.

Keywords

Secretory leukocyte protease inhibitor; SLPI; Anal intraepithelial neoplasia; Human immunodeficiency virus; human papillomavirus; HPV

INTRODUCTION

Secretory leukocyte protease inhibitor (SLPI) is an 11.7 kDa serine protease inhibitor, which is produced by different epithelial cell types and in salivary glands, and hence is found in high concentrations in secretions from the cervix, nasal cavity, upper respiratory tract, prostate, and in the saliva.[1-3] Moreover, there is evidence that the protease activity of SLPI likely plays a role in the protection of the mucosa against proteolysis.[2,4] Since many viruses require protease activity for infectivity, it is not surprising that SLPI has been demonstrated to inhibit viral infections, including those by HIV-1 and influenza A.[5-11] Interestingly, however, SLPI protection against HIV-1 infection has been shown to be independent of its anti-protease activity,[12-14] which suggests that SLPI may block the interaction between HIV and a cell surface receptor. Particularly, SLPI blocked HIV-1 entry into macrophages through an interaction with annexin A2,[15] which exists at the cell surface with S100A10 as a heterotetrameric receptor (A2t).[16] Furthermore, SLPI has been shown to block human papillomavirus (HPV) entry via annexin A2 in both epithelial and Langerhans cells *in vitro*.[17,18] Conversely, reductions in the amount of SLPI secreted by epithelial cells was shown to promote HPV16 infection through annexin A2 *in vitro*.[19]

Interestingly, several studies have reported a direct correlation between the presence of HPV and low expression levels of SLPI *in vivo* in head and neck squamous cell carcinoma (HNSCC) patients.[20-23] In particular, it was shown that SLPI expression at the gene and protein level was significantly lower in metastatic versus non-metastatic HNSCC tissues,[20] and that high levels of SLPI were correlated with protection against HPV infection.[21] Additionally, high-risk HPV-positive (HPV^+) HNSCC tumors were shown to express higher levels of the HPV receptor annexin A2 than those of HPV-negative tumors, suggesting that in HNSCC, higher levels of SLPI may be protective against HPV infection through annexin A2.[23] Importantly, these studies demonstrate that there is a correlation between SLPI expression and HPV infection in HNSCC *in vivo*, though it is unknown if such a correlation exists in other HPV-associated lesions and or/cancers.

Anal intraepithelial neoplasia (AIN) is a precursor to anal cancer and is caused by persistent high-risk HPV infections.[24] Though the incidence of AIN and anal cancer are very low in the general population, both are relatively common in individuals infected with HIV-1.[25] It is well established that HPV can be found in the dysplastic squamous epithelium of AIN lesions, whereas HIV-1 is more often found in the underlying stromal cells including stromal

macrophages.[26-29] However, to date, there has been no prior research regarding SLPI distribution patterns or expression levels in the anal epithelium of AIN lesions. Additionally, while previous studies investigating SLPI have employed enzyme linked immunosorbent assays (ELISA) or western blot analyses to analyze SLPI levels,[29-34] immunohistochemical analyses provide more information with respect to the cells of origin of SLPI, and its modulation in tissues during viral infection. Given that the expression of SLPI is associated with susceptibility to HPV infection in HNSCC, and that HPV-induced AIN is more common in HIV^+ individuals, the purpose of the present study was to perform an immunohistochemical analysis to determine SLPI distribution patterns and expression levels in anal biopsies from HIV^+ individuals, and compare the resultant data to AIN diagnoses and HPV status.

METHODS

Study population and Tissue samples

The study material consisted of anorectal tissue samples obtained from HIV^+ subjects at the DST/AIDS Clinical Laboratory (LapClin-AIDS-INI) of the Evandro Chagas National Institute of Infectious Diseases (INI), Oswaldo Cruz Foundation (FIOCRUZ), Rio de Janeiro, Brazil. Eight patients underwent additional follow up biopsies at 1 and 5 months from baseline. All patients were followed up in order to monitor the progression of the lesion. Additionally, clinical parameters including CD4 counts and HIV RNA status were assessed at the time of tissue biopsy. The anorectal specimens were used to construct two tissue microarray (TMA) paraffin blocks. The biopsies were used only if sufficient tissue was present to submit as part of the TMAs. Biopsies were evaluated by an expert pathologist and classified as low-grade anal intraepithelial neoplasia (AIN1), high-grade anal intraepithelial neoplasia (AIN2/3), or normal squamous epithelium. In total, 54 tissue samples fit the criteria for inclusion, which included those from 31 males and 23 females. Additionally, laboratory and clinical variables including age at the time of the biopsy, gender, and smoking status were obtained from the cohort studies databank of LapClin-AIDS-INI. Written informed consent was obtained from all participants in strict compliance with the ethical guidelines involving human subjects in Brazil as required in the Resolution n.466/2012 of the National Health Council. The study was approved by the INI FIOCRUZ Institutional Review Board (IRB).

Immunohistochemical analysis

The preparation of the TMA blocks was performed as previously described.[35] Following the construction of the array blocks, 4 μm sections were cut with a microtome and placed on silane-coated slides for immunohistochemical analysis following published procedures.[26-29] Briefly, the 4 μm paraffin-embedded sections were dehydrated, incubated in 3% hydrogen peroxide for 10 min, and incubated in trypsin for 20 min. The sections were blocked with 10% goat serum at room temperature for 20 min and treated with a goat anti-human SLPI antibody (1:100; Minneapolis, MN, USA) at for 1h. After rinsing, the sections were treated with biotin-conjugated antibodies from the MACH 4 Kit (Biocare Medical, Concord, CA, USA) for 20 min, and streptavidin immune complexes were identified with a diaminobenzidine (DAB) substrate immunochemistry (Biocare) and hematoxylin stain.

Sections were mounted, dehydrated, and sealed with a coverslip. For the negative control, sections were treated identically except that the primary antibody was replaced with goat IgG. All steps were performed at room temperature. Semiquantitative analysis of SLPI stained cells was performed by one expert pathologist (GJN). SLPI staining was scored as 0, 1+, 2+, or 3+ based on the percent of positive target cells (normal squamous epithelia in the controls and dysplastic squamous cells in the AIN lesions) in each specimen as follows: 0 = 0%; 1-19% = 1+; 20-49% = 2+; and ≥50% = 3+, respectively. Histology slides of normal cervical, brain, and placental tissues obtained from a tissue block were stained for SLPI to validate the antibody.

HPV genotyping

Tissue biopsies were stored in liquid nitrogen before embedding in Tissue-Tek medium (Sakura Finetek, Torrance, CA, USA). Using a cryostat, three 5 μm thick sections were cut for DNA testing. DNA extraction was performed using the Illustra Tissue Genomic Prep Mini Spin Kit (GE Healthcare, Buckinghamshire, United Kingdom) according to the manufacturer's protocol. The purified DNA was quantified by spectrophotometry using the NanoDrop spectrophotometer (ND 1000, Thermo Fisher Scientific, Wilmington, MA, USA). All samples were tested for the amplification of a 110 base pair fragment of the β-globin gene using primers PC03 (ACACAACTGTGTTCACTAGC) and PC04 (CAACTTCATCCACGTTCACC). Samples negative for β-globin were excluded from the HPV DNA analysis. HPV DNA (100 ng) isolated from HeLa cells was used a positive control, and the addition of no DNA served as the negative control. DNA samples with concentrations below 3 ng/μL were subjected to thermostable amplification of total DNA with Phi29 DNA polymerase using the GenomePhi V2 DNA Amplification Kit (GE Healthcare). For detection of HPV DNA, genomic material was subjected to polymerase chain reaction (PCR) for amplification of conserved regions of the L1 gene for low- and high-risk HPV genotypes using primer pairs flanking these regions. Oligonucleotides (dNTPs) used for all reactions were acquired from Invitrogen (Sao Paulo, Brazil). Samples negative for HPV in the first reaction were then subjected to nested PCR using the GP5+ and GP6+ primers to amplify HPV DNA fragments of approximately 155 base pairs as previously reported.[36] The PCR products were purified using the NucleoSpin Gel and PCR Clean-up Kit (Macherey Nagel, Düren, Germany). Sequencing was carried out using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and analyzed with the ABI Prism 3730 Genetic Analyzer (Applied Biosystems). The obtained sequences were further analyzed using MEGA software version 6.0 (available at http://www.megasoftware.net/, and BLAST software (available http://blast.ncbi.nlm.nih.gov/Blast.cgi). We assumed that sequences with double peaks in the chromatogram, i.e. more than one base at the same position, were indicative of multiple infections. In these cases, multiple infections were analyzed using the High+Low PapillomaStrip Kit (Operon, Zaragoza, Spain), which identifies 18 low-risk and 19 high-risk HPV types.

Statistical analysis

Analyses were performed based on SLPI expression, HPV status, histopathological diagnosis, and clinical parameters. Fisher's exact and Pearson's chi-square tests were performed to assess differences between groups using SPSS 20 software (SPSS Inc.,

Chicago, IL, USA). All tests were two-sided, and p<0.05 was considered statistically significant.

RESULTS

Patient characteristics

Clinical and laboratory data provided by the cohort studies databank at LapClin-AIDS-INI identified individuals who were seropositive for HIV-1, and who had concurrent anal biopsies. It was from this data bank that we obtained the tissues used in this study. Of the 54 study participants, 31 (57.4%) were male, 23 (42.6%) were female, with ages ranging from 24.7 to 66.8 years old, and all were seropositive for HIV-1. The histopathological diagnoses of the tissue samples were as follows: 17 cases of AIN1, 17 cases of AIN2/3, and 20 histologically normal squamous epithelial samples that were used as controls. The average age was 42.5±9.1 years for individuals with normal squamous epithelium; 43.1±6.7 years for AIN1; and 44.2±11.6 years for AIN2/3 with no statistical differences in age among the groups (p=0.963 by Kruskal-Wallis test). The age, sex, HPV type detected, CD4 counts, HIV RNA status, and current smoking status of the study population are summarized in Table 1. As seen in the table, the vast majority of the study population was HPV^+ (only 1 negative, and 1 unknown), though the detection of low-risk, high-risk, or a combination of both low- and high-risk HPV genotypes was variable.

HPV DNA genotyping

HPV DNA was detected in 96.3% (52/54) of the total anal biopsies analyzed. By sequencing, we identified eight high-risk oncogenic HPV types (16, 18, 31, 33, 35, 51, 58, and 59); six low-risk HPV types (6, 11, 40, 42, 43, 54); and three HPV types classified as probable high-risk (53, 66, and 69). The most common type found in the anal biopsies was high-risk HPV16, which was detected in 20.4% (11/54) of the samples; followed by low-risk HPV6, which was detected in 16.7% (9/54). Multiple HPV infections were found in 4 specimens (7.4%).

SLPI expression in normal anal squamous epithelium and AIN tissues

Prior to immunohistochemical analysis of normal anal squamous epithelium and AIN tissues, the SLPI antibody was validated by staining normal cervical transformation zone, which exhibited an intense SLPI signal in the epithelia, whereas brain, and kidney tissues each showed no signal in agreement with previous reports (data not shown).[2] We next evaluated the distribution of SLPI in the histologically normal anorectal epithelium. Each of these tissues showed histologically normal squamous epithelium and many showed adjacent normal columnar epithelia of the rectum. In these normal tissues, SLPI tended to be more highly expressed in the more differentiated squamous cells whereas there was weaker SLPI staining towards the basal zone (Figure 1A). Overall, 17/20 (85.0%) of these samples had high SLPI expression scores defined as either 2+ (4/20) or 3+ (13/20) (Table 2 and Figure 2A). We next evaluated the expression and distribution patterns of SLPI in the AIN biopsies. AIN1 lesions were found to have generally weak SLPI staining in the middle and upper thirds of the lesion (Figure 1B). Specifically, only 9/17 (52.9%) of the AIN1 tissues received high SLPI expression (2+/3+) scores (Table 2 and Figure 2A). In the AIN2/3 lesions, no

(7/17) or weak (5/17) SLPI staining was observed in the majority of the tissues (Figure 1C), whereas only 5/17 (29.4%) of the samples received high SLPI expression scores (Table 2 and Figure 2A). Moreover, the number of samples receiving high SLPI expression scores from AIN tissues was significantly lower compared to that from histologically normal anorectal tissues ($p<0.005$).

SLPI expression across HPV status and other clinical parameters

Next, we evaluated the distribution of SLPI expression scores in regards to HPV status as well as other well-established immune biomarkers of HIV infection, in particular CD4 counts and HIV viral load. We found that there was a significantly greater proportion of high SLPI expression (10/12) in tissues where low-risk HPV types were detected compared to tissues where high-risk HPV types were detected (20/40) ($p<0.040$) (Figure 2B). Hence there was an inverse correlation between high-risk HPV infection and the level of SLPI expression, though this association was less apparent when viewed across individualized SLPI expression scores, which was likely due to the small sample size (Table 2). While the majority of samples were found to be HPV^+, only one sample was found to be HPV negative and was histologically normal as expected. Additionally, one AIN2/3 sample was found to be of unknown HPV type, which may have been due to the presence of a high-risk HPV genotype that was not covered in the primers used herein. No associations with SLPI signal were evident between other clinical parameters that could have impacted SLPI protein expression, such as smoking, CD4 counts or HIV RNA status (Table 2). Interestingly, while smoking is known to play a role in the development of anogenital cancers,[37] and we previously reported a correlation between SLPI expression and smoking status,[21] no such correlation was observed herein ($p=0.767$), which may have been due to too few of smokers (N=14) in the current study population.

AIN diagnoses across HPV type

The distribution of AIN diagnoses across HPV types was assessed, and the Fisher's exact test revealed that there was no statistical correlation between AIN status and HPV type ($p=1.000$). For example, there was a similar distribution of high-risk HPV^+ samples among normal (14/36), AIN1 (10/36), and AIN2/3 (12/36) (Table 3).

DISCUSSION

There are several sites in the human body where different types of epithelial tissue meet at distinct transitions. These sites are typically referred to as transformation zones, such as when the squamous epithelium distal to mucosal sites merge with the glandular/columnar epithelium typical of mucosal layers.[38] The two most studied transformation zones are those of the cervix and anorectal junction, and each of these are classic sites of transmission for HIV-1 and HPV.[26-28] The target cells for HIV-1 in the transformation zone are mucosal Langerhans cells, which can transmit the virus to underlying macrophages or disseminate the virus directly to T cells.[26,27] In comparison, the target cells for HPV are the squamous metaplastic cells, which reside only in the "active" area of the transformation zone where over 95% of cervical cancers occur.[28] Thus, many investigators have examined the levels of SLPI in cervical secretions given its role in viral infections,[29,31,39] while less is known about

SLPI expression in anal epithelium. Data regarding the influence of HIV-1 infection on SLPI concentrations in bodily fluids is conflicting. For example, several studies have concluded that SLPI levels are decreased in cervical secretions and saliva of HIV-1 infected individuals.[29-33] However, other studies have indicated that HIV-1 infection may increase salivary SLPI.[40,41] For these reasons, in the current study we chose to investigate SLPI levels in an HIV^+ cohort to reduce potential confounding. Moreover, this cohort is far more afflicted by precancerous anal lesions and anal cancer, which was the focus of this study.

One of the observations from the current study was that epithelial cells in the anorectal junction were the primary source of SLPI expression. Moreover, the squamous epithelium was observed to produce abundant SLPI levels in all layers except the basal layer. Interestingly, SLPI is a ligand for annexin A2,[15] which we have shown is a receptor for HPV,[17,42] and annexin A2 expression is restricted to the basal and suprabasal layers of stratified epithelium,[43] matching the tropism of HPV infection. This may suggest that even small overall reductions of SLPI expression in the epithelial layer may dramatically affect the amount of SLPI bound to annexin A2 at the basal cell surface, thus promoting HPV infection through its putative receptor. This notion is further supported by our data showing the significantly reduced levels of SLPI observed in tissues infected by high-risk HPV types and in high-grade AIN lesions. Additionally, glandular epithelium expressed much less SLPI than squamous epithelium in the anorectal junction, and minimal SLPI was observed in the stroma. As epithelial cells can secrete directly into the mucosal lumen, it can be inferred that inhibition by SLPI in the anorectal junction occurs primarily in the epithelial layers and subsequently in the lumen to which they secrete.

As mentioned, our cooperating group previously reported that SLPI expression levels were inversely correlated with HPV^+ HNSCC.[20,21] Specifically, it was demonstrated that HNSCC patients with high-risk HPV infections showed little to no SLPI expression, whereas HNSCC patients without HPV infections had high SLPI expression.[21] However, in the current study, such a direct comparison could not be performed as HPV was detected in all but one patient. This is likely because highly sensitive PCR methods can detect subclinical HPV infection as well as clinical HPV infection that has resulted in cytological changes in the epithelium. This particular study population is known to be a high-exposure high-risk group,[44-46] therefore the high prevalence of HPV infection was not surprising. As such, we evaluated the potential relationship between the proportions of samples with different SLPI expression levels and either low- or high-risk HPV infection. Through this analysis, we found that there was an inverse correlation between high-risk HPV infection and the level of SLPI expression, which is in agreement with our previous results.[20,21] Interestingly, we also previously found that smoking increased SLPI expression, and as such, smokers were less likely to develop HPV^+ HNSCC.[21] However, no such correlation was observed herein, which may have been due to the small numbers of current smokers in the study cohort, or potentially because smoking has less of an influence on SLPI levels in the anorectal region compared to the head and neck region.

A question that remains is whether reduced SLPI expression results in HPV infection, as in those with low expression levels have increased susceptibility leading to AIN development, or whether HPV infection itself actively down-regulates SLPI. Since participants of this

study had both HPV and HIV-1 infections, it is difficult to assess if reduced SLPI expression in AIN represents an effect of viral infection. When comparing AIN grade to SLPI expression, it was clear that in less differentiated squamous cells, there was generally weaker SLPI staining, which may suggest that the de-differentiation of squamous cells during AIN progression leads to reduced SLPI. However, future studies with larger sample sizes will allow for multivariate analyses to address whether certain factors influence both AIN and SLPI levels, since this was a limitation of the current study. In any case, a scenario where SLPI expression is reduced likely increases the potential for HPV to infect more cells, and hence promotes AIN development. Though the complete ramifications of reduced SLPI expression in AIN awaits further study, we speculate that it may permit more effective HPV co-infection due to the increased availability of the putative annexin A2 receptor in the basal epithelium of anorectal lesions.

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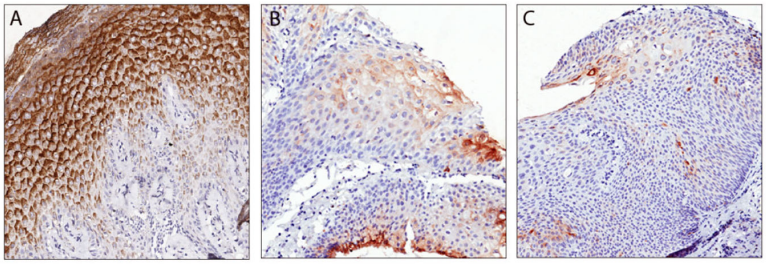

Figure 1. Immunohistochemical staining of SLPI in anal biopsies

A. Histologically normal squamous epithelium that lines the anus shows strong SLPI staining (brown) in the more differentiated squamous cells in the middle and superficial layers, and less SLPI expression in the basal and stromal layers (40× magnification). **B.** Epithelium from an AIN1 lesion shows weaker SLPI staining in dysplastic squamous cells (40× magnification). **C.** Epithelium from an AIN3 lesion shows a further reduction in SLPI staining in dysplastic squamous cells (20× magnification).

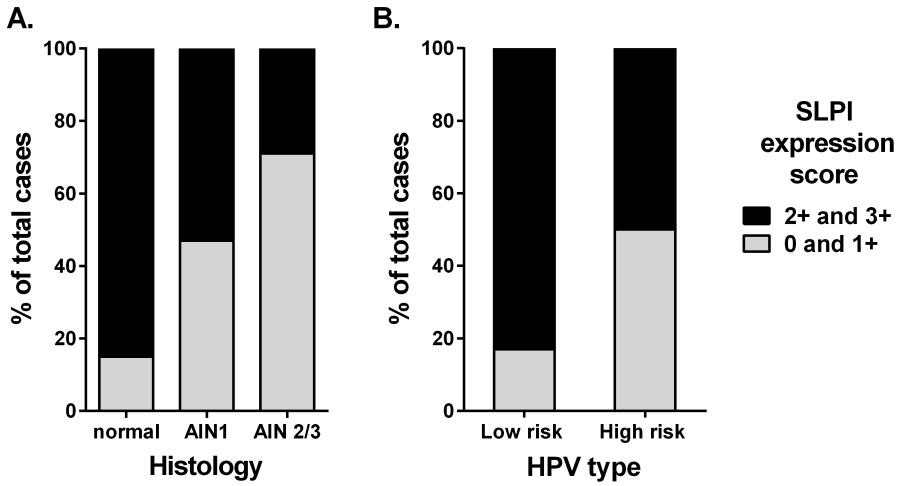

Figure 2. Proportion of SLPI expression scores according to anal histology and HPV type

A. The proportion of high SLPI expression scores (2+ and 3+) and low SLPI expression scores (0 and 1+) for normal anal squamous epithelium, AIN1, and AIN2/3. The proportions were found to be statistically different by chi-squared test (p=0.005). **B.** The proportion of high SLPI expression scores (2+ and 3+) and low SLPI expression scores (0 and 1+) for samples with low-risk and high-risk HPV infections. Samples where both low- and high-risk HPV types were detected were counted as high-risk. The proportions were found to be statistically different by chi-squared test (p=0.040).

Table 1

Patient characteristics

Characteristic		N (%)
Number of patients		54 (100%)
Age	< 40 years	19 (35.2)
	40-49 years	23 (42.6)
	> 50 years	12 (22.2)
Sex	Male	31 (57.4)
	Female	23 (42.6)
HPV type	Negative	1 (1.9)
	Low risk[a]	12 (22.2)
	High risk[b]	36 (66.7)
	Low & High risk[c]	4 (7.4)
	Unknown	1 (1.9)
Current CD4	< 50	1 (1.9)
	50-99	1 (1.9)
	100-199	2 (3.7)
	200-349	12 (22.2)
	350-499	17 (31.5)
	≥ 500	21 (38.9)
HIV RNA viral load	Undetectable	35 (64.8)
	Detectable	19 (35.2)
Current Smoking	No	39 (72.2)
	Yes	14 (25.9)
	Unknown	1 (1.9)

[a] Low risk HPV types detected: 6, 11, 40, 42, 43, and 54

[b] High risk HPV types detected: 16, 18, 31, 33, 35, 51, 58, and 59

[c] Both low and high risk HPV types were detected in these specimens

Table 2

SLPI expression scores across different characteristics

Characteristic		SLPI signal					
		0	1+	2+	3+	Total	*p* value[a]
		N (%)	N (%)	N (%)	N (%)	N (%)	
Histopathology	Normal	-	3 (15.0)	4 (20.0)	13 (65.0)	20 (100.0)	0.003*
	AIN1	4 (23.5)	4 (23.5)	6 (35.3)	3 (17.6)	17 (100.0)	
	AIN2/3	7 (41.2)	5 (29.4)	2 (11.8)	3 (17.6)	17 (100.0)	
	Total	11 (20.4)	12 (22.2)	12 (22.2)	19 (35.2)	54 (100.0)	
HPV Type[b]	Low risk	-	2 (16.7)	4 (33.3)	6 (50.0)	12 (100.0)	0.177
	High risk	10 (25.0)	10 (25.0)	8 (20.0)	12 (30.0)	40 (100.0)	
	Total	10 (19.2)	12 (23.1)	12 (23.1)	18 (34.6)	52 (100.0)	
Current CD4	< 200	1 (25.0)	2 (50.0)	1 (25.0)	-	4 (100.0)	0.590
	200-350	2 (16.7)	2 (16.7)	4 (33.3)	4 (33.3)	12 (100.0)	
	> 350	8 (21.1)	8 (21.1)	7 (18.4)	15 (39.5)	38 (100.0)	
	Total	11 (20.4)	12 (22.2)	12 (22.2)	19 (35.2)	54 (100.0)	
HIV RNA viral load	Undetectable	5 (14.3)	9 (25.7)	8 (22.9)	13 (37.1)	35 (100.0)	0.526
	Detectable	6 (31.6)	3 (15.8)	4 (21.1)	6 (31.6)	19 (100.0)	
	Total	11 (20.4)	12 (22.2)	12 (22.2)	19 (35.2)	54 (100.0)	
Current smoking status[c]	Non-smokers	9 (23.1)	8 (20.5)	8 (20.5)	14 (35.6)	39 (100.0)	0.767
	Smokers	2 (14.3)	4 (28.6)	4 (28.6)	4 (28.6)	14 (100.0)	
	Unknown	-	-	-	1 (100.0)	1 (100.0)	
	Total	11 (20.4)	12 (22.2)	12 (22.2)	19 (35.2)	54 (100.0)	

[a] Fisher's exact test

[b] HPV-negative (N = 1, 1.9%) and missing data (N = 1, 1.9%) samples were excluded from analysis

[c] Missing data (N = 1, 1.9%) sample was excluded from analysis

* $p<0.05$

Table 3

Anal histopathology status across HPV type

HPV type	Histopathology				
	Normal	AIN1	AIN2/3	Total	*p* value[a]
	N (%)	N (%)	N (%)	N (%)	
Low risk	4 (33.3)	4 (33.3)	4 (33.3)	12 (100.0)	
High risk	15 (37.5)	12 (30.0)	13 (32.5)	40 (100.0)	1.000[b]
Total	19 (36.5)	16 (30.8)	17 (32.7)	52 (100.0)	

[a] Fisher's exact test

[b] HPV-negative (N = 1, 1.9%) and missing data (N = 1, 1.9%) samples were excluded from analysis