Human papillomavirus 16 L1 gene methylation as a potential biomarker for predicting anal intraepithelial neoplasia in men who have sex with men (MSM)

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Abstract

The human papillomavirus (HPV) 16 early promoter and L1 gene methylation were quantitatively measured using pyrosequencing assay in anal cells collected from men who have sex with men (MSM) to determine potential biomarkers for HPV-related anal cancer. The methylation patterns of HPV16 genes, including the early promoter (CpG 31, 37, 43, 52, and 58) and L1 genes (CpG 5600, 5606, 5609, 5615, 7136, and 7145), were analyzed in 178 anal samples. The samples were diagnosed as normal, anal intraepithelial neoplasia (AIN) 1, AIN2, and AIN3. Low methylation levels of the early promoter (<10%) and L1 genes (<20%) were found in all detected normal anal cells. In comparison, medium to high methylation (≥20–60%) in the early promoter was found in 1.5% (1/67) and 5% (2/40) of AIN1 and AIN2-3 samples, respectively. Interestingly, slightly increased L1 gene methylation levels (≥20–60%), especially at the HPV16 5’L1 regions CpGs 5600 and 5609, were demonstrated in AIN2-3 specimen. Moreover, a negative correlation between high HPV16 L1 gene methylation at CpGs 5600, 5609, 5615, and 7145 and a percentual CD4 count was found in AIN3 HIV positive cases. When comparing the methylation status of AIN2-3 to that of normal/AIN1 lesions, the results indicated the potential of using HPV16 L1 gene methylation as a biomarker for HPV-related cancer screening.

Introduction

Anal carcinoma is a rare disease found globally in men and women, with an incidence of <1–2 cases per 100,000 [1]. However, a high incidence of anal cancer was present in HIV-infected women and HIV-infected men who have sex with men (MSM), accounting for 30/100,000 and 131/100,000, respectively [2]. There is an association between human papilloma-virus (HPV) infection and anal carcinoma; HPV DNA was found in men (68.7–91.2%) and
women (90.4–90.9%) with anal carcinoma worldwide [3]. Studies showed that HPV16 was the most prevalent type found in anal carcinoma that detected in 70–71.6% of men and 74–83.4% of women [2,4,5].

A high prevalence of HPV infection was reported in anal cells collected particularly from HIV-infected MSM [6–11]. The worldwide HPV prevalence in anal cells of HIV-infected and HIV-uninfected MSM was 92.6% and 63.9%, respectively, and HPV16 was found in 35.4% and 12.5% of HIV-infected and HIV-uninfected MSM, respectively [12]. Recent studies in Asian countries, for example, in China reported a high prevalence of HPV infection among HIV-infected MSM (82.69%) compared to HIV-uninfected MSM (62.81%) [13], in Korea and Japan reported HPV infection rate were 82.7% and 75.9% of HIV-infected MSM, respectively [14,15]. In Bangkok, Thailand, anal HPV infections were found in 85% of HIV-infected MSM compared to 58.5% in HIV-uninfected MSM; HPV16 was detected in 22.5% and 9.8% of HIV-infected and HIV-uninfected MSM, respectively [16]. One study reported a high prevalence of HPV16 infection in both HIV-infected (54.9%) and HIV-uninfected (61.1%) MSM with a histological diagnosis of AIN3 [18]. It was reported that HPV16 was the most persistent high-risk HPV type [19,20] and less likely to spontaneously regress from cervical intraepithelial neoplasia (CIN)2-3 to normal when compared to other HPV types [21,22]. The study in HIV-uninfected MSM [23] and HIV-infected MSM [6,24] revealed that HPV16 showed the longest duration of infection with the lowest rate of viral clearance compared to low-risk and other high-risk HPV types.

High-risk HPV is considered to be the causative agent of cervical cancer and other HPV-related cancers such as vulva, anal, head, and neck cancer. The viral oncoproteins E6 and E7 disrupt the normal function of host proteins involved in cell cycle regulation, where E6 causes p53 degradation and E7 inactivates retinoblastoma proteins [25–27]. However, HPV-related cancer development takes more than 10–20 years, while most HPV-infected populations spontaneously regress [28,29]. The up-regulation of the viral oncogenes E6 and E7 [30–32] and the down-regulation of viral proteins involved in viral particle assembly, such as the L1/L2 proteins [33–35], are correlated with cancer progression. Epigenetic modifications such as methylation of the HPV genome is considered to be one factor that controls the expression of viral genes during productive and transforming infections [36].

Differential methylation of the HPV16 genome has been reported in cervical samples during productive infections [36,37], the HPV16 early promoter was unmethylated in basal and intermediate cells at the proximal E2 binding sites 2–4 (E2BS) but became highly methylated in superficial cells at the upper part of the epithelium [36]. In latent HPV16 infection, the viral long control region (LCR), which encompasses the early promoter, was highly methylated throughout the epithelium. In transforming HPV16-infected cells, the distal E2BS (E2BS1) and enhancer regions were found to be methylated, while the early promoter was unmethylated [36]. One study showed that the HPV16 p670 late promoter was highly methylated in cervical carcinoma cases [38]. The low expression of the early viral gene and lack of the capsid L1/L2 proteins expression in undifferentiated basal cells prevented the activation of an immune response to viral infection [39].

The methylation pattern in early promoters, especially HPV16 E2BS (CpG positions 31, 37, 43, 52, and 58), has been widely studied in cervical cells, and the observed methylation patterns were either progressive hypomethylation [40–42] or progressive hypermethylation [38,43,44]. One study showed that a high methylation of E2BS was correlated with the episomal form and multiple copies of integrated HPV genome in high-grade cervical lesions and cervical
carcinoma [45]. HPV 16 L1 gene hypermethylation was correlated with severe cervical lesions and cervical carcinoma [46–49]. It was reported that the HPV16 3’L1 CpG positions 7136 and 7145 [50] and the 5’L1 CpG positions 5600, 5606, 5609, and 5615 (some published paper mentioned 5602, 5608, 5611, and 5617, respectively, according to reference sequence used in the studies [51,52]) were highly methylated in cervical cancers [49,51–54]. A recent study reported that a high methylation at CpG 5611 and 7145 respectively predicted the presence of CIN2 + and CIN3+ with high accuracy [55]. Our group has previously reported an association between high methylation of the HPV16 L1 gene, especially at the CpG sites 5600 and 5609, and high-grade cervical lesions and cervical carcinoma [49]. However, there is a limited number of studies on the HPV16 genome methylation in anal cells [56–58], and to the best of our knowledge, there has been no HPV methylation study in anal cells collected from Asian countries. Therefore, the investigation of the HPV16 methylation status of these CpG positions in anal cells is of scientific interest. We aimed to detect the methylation pattern of the HPV16 genome in the CpG positions within the early promoter and L1 regions in anal cells obtained from the Thai men who have sex with men (MSM), analyzed by a quantitative pyrosequencing assay.

**Materials and methods**

**Clinical samples and cell lines**

This study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (COA No. 053/2016). The present study is a retrospective study of 178 archived HPV16-positive DNA samples extracted from anal cells; therefore, no informed consent was required from the patients. These samples were collected from MSM at the Thai Red Cross AIDS Research Centre (TRC-ARC) Bangkok, Thailand, between May 2013 and December 2013. All samples were anonymized. There were 134 HIV-infected cases and 44 HIV-uninfected cases. The percentages of CD4+ results were only obtained from HIV-infected men. The DNA was extracted from human cervical cancer cell lines that contained integrated HPV 16. CaSki (CRL-1550 Lot No.3794357) and SiHa (HTB-35 Lot No.4031219) were used as positive controls for the amplification and pyrosequencing. They respectively contained approximately 500–600 copies or 1–2 copies per cell.

**Specimen collection and DNA extraction**

A moistened, non-lubricated flocked swab (Rovers EndoCervex-Brush, Rover Medical Devices B.V., Netherlands or FLOQSwabs, Copan Italia S.p.A., Italy) was used to collect anal cells from the anal canal surfaces. After sample collection, the anal swab was placed and kept in a liquid-based cytology (LBC) fluid (Liqui-PREP™ LGM International, Inc., Florida, USA) at 2–8°C until the DNA extraction within 7 days. DNA was extracted from anal cells according to the manufacturer’s protocol using the AmpliLute Liquid Media Extraction kit (Roche Molecular Diagnostics, California, and USA) and DNA was collected in 120 μL of elution buffer.

**HPV DNA detection**

Extracted DNA samples from LBC were subjected to HPV genotyping using the Linear Array HPV genotype test (Roche Molecular System, Inc., Mannheim, Germany). Extracted DNA samples were amplified for HPV genotypes and the beta-globin gene. HPV and beta-globin amplicons were hybridized with oligonucleotide probes for specific HPV and beta-globin and detected by colorimetric determination. The test kit could detect the following 37 HPV genotypes: 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84, IS39, and CP6108.
HIV detection and CD4 cell count

At an anonymous clinic, the Architect HIV Ag/Ab combo kit (Abbott Laboratories, GmBH, Wiesbaden, Germany) was used as the screening test, followed by Alere DetermineTM HIV ½ (Abbott Laboratories) and Serodia HIV ½ (Fujirebio, Tokyo, Japan) as the confirmatory test. BD FACSCount™ CD4 reagents (BD Biosciences, San Jose, CA, USA) were used to enumerate the absolute counts and percentages of CD4 T lymphocytes in unlysed whole blood (CD4 counts and CD4 percentages). The reagents were intended for in vitro diagnostic on a BD FACSCount™ instrument.

Methylation analysis by a pyrosequencing assay

The extracted DNA (100–1000 ng) from anal cells was subjected to bisulfite conversion by using the EZ kit Gold Bisulfite Conversion Kit (Zymo Research) according to the manufacturer’s instructions. The sequences of forward, reverse, and sequencing primers for the early promoter CpG positions 31, 37, 43, 52, and 58, the 3’L1 CpG positions 7136, and 7145, and the 5’L1 CpG positions 5600, 5606, 5609, and 5615 are shown in Table 1.

The PCR amplification protocol was as follows: 1x PCR buffer, 2.5 mM MgCl2, 250 μM dNTP, 12.5 PM of each forward and reverse primer, 1 Unit DNA polymerase (HotStart HiFi-delicity Polymerase, Affymetrix, USA), 2.5 μl of bisulfite-treated DNA and DNase/RNase-free water were added to the final volume of 25 μl. The PCR amplification was started with an initial denaturing at 95˚C for 10 minutes, followed by 50 cycles of 95˚C for 30 seconds, 55˚C for 1 minute, and 72˚C for 1 minute, and a cycle for the final extension at 72˚C for 10 minutes. The PCR products were detected by 1.5% agarose gel electrophoresis. Prior to pyrosequencing, all reagents including 70% ethanol, denaturation buffer and washing buffer were prepared in Milli-Q water and placed on the PyroMark Q96 Vacuum Workstation. For sample preparation, 20 μl of biotin-labeled amplification products were mixed with 2 μl of streptavidin sepharose beads (GE Healthcare Bio-science AB, Uppsala, Sweden) in 40 μl of PyroMark binding buffer (QIAGEN, Hilden, Germany), added Mill-Q water to a total volume of 80 μl and then agitated at 1400 rpm for at least 10 minutes. After switch on the vacuum pump, the filter probes were placed into the tube containing the beads, after all liquid was aspirated and beads were captured onto the filter probed. Next, the filter probes were washed by flushing

| Target gene | Nucleotide sequences | Size (bp) | Reference |
|-------------|----------------------|-----------|-----------|
| Early promoter: CpG 31, 37, 43, 52, and 58 | FW: 5’-TTGTAAAATTTGTATAATGGGTTTG-3’ RV: Biotin-5’-AAATCCTAAACATTA GAATTCCT-3’ Sequencing primer: 400S1: 5’-AATTTTATATGTTAAATTTAGGG-3’ Sequence to analyze: YGTAAYGAAATYGGTTGAATGAGTTAGTA | 192 | [59] |
| 3’L1: CpG 7136, and 7145 | FW: Biotin-5’-GGTTAATTAAAAATTTAATAGGAAA-3’ RV: 5’-AAAACATATCAACCAACAACACACTAACTTC-3’ Sequencing primer: 800: 5’-TACATACAATTACCTCAGCT-3’ Sequence to analyze: TACCTTTTTTACCTTTTACATATAAAA | 140 | [49] |
| 5’L1: CpG 5600, 5606, 5609, and 5615 | FW Biotin 5’-TAAATATATTTAGTTGGATTGAT-3’ RV 5’-AAGTTAACCCACTAAACACACAAA-3’ Sequencing primer: 5600: 5’-AAGAAAACATCTAAAAAATATAATA-3’ Sequence to analyze: AACRTTACRTCRTTTTCRTAACAT | 130 | [49] |

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with 70% ethanol for 5 seconds, denatured in the PyroMark denaturation solution (QIAGEN, Hilden, Germany) for 5 seconds. The filter probes were then flushed again in PyroMark wash buffer (QIAGEN, Hilden, Germany) for 10 seconds. Switch off the vacuum pump, mixed the beads captured PCR products with 0.4 μM of the sequencing primers in 40 μL of PyroMark annealing buffer (QIAGEN, Hilden, Germany) prepared in pyromark plate low, after that, placed the plate on a heating block at 80°C for 2 min and cooled down to room temperature for at least 5 min. Then, the PyroMark nucleotides, substrate and enzyme mixture (QIAGEN, Hilden, Germany) were loaded into cartridge according to the calculated volume after setting up the run in the PyroMark Q96 Software. Finally, the pyromark plate low and filled reagent cartridge were loaded onto the PyroMark™ Q96 machine (Qiagen, Hilden, Germany). After the run, the software will measure the percent methylation value of each CpG site shown in the analyzed pyrogram. The bisulfite conversion control (Single cytosine was completely converted to uracil) was highlighted in yellow bar must be lacking an intensity signal, thus, no peak was found within the analyzed sequence. The gray bar represents the analyzed CpG sites within the sequence. The percent methylation value of each CpG site that perfectly pass quality control are indicated in blue on the top of the gray bar (S1–S3 Figs).

Statistical analysis

The Kruskal–Wallis test was used to analyze the differences in the mean methylation values among the groups of specimens. The Fisher exact test was used to examine the significant differences of the sample proportions with a methylation ≥ 20% between normal/AIN1 and AIN2-3. Pearson’s correlation coefficient (r) was used to analyze the association between the percentage of methylation and the CD4 count. A P value less than 0.05 was considered a statistically significant difference.

Results

Methylation levels of the HPV16 early promoter and the L1 gene in cervical cancer cell lines

Of 178 HPV16 positive samples, including 134 HIV-infected and 44 HIV-uninfected samples, the mean patient age was 31.23 years. Histology results were obtained from 123 samples classified as normal (n = 16), AIN1 (n = 67), AIN2 (n = 12), and AIN3 (n = 28). The methylation patterns in the HPV16 early promoter and the two regions within the HPV16 L1 gene were analyzed. The methylation levels of the early promoter comprising the 5 CpGs (31, 37, 43, 52, and 58), including the proximal E2 binding sites (E2BSs) and the Sp1 binding site of CaSki, were 70%, 60.5%, 73.5%, 66.5%, 77%, respectively. For SiHa cells, the methylation levels were 0–1% for all 5 CpGs. The methylation levels of the L1 gene (CpG 5600, 5606, 5609, 5615, 7136, and 7145) were 84%, 59%, 76%, 65%, 69%, and 67% for CaSki and 95%, 96%, 80%, 80%, 69%, and 76% for SiHa, respectively, as shown in Fig 1. A high methylation of the L1 gene was detected in both cervical cell lines regardless of the copy number of integrated HPV16. Pyrogram of hyper-methylated and hypo-methylated HPV16 genome was shown in S1–S3 Figs.

Methylation levels of the HPV16 early promoter and the L1 gene in anal cells

The methylation levels of the early promoter in normal, AIN1, AIN2, and AIN3 are shown in Fig 2. The methylation level was < 10% in all normal anal samples, while an intermediate to high methylation level (≥ 20–60%) was found in 1.5% (1/67) and 5% (2/40) in AIN1 and AIN2-3 samples. The majority of AIN1, AIN2, and AIN3 showed a low methylation level in the early
promoter (< 10%). For the L1 gene, a low methylation level was found in all detected normal anal samples (< 20%). There was a slight increase in L1 gene methylation from normal to AIN3, especially at CpG 5600 and 5609, for which the methylation levels were higher than for other CpGs (5606, 5615, 7136, and 7145) (Fig 3). Intermediate to high methylation levels (≥ 20–60%) of CpG 5600 were found in 0% (0/16) of normal, 10.5% (7/67) of AIN1, 25% (3/12) of AIN2, and 28.6% (8/28) of AIN3 (P < 0.05) cases, while CpG 5609 methylation was found in 0% (0/16) of normal, 3% (2/67) of AIN1, 16.7% (2/12) of AIN2 and 7.1% (2/28) of AIN3 cases (P > 0.05) (Table 2).

Correlation between the CD4+ percentage and HPV16 gene methylation
There were no statistically significant differences in the mean HPV16 methylation percentage between HIV-uninfected and HIV-infected cases (Table 3). There was no correlation between the HPV16 L1 gene methylation and a low CD4 count in normal, AIN1, and AIN2 cases. Interestingly, high gene methylation of HPV16 L1 was moderately correlated with a low percentual CD4 count in AIN3 HIV-infected cases, especially at CpGs 5600, 5609, 5615, and 7145 (R = 0.4692–0.5412) (Fig 4).

Discussion
In the present study, the methylation patterns of the HPV16 early promoter and the L1 region in anal cells were studied using a pyrosequencing assay. The quantitative methylation analysis
was first analyzed in CaSki and SiHa control cell lines, where the methylation levels were consistent with previous reports [47,59–62]. HPV16 and 18 L1 gene hypermethylation have been reported previously in cervical carcinoma, vulva intraepithelial neoplasia (VIN), oral carcinoma, and penile carcinoma [47,48,63–68]. Hypermethylation in the L1 gene was found to be correlated with an integration form of HPV16 [65,69]. The present study found a high methylation of the HPV16 L1 gene in some of the AIN2/3 samples compared to normal anal cells. We also found that the CpG sites 5600 and 5609 showed a higher methylation (≥20%) compared to the other CpG sites in the L1 region (5606, 5615, 7136, and 7145) (Fig 1 and Table 2). Previous reports in cervical cells showed that the CpG sites 5600 and 5609 were the best sites for separation of normal cervical cells and high-grade dysplasia [47,49,54]. The methylation patterns of the HPV16 L1 gene in anal cells were similar to cervical cells and might therefore be used to distinguish normal cells from HPV-related abnormal cells. Anal samples with intermediate to high methylation levels (≥20%) in the present study may indicate an increased risk of more rapid progression compared to those with low methylation levels (<20%).

Methylation patterns of the HPV16 early promoter have been widely studied in cervical cells. Nevertheless, controversial results were found. Some studies reported hypomethylation of HPV16 early promoter in cervical carcinoma or so-called progressive hypomethylation [40,41]. Other studies reported hypermethylation or progressive hypermethylation of the early promoter in cervical carcinoma [38,43,44,48,50]. The physical state and copy number of integrated HPV16 genomes were the main reason for these methylation differences within the early promoter, as shown in CaSki and SiHa cells. There was evidence for closed chromatin within the viral oncogene promoter region due to hypermethylation of multiple copies of the integrated HPV, leading to controlling the expression level of viral oncogenes to be optimal that facilitates the survival of cancer cells [70,71]. An episomal form of the HPV16 genome was found in high-grade cervical lesions, and cervical carcinoma displayed high methylation levels at E2BS in the early promoter compared to a single integrated HPV16 genome [45]. One study showed a high methylation of the early promoter in high-grade anal cells [58]. The E2
protein plays a role in controlling viral oncogene expression by either activating or suppressing viral oncogene promoter depending on the E2 protein concentration [72–74]. It was hypothesized that the E2BS methylation of episomal HPV16 with an intact E2 gene could prevent the binding of the E2 protein at the proximal E6/E7 oncogene promoter, thus preventing the suppressive activity of E2 protein leading to the overexpression of viral oncogenes [75].

An anal cytology assay has been used for screening of abnormal anal cells [76,77]; however, there was a wide range of assay sensitivity, varying from 19–89%, for detecting high-grade AIN [78–81]. The pooled specificity of the HR-HPV for detecting anal cells diagnosed as AIN2+ was only 33.1% [82]. The combination of the established methods with a more specific assay, such as HPV16 L1 methylation, would improve the specificity to detect abnormal anal cells as reported in high-grade cervical lesions and cervical carcinoma [45,55,83].
Table 2. Patient age and methylation status of the HPV16 early promoter and the L1 genes in anal cells with various grades of lesions.

| Group | Normal | AIN1 | AIN2 | AIN3 | P value |
|-------|--------|------|------|------|---------|
| No. (123) | 16 | 67 | 12 | 28 | > 0.05 |
| Age (years): | Mean | 27.93 | 31.06 | 31.3 | 32.52 | > 0.05 |
| | SD | 6.82 | 6.66 | 5.66 | 6.27 | |
| | Range | 22–47 | 19–50 | 25–42 | 22–52 | |
| % Methylation level: | CpG 31 | | | | |
| Mean | 0.300 | 1.597 | 0.667 | 3.00 | > 0.05 |
| SD | 0.483 | 4.568 | 0.888 | 7.299 | |
| Range | 0–1 | 0–34 | 0–2 | 0–28 | |
| % samples ≥ 20% | 0% | 1.5% | 0% | 7.1% | > 0.05 |
| % samples < 20% | 100% | 98.5% | 100% | 92.9% | |
| CpG 37 | | | | | |
| Mean | 0.400 | 1.016 | 0.583 | 2.269 | > 0.05 |
| SD | 0.516 | 3.011 | 0.900 | 5.625 | |
| Range | 0–1 | 0–23 | 0–3 | 0–23 | |
| % samples ≥ 20% | 0% | 1.5% | 0% | 7.1% | > 0.05 |
| % samples < 20% | 100% | 98.5% | 100% | 92.9% | |
| CpG 43 | | | | | |
| Mean | 0.600 | 1.065 | 0.250 | 2.538 | > 0.05 |
| SD | 0.699 | 4.745 | 0.452 | 7.824 | |
| Range | 0–2 | 0–37 | 0–1 | 0–31 | |
| % samples ≥ 20% | 0% | 1.5% | 0% | 7.1% | > 0.05 |
| % samples < 20% | 100% | 98.5% | 100% | 92.9% | |
| CpG 52 | | | | | |
| Mean | 0.700 | 1.532 | 1.083 | 3.115 | > 0.05 |
| SD | 0.823 | 3.745 | 1.084 | 6.364 | |
| Range | 0–2 | 0–28 | 0–3 | 0–26 | |
| % samples ≥ 20% | 0% | 1.5% | 0% | 7.1% | > 0.05 |
| % samples < 20% | 100% | 98.5% | 100% | 92.9% | |
| CpG 58 | | | | | |
| Mean | 0.400 | 1.500 | 0.333 | 3.231 | > 0.05 |
| SD | 0.516 | 5.331 | 0.492 | 8.878 | |
| Range | 0–1 | 0–41 | 0–1 | 0–35 | |
| % samples ≥ 20% | 0% | 1.5% | 0% | 7% | > 0.05 |
| % samples < 20% | 100% | 98.5% | 100% | 93% | |
| CpG 5600 | | | | | |
| Mean | 8.111 | 9.00 | 11.500 | 14.346 | > 0.05 |
| SD | 3.887 | 8.976 | 12.494 | 13.069 | |
| Range | 0–13 | 0–50 | 0–40 | 0–57 | |
| % samples ≥ 20% | 0% | 10.5% | 25% | 28.6% | < 0.05* |
| % samples < 20% | 100% | 89.5% | 75% | 71.4% | |
| CpG 5606 | | | | | |
| Mean | 6.889 | 3.593 | 4.417 | 4.000 | > 0.05 |
| SD | 5.183 | 3.616 | 5.017 | 4.699 | |
| Range | 0–15 | 0–16 | 0–14 | 0–23 | |
| % samples ≥ 20% | 22% | 0% | 0% | 3.6% | > 0.05 |

(Continued)
### Table 2. (Continued)

| Group     | Normal | AIN1 | AIN2 | AIN3 | P value |
|-----------|--------|------|------|------|---------|
| % samples < 20% | 78%   | 100% | 100% | 96.4% |         |
| CpG 5609  |        |      |      |      |         |
| Mean      | 3.667  | 5.833| 6.833| 8.577| > 0.05  |
| SD        | 3.841  | 6.279| 8.537| 8.732|         |
| Range     | 0–10   | 0–37 | 0–24 | 0–35 |         |
| % samples ≥ 20% | 0%   | 3%   | 16.7%| 7.1% | > 0.05  |
| % samples < 20% | 100% | 97%  | 83.3%| 92.9%|         |
| CpG 5615  |        |      |      |      |         |
| Mean      | 6.00   | 2.648| 3.250| 3.577| > 0.05  |
| SD        | 3.12   | 2.789| 3.251| 4.032|         |
| Range     | 2–12   | 0–13 | 0–9  | 0–20 |         |
| % samples ≥ 20% | 0%   | 0%   | 0%   | 3.6% | > 0.05  |
| % samples < 20% | 100% | 100% | 100% | 96.4%|         |
| CpG 7136  |        |      |      |      |         |
| Mean      | 1.583  | 2.063| 1.917| 3.680| > 0.05  |
| SD        | 2.193  | 2.945| 1.621| 3.945|         |
| Range     | 0–8    | 0–17 | 0–5  | 0–16 |         |
| % samples ≥ 20% | 0%   | 0%   | 0%   | 8%   | > 0.05  |
| % samples < 20% | 100% | 100% | 100% | 92%   |         |
| CpG 7145  |        |      |      |      |         |
| Mean      | 2.167  | 1.365| 1.333| 2.040| > 0.05  |
| SD        | 3.460  | 2.180| 1.557| 3.089|         |
| Range     | 0–10   | 0–12 | 0–5  | 0–12 |         |
| % samples ≥ 20% | 0%   | 0%   | 0%   | 8%   | > 0.05  |
| % samples < 20% | 100% | 100% | 100% | 92%   |         |

The P-values were calculated using the Kruskal-Wallis and Fisher Exact tests. * indicated a significant difference (P < 0.05).

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### Table 3. Mean HPV16 gene methylation level of each CpG and the percentual CD4 count between HIV-infected and HIV-uninfected patients.

| Group     | Normal (16) | AIN1(67) | AIN2(12) | AIN3(28) | P value |
|-----------|-------------|----------|----------|----------|---------|
| HIV       | uninfected  | infected | uninfected| infected | uninfected| infected | uninfected| infected |
| No. (123) | 4           | 12       | 18       | 49       | 2        | 10       | 1         | 27       |
| Mean %CD4+, Mean cell count | NA 19.5%, 389 | NA 16.83%, 315 | NA 19.2%, 359 | NA 16.93%, 326 | > 0.05 |
| Mean % Methylation | CpG 31 0% 0.43% 2.88% 1.11% 1.5% 0.5% 0.9% 3.12% > 0.05 | CpG 37 0% 0.57% 2.0% 0.64% 2.0% 0.3% 0.5% 2.36% > 0.05 | CpG 43 0% 0.86% 2.65% 0.47% 0% 0.3% 0% 2.64% > 0.05 | CpG 52 0% 1% 2.47% 1.18% 2.0% 0.9% 0% 3.24% > 0.05 | CpG 58 0% 0.57% 3.1% 0.91% 0.5% 0.3% 0% 3.36% > 0.05 | CpG 5600 10.3% 7% 12.33% 7.72% 5.5% 12.7% 14% 14.36% > 0.05 | CpG 5606 6.67% 7% 3.73% 3.54% 0% 5.3% 6% 3.92% > 0.05 | CpG 5609 7% 2% 8.07% 4.98% 1.0% 8.0% 10% 8.52% > 0.05 | CpG 5615 4.67% 6.67% 3.13% 2.46% 0% 3.9% 5% 3.52% > 0.05 | CpG 7136 3% 1.11% 2.5% 1.92% 0.5% 2.2% 0% 3.83% > 0.05 | CpG 7145 3.67% 1.67% 1.69% 1.26% 1.0% 1.4% 0% 2.13% > 0.05 |
| NA: Not applicable. |

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It has been reported that HIV-infected patients were susceptible to HPV infection. One study reported that HIV-infected women with a CD4 count of fewer than 200 cells/mm³ have 59.3% of high-risk HPV infections, correlating with increasing severity of cervical lesions [84].

The study of oral samples reported that a low CD4 count (< 200 cells/mm³) increased the risk for oral HPV infections in HIV-infected patients [85]. There was an association between a low...
CD4 count (< 200 cells/mm³) and rapid anal cancer progression in HIV-infected MSM [86]. In the present study, a combined HPV16 L1 gene methylation (≥ 20%) and a low percentage of a CD4 count might be beneficial to differentiate HIV-infected MSM who are at risk to rapidly progress to high-grade AIN and carcinoma. The limitation of the present study was that anal carcinoma samples could not be included due to the very low incidence of anal cancer. The low normal sample size may limit the significance of the statistical comparison between normal and high-grade AIN.

Conclusions
The methylation patterns of the HPV16 genome in anal intraepithelial neoplasia were similar to those of abnormal cervical cells. Hypermethylation of the HPV16 L1 gene, especially at CpG 5600 and 5609, found in AIN2/3, could be a biomarker for predicting HPV-related abnormal anal cells. Moreover, the combination of HPV16 L1 gene hypermethylation together with a low CD4 count in HIV-infected patients might be used as a biomarker for rapid progression to more severe lesions and anal cancer than those with a low methylation and high CD4 count. Thus, in order to employ methylation of specific CpG sites for screening of HPV-related abnormal lesions and cancer, a large sample size including normal and anal carcinoma samples should be further studied and evaluated.

Supporting information
S1 Fig. Pyrogram from quantification of methylation by pyrosequencing of HPV16 early promoter. The highlighted yellow bar represents the internal control (No intensity signal was found when cytosine was completely converted to uracil) within the analyzed sequence. The percent methylation value of each CpG site that perfectly pass quality control were indicated in blue box on the top of the gray bar. (TIF)

S2 Fig. Pyrogram from quantification of methylation by pyrosequencing of HPV16 5' L1 regions. The highlighted yellow bar represents the internal control (No intensity signal was found when cytosine was completely converted to uracil) within the analyzed sequence. The percent methylation value of each CpG site that perfectly pass quality control were indicated in blue box on the top of the gray bar. (TIF)

S3 Fig. Pyrogram from quantification of methylation by pyrosequencing of HPV16 3' L1 regions. The highlighted yellow bar represents the internal control (No intensity signal was found when cytosine was completely converted to uracil) within the analyzed sequence. The percent methylation value of each CpG site that perfectly pass quality control were indicated in blue box on the top of the gray bar. (TIF)

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References

1. Flejou JF. An update on anal neoplasia. Histopathology. 2015; 66(1):147–60. https://doi.org/10.1111/ his.12574 PMID: 25283345

2. Wang CJ, Sparano J, Palefsky JM. Human Immunodeficiency Virus/AIDS, Human Papillomavirus, and Anal Cancer. Surg Oncol Clin N Am. 2017; 26(1):17–31. https://doi.org/10.1016/j.soc.2016.07.010 PMID: 27889034

3. De Vuyst H, Clifford GM, Nascimento MC, Madeleine MM, Franceschi S. Prevalence and type distribution of human papillomavirus in carcinoma and intraepithelial neoplasia of the vulva, vagina and anus: a meta-analysis. Int J Cancer. 2009; 124(7):1626–36. https://doi.org/10.1002/ijc.24116 PMID: 19115209

4. Serrano B, de Sanjose S, Tous S, Quirós B, Munoz N, Bosch X, et al. Human papillomavirus genotype attribution for HPV6, 11, 16, 18, 31, 33, 45, 52 and 58 in female anogenital lesions. Eur J Cancer. 2015. https://doi.org/10.1016/j.ejca.2015.06.001 PMID: 26121913

5. Daling JR, Madeleine MM, Johnson LG, Schwartz SM, Shera KA, Wurscher MA, et al. Human papillomavirus, smoking, and sexual practices in the etiology of anal cancer. Cancer. 2004; 101(2):270–80. https://doi.org/10.1002/cncr.20365 PMID: 24651691

6. Darwich L, Cañas MP, Videla S, Coll J, Molina-López RA, Sirera G, et al. Prevalence, clearance, and incidence of human papillomavirus type-specific infection at the anal and penile site of HIV-infected men. Sex Transm Dis. 2013; 40(8):611–8. https://doi.org/10.1097/01.OLQ.0000430798.61475.08 PMID: 23859907

7. Videla S, Darwich L, Cañas MP, Coll J, Piñol M, García-Cuyás F, et al. Natural history of human papillomavirus infections involving anal, penile, and oral sites among HIV-positive men. Sex Transm Dis. 2013; 40(1):3–10. https://doi.org/10.1097/OLQ.0b013e31827e87bd PMID: 23250297

8. van Rijn VM, Mooij SH, Mollers M, Snijders PJ, Speksnijder AG, King AJ, et al. Anal, penile, and oral high-risk HPV infections and HPV seropositivity in HIV-positive and HIV-negative men who have sex with men. PloS one. 2014; 9(3):e92208. https://doi.org/10.1371/journal.pone.0092208 PMID: 24651691

9. Ucciferri C, Tamburro M, Falasca K, Sgambaro ML, Ripabelli G, Vecchiet J. Prevalence of anal, oral, penile and urethral Human Papillomavirus in HIV infected and HIV uninfected men who have sex with men. J Med Virol. 2018; 90(2):358–66. https://doi.org/10.1002/jmv.24943 PMID: 28906006

10. Welling CA, Mooij SH, van der Sande MA, van Rooijen MS, Vermeulen-Oost WF, King AJ, et al. Association of HIV Infection With Anal and Penile Low-Risk Human Papillomavirus Infections Among Men Who Have Sex With Men in Amsterdam: The HIV & HPV in MSM Study. Sex Transm Dis. 2015; 42(6):297–304. https://doi.org/10.1097/OLQ.000000000000278 PMID: 25970305

11. Remis RS, Liu J, Loutfy MR, Tharaor W, Rebbapragada A, Hulbner S, et al. Prevalence of Sexually Transmitted Viral and Bacterial Infections in HIV-Positive and HIV-Negative Men Who Have Sex with Men in Toronto. PloS one. 2016; 11(7):e0158090. https://doi.org/10.1371/journal.pone.0158090 PMID: 27391265

12. Machalek DA, Poynent M, Jin F, Fairley CK, Farnsworth A, Garland SM, et al. Anal human papillomavirus infection and associated neoplastic lesions in men who have sex with men: a systematic review and meta-analysis. Lancet Oncol. 2012; 13(5):487–500. https://doi.org/10.1016/S1470-2045(12)70080-3 PMID: 22445259

13. Li X, Li M, Yang Y, Zhong X, Feng B, Xin H, et al. Anal HPV/HIV co-infection among Men Who Have Sex with Men: a cross-sectional survey from three cities in China. Sci Rep. 2016; 6:21368. https://doi.org/10.1038/srep21368 PMID: 26882938
14. Lee CH, Lee SH, Lee S, Cho H, Kim KH, Lee JE, et al. Anal Human Papillomavirus Infection among HIV-Infected Men in Korea. PloS one. 2016; 11(8):e0161460. https://doi.org/10.1371/journal.pone.0161460 PMID: 2754632

15. Nagata N, Watanabe K, Nishijima T, Tadokoro K, Watanabe K, Shimbo T, et al. Prevalence of Anal Human Papillomavirus Infection and Risk Factors among HIV-positive Patients in Tokyo, Japan. PloS one. 2015; 10(9):e0137434. https://doi.org/10.1371/journal.pone.0137434 PMID: 26368294

16. Phanuphak N, Teeratakulpisarn N, Pankam T, Kerr SJ, Barisri J, Deesu a A, et al. Anal human papillomavirus infection among Thai men who have sex with men and without HIV infection: prevalence, incidence, and persistence. J Acquir Immune Defic Syndr. 2013; 63(4):472–9. https://doi.org/10.1097/QAI.0b013e3182918a5a PMID: 23514956

17. Supindham T, Chariyalatsak S, Utaipat U, Miura T, Ruanpeng D, Chotirosnimmit N, et al. High Prevalence and Genotype Diversity of Anal HPV Infection among MSM in Northern Thailand. PloS one. 2015; 10(5):e0124499. https://doi.org/10.1371/journal.pone.0124499 PMID: 25932915

18. Machalek DA, Jin F, Poynten IM, Hillman RJ, Templeton DJ, Law C, et al. Prevalence and risk factors associated with high-grade anal squamous intraepithelial lesions (HSIL)-AIN2 and HSIL-AIN3 in homosexual men. Papillomavirus Res. 2016; 2:97–105. https://doi.org/10.1016/j.pvr.2016.05.003 PMID: 28074193

19. Cho HW, So KA, Lee JK, Hong JH. Type-specific persistence or regression of human papillomavirus genotypes in women with cervical intraepithelial neoplasia 1: A prospective cohort study. Obstet Gynecol Sci. 2015; 58(1):40–5. https://doi.org/10.5468/ogs.2015.58.1.40 PMID: 25629017

20. Bruno MT, Cassaro N, Garofalo S, Boemi S. HPV16 persistent infection and recurrent disease after LEEP. Virol J. 2019; 16(1):148. https://doi.org/10.1186/s12985-019-1252-3 PMID: 31775792

21. Trimble CL, Piantadosi S, Gravitt P, Ronnett B, Pizer E, Elko A, et al. Spontaneous regression of high-grade cervical dysplasia: effects of human papillomavirus type and HLA phenotype. Clin Cancer Res. 2005; 11(13):4717–23. https://doi.org/10.1158/1078-0432.CCR-04-2599 PMID: 16005666

22. Schlecht NF, Platt RW, Duarte-Franco E, Costa MC, Sobrinho JP, Prado JC, et al. Human papillomavirus infection and time to progression and regression of cervical intraepithelial neoplasia. J Natl Cancer Inst. 2003; 95(17):1336–43. https://doi.org/10.1093/jnci/dg307 PMID: 12953088

23. Dona MG, Vescio MF, Latini A, Giglio A, Moretto D, Frasca M, et al. Anal human papillomavirus in HIV-uninfected men who have sex with men: incidence and clearance rates, duration of infection, and risk factors. Clin Microbiol Infect. 2016; 22(12):1004 e1–e7. https://doi.org/10.1016/j.cmi.2016.08.011.

24. Alberts CJ, Heard I, Canestri A, Marchand L, Flé jou JF, Piroth L, et al. Incidence and Clearance of Anal Human Papillomavirus (HPV)-16 and HPV-18 Infection, and Their Determinants, Among Human Immunodeficiency Virus-Infected Men Who Have Sex With Men in France. J Infect Dis. 2020; 221(9):1488–93. https://doi.org/10.1093/infectdis/jiz623 PMID: 31754686

25. Munger K, Baldwin A, Edwards KM, Hayakawa H, Nguyen CL, Owens M, et al. Mechanisms of human papillomavirus-induced oncogenesis. J Virol. 2004; 78(21):11451–60. https://doi.org/10.1128/JVI.78.21.11451-11460.2004 PMID: 15479788

26. Munger K, Basile JR, Duensing S, Eichten A, Gonzalez SL, Grace M, et al. Biological activities and molecular targets of the human papillomavirus E7 oncoprotein. Oncogene. 2001; 20(54):7888–98. https://doi.org/10.1038/sj.onc.1204860 PMID: 11753671

27. DeFilippis RA, Goodwin EC, Wu L, DiMaio D. Endogenous human papillomavirus E6 and E7 proteins differentially regulate proliferation, senescence, and apoptosis in HeLa cervical carcinoma cells. J Virol. 2003; 77(2):1551–63. https://doi.org/10.1128/JVI.77.2.1551-1563.2003 PMID: 12502868

28. Chen HC, Schiffman M, Lin CY, Pan MH, You SL, Chuang LC, et al. Persistence of type-specific human papillomavirus infection and increased long-term risk of cervical cancer. J Natl Cancer Inst. 2011; 103(18):1387–96. https://doi.org/10.1093/jnci/djr283 PMID: 21900119

29. Moscicki AB, Shiboski S, Hills NK, Powell KJ, Jay N, Hanson EN, et al. Regression of low-grade squamous intra-epithelial lesions in young women. Lancet. 2004; 364(9446):1678–83. https://doi.org/10.1016/S0140-6736(04)17354-6 PMID: 15530628

30. Origon M, Cristoforoni P, Carminati G, Stefani C, Costa S, Sandri MT, et al. E6/E7 mRNA testing for human papilloma virus-induced high-grade cervical intraepithelial disease (CIN2/CIN3): a promising perspective. Ecancermedicalscience. 2015; 9:533. https://doi.org/10.3332/ecancer.2015.533 PMID: 26015862

31. Cattani P, Zannoni GF, Ricci C, D’Onghia S, Trivellizzi IN, Di Franco A, et al. Clinical performance of human papillomavirus E6 and E7 mRNA testing for high-grade lesions of the cervix. J Clin Microbiol. 2008; 47(12):3895–901. https://doi.org/10.1128/JCM.01275-09 PMID: 19827389

32. Phanuphak N, Teeratakulpisarn N, Keelawat S, Pankam T, Barisri J, Triratanachat S, et al. Use of human papillomavirus DNA, E6/E7 mRNA, and p16 immunocytochemistry to detect and predict anal high-grade squamous intraepithelial lesions in HIV-positive and HIV-negative men who have sex with men. PloS one. 2013; 8(11):e78291. https://doi.org/10.1371/journal.pone.0078291 PMID: 24265682
33. Ki EY, Park JS, Lee A, Kim TJ, Jin HT, Seo YB, et al. Utility of human papillomavirus L1 capsid protein and HPV test as prognostic markers for cervical intraepithelial neoplasia 2+ in women with persistent ASCUS/LSIL cervical cytology. Int J Med Sci. 2019; 16(8):1096–101. https://doi.org/10.7150/ijms.31163 PMID: 31523171

34. Choi YS, Kang WD, Kim SM, Choi YD, Nam JH, Park CS, et al. Human papillomavirus L1 capsid protein and human papillomavirus type 16 as prognostic markers in cervical intraepithelial neoplasia 1. Int J Gynecol Cancer. 2010; 20(2):288–93. https://doi.org/10.1111/j.1365-2583.2013.05477.x PMID: 20134272

35. Hu H, Zhao J, Yu W, Zhao J, Wang Z, Jin L, et al. Human papillomavirus DNA, HPV L1 capsid protein and p16(INK4a) protein as markers to predict cervical lesion progression. Arch Gynecol Obstet. 2019; 299(1):141–9. https://doi.org/10.1007/s00404-018-4931-1 PMID: 30390110

36. Vinokurova S, von Knebel Doeberitz M. Differential methylation of the HPV 16 upstream regulatory region during epithelial differentiation and neoplastic transformation. PLoS One. 2011; 6(9):e24451. https://doi.org/10.1371/journal.pone.0024451 PMID: 21915330

37. Kiallantari M, Lee D, Calleja-Macias IE, Lambert PF, Bernard HU. Effects of cellular differentiation, chromosomal integration and 5-aza-2'-deoxycytidine treatment on human papillomavirus-16 DNA methylation in cultured cell lines. Virology. 2008; 374(2):292–303. https://doi.org/10.1016/j.virol.2007.12.016 PMID: 18242658

38. Baedyananda F, Chaiwongkot A, Bhattarakosol P. Elevated HPV16 E1 Expression Is Associated with Human papillomavirus type 16 DNA and risk of cervical intraepithelial neoplasia grades 2 or 3. PloS one. 2011; 6(8):e23897. https://doi.org/10.1371/journal.pone.0023897 PMID: 21887341

39. Burley M, Roberts S, Parish JL. Epigenetic regulation of human papillomavirus transcription in the productive virus life cycle. Semin Immunopathol. 2020; 42(2):159–71. https://doi.org/10.1007/s00281-019-00773-0 PMID: 31919577

40. Badal V, Chuang LS, Tan EH, Badal S, Villa LL, Wheeler CM, et al. CpG methylation of human papillomavirus type 16 DNA in cervical cancer cell lines and in clinical specimens: genomic hypomethylation correlates with carcinogenic progression. J Virol. 2003; 77(11):6227–34. https://doi.org/10.1128/jvi.77.11.6227-6234.2003 PMID: 12743279

41. Piyathilake CJ, Macaluso M, Alvarez RD, Chen M, Badiga S, Edberg JC, et al. A higher degree of methylation of the HPV 16 E5 gene is associated with a lower likelihood of being diagnosed with cervical intraepithelial neoplasia. Cancer. 2011; 117(5):957–63. https://doi.org/10.1002/cncr.25511 PMID: 20945322

42. Xi LF, Jiang M, Shen Z, Hulbert A, Zhou XH, Lin YY, et al. Inverse association between methylation of human papillomavirus type 16 DNA and risk of cervical intraepithelial neoplasia grades 2 or 3. PloS one. 2011; 6(8):e23897. https://doi.org/10.1371/journal.pone.0023897 PMID: 21887341

43. Bhattacharjee B, Sengupta S. CpG methylation of HPV 16 LCR at E2 binding site proximal to P97 is associated with cervical cancer in presence of intact E2. Virology. 2006; 354(2):280–5. https://doi.org/10.1016/j.virol.2006.06.018 PMID: 16905170

44. Ding DC, Chiang MH, Lai HC, Hsiung CA, Hsieh CY, Chu TY. Methylation of the long control region of human papillomavirus type 16 DNA and risk of cervical intraepithelial neoplasia grades 2 or 3. J Clin Virol. 2014; 59(1):24–9. https://doi.org/10.1016/j.jcv.2013.10.029 PMID: 24268385

45. Fernandez AF, Rosales C, Lopez-Nieves P, Grana O, Ballestar E, Ropero S, et al. The dynamic DNA methylation patterns of double-stranded DNA viruses associated with human cancer. Genome Res. 2009; 19(3):438–51. https://doi.org/10.1101/gr.083550.108 PMID: 19208682

46. Chaiwongkot A, Niruthisard S, Kitkumthorn N, Bhattarakosol P. Quantitative methylation analysis of human papillomavirus 16L1 gene reveals potential biomarker for cervical cancer progression. Diagn Microbiol Infect Dis. 2017; 89(4):265–70. https://doi.org/10.1016/j.diagmicrobio.2017.08.010 PMID: 28985972

47. Bryant D, Tristram A, Liloglou T, Hibbits S, Fianter A, Powell N. Quantitative measurement of Human Papillomavirus type 16 L1/L2 DNA methylation correlates with cervical disease grade. J Clin Virol. 2014; 59(1):24–9. https://doi.org/10.1016/j.jcv.2013.10.029 PMID: 24268385

48. Fernandez AF, Rosales C, Lopez-Nieves P, Grana O, Ballestar E, Ropero S, et al. The dynamic DNA methylation patterns of double-stranded DNA viruses associated with human cancer. Genome Res. 2009; 19(3):438–51. https://doi.org/10.1101/gr.083550.108 PMID: 19208682

49. Chaiwongkot A, Niruthisard S, Kitkumthorn N, Bhattarakosol P. Quantitative methylation analysis of human papillomavirus 16L1 gene reveals potential biomarker for cervical cancer progression. Diagn Microbiol Infect Dis. 2017; 89(4):265–70. https://doi.org/10.1016/j.diagmicrobio.2017.08.010 PMID: 28985972

50. Kiallantari M, Calleja-Macias IE, Tewari D, Hagmar B, Lie K, Barrera-Saldana HA, et al. Conserved methylation patterns of human papillomavirus type 16 DNA in asymptomatic infection and cervical
neoplasia. J Virol. 2004; 78(23):12762–72. https://doi.org/10.1128/JVI.78.23.12762-12772.2004 PMID: 15542628

51. Clarke MA, Gradissimo A, Schiffman M, Lam J, Sollecito CC, Fetterman B, et al. Human Papillomavirus DNA Methylation as a Biomarker for Cervical Precancer: Consistency across 12 Genotypes and Potential Impact on Management of HPV-Positive Women. Clin Cancer Res. 2018; 24(9):2194–202. https://doi.org/10.1158/1078-0432.CCR-17-3251 PMID: 29420222

52. Dong L, Zhang L, Hu S-Y, Feng R-M, Zhao X-L, Zhang Q, et al. Risk stratification of HPV 16 DNA methylation combined with E6 oncoprotein in cervical cancer screening: a 10-year prospective cohort study. Clin Epigenetics. 2020; 12(1):62. https://doi.org/10.1186/s13148-020-00853-1 PMID: 32381054

53. Mirabello L, Frimer M, Harari A, McAndrew T, Smith B, Chen Z, et al. HPV16 methyl-haplotype determined by a novel next-generation sequencing method are associated with cervical precursor. Int J Cancer. 2015; 136(4):E146–53. https://doi.org/10.1002/ijc.29119 PMID: 25081507

54. Bryant D, Hibbitts S, Almonte M, Tristram A, Fiander A, Powell N. Human papillomavirus type 16 L1/L2 DNA methylation shows weak association with cervical disease grade in young women. J Clin Virol. 2015; 66:66–71. https://doi.org/10.1016/j.jcv.2015.03.001 PMID: 25866341

55. Kottaridi C, Kyrgiou M, Pouliakis A, Magkana M, Aga E, Spathis A, et al. Quantitative Measurement of L1 Human Papillomavirus Type 16 Methylation for the Prediction of Preinvasive and Invasive Cervical Disease. J Infect Dis. 2017; 215(5):764–71. https://doi.org/10.1093/infdis/jiw454 PMID: 28170039

56. Lorincz AT, Nathan M, Reuter C, Warman R, Thaha MA, Sheaff M, et al. Methylation of HPV and a tumor suppressor gene reveals anal cancer and precursor lesions. Oncotarget. 2017; 8(31):50510–20. https://doi.org/10.18632/oncotarget.17984 PMID: 28881579

57. Molano M, Tabrizi SN, Garland SM, Roberts JM, Machalek DA, Phillips S, et al. CpG Methylation Analysis of HPV16 in Laser Capture Microdissected Archival Tissue and Whole Tissue Sections from High Grade Anal Squamous Intraepithelial Lesions: A Potential Disease Biomarker. PloS one. 2016; 11(8):e0160673. https://doi.org/10.1371/journal.pone.0160673 PMID: 27529629

58. Wiley DJ, Huh J, Rao JY, Chang C, Goetz M, Poulter M, et al. Methylation of human papillomavirus genomes in cells of anal epithelium of HIV-infected men. J Acquir Immune Defic Syndr. 2005; 39(2):143–51. https://doi.org/10.1097/01.qai.0000160405.01300.80. PMID: 15905729

59. Rajeevan MS, Swan DC, Duncan K, Lee DR, Limor JR, Unger ER. Quantitation of site-specific HPV 16 DNA methylation by pyrosequencing. J Virol Methods. 2006; 138(1–2):1–6. https://doi.org/10.1016/j.jvymet.2006.08.012 PMID: 17045346

60. Kalantari M, Osann K, Calleja-Macias IE, Kim S, Yan B, Jordan S, et al. Methylation of human papillomavirus 16, 18, 31, and 45 L2 and L1 genes and the cellular DAPK gene: Considerations for use as biomarkers of the progression of cervical neoplasia. Virology. 2014; 448:314–21. https://doi.org/10.1016/j.virol.2013.10.032 PMID: 24314662

61. Lorincz AT, Brentnall AR, Vasilijevic N, Scibior-Bentkowska D, Castanon A, Fiander A, et al. HPV16 L1 and L2 DNA methylation predicts high-grade cervical intraepithelial neoplasia in women with mildly abnormal cervical cytology. Int J Cancer. 2013; 133(9):637–44. https://doi.org/10.1002/ijc.28050 PMID: 23335178

62. Brandsma JL, Harigopal M, Kivist NB, Sun Y, Deng Y, Zelterman D, et al. Methylation of Twelve CpGs in Human Papillomavirus Type 16 (HPV16) as an Informative Biomarker for the Triage of Women Positive for HPV16 Infection. Cancer Prev Res. 2014; 7(10):12762–72. https://doi.org/10.1158/1940-6207.CPR-13-0354 PMID: 24556390

63. Kalantari M, Villa LL, Calleja-Macias IE, Bernard HU. Human papillomavirus-16 and -18 in penile carcinomas: DNA methylation, chromosomal recombination and genomic variation. Int J Cancer. 2008; 123(8):1832–40. https://doi.org/10.1002/ijc.23707 PMID: 18688866

64. Balderas-Loaeza A, Anaya-Saavedra G, Ramirez-Amador VA, Guido-Jimenez MC, Kalantari M, Calleja-Macias IE, et al. Human papillomavirus-16 DNA methylation patterns support a causal association of the virus with oral squamous cell carcinomas. Int J Cancer. 2007; 120(10):2165–9. https://doi.org/10.1002/ijc.20550 PMID: 17278110

65. Bryant D, Onions T, Raybould R, Jones S, Tristram A, Hibbitts S, et al. Increased methylation of Human Papillomavirus type 16 DNA correlates with viral integration in Vulval Intraepithelial Neoplasia. J Clin Virol. 2014; 61(3):393–9. https://doi.org/10.1016/j.jcv.2014.08.006 PMID: 25218242

66. Badal S, Badal V, Calleja-Macias IE, Kalantari M, Chuang LS, Li BF, et al. The human papillomavirus-18 genome is efficiently targeted by cellular DNA methylation. Virology. 2004; 324(2):483–92. https://doi.org/10.1016/j.virol.2004.04.002 PMID: 15207633

67. Sun C, Reimers LL, Burk RD. Methylation of HPV16 genome CpG sites is associated with cervical precursor and cancer. Gynecol Oncol. 2011; 121(1):59–63. https://doi.org/10.1016/j.ygyno.2011.01.013 PMID: 21306759
68. Turan T, Kalantari M, Calleja-Macias IE, Cubie HA, Cuschierei K, Villa LL, et al. Methylation of the human papillomavirus-18 L1 gene: a biomarker of neoplastic progression?. Virolology. 2006; 349 (1):175–83. https://doi.org/10.1016/j.virol.2005.12.033 PMID: 16472835

69. Kalantari M, Chase DM, Tewari KS, Bernard HU. Recombination of human papillomavirus-16 and host DNA in exfoliated cervical cells: a pilot study of L1 gene methylation and chromosomal integration as biomarkers of carcinogenic progression. J Med Virol. 2010; 82(2):311–20. https://doi.org/10.1002/jmv.21676 PMID: 20029805

70. Van Tine BA, Kappes JC, Banerjee NS, Knops J, Lai L, Steenbergen RD, et al. Clonal selection for transcriptionally active viral oncogenes during progression to cancer. J Virol. 2004; 78(20):11172–86. https://doi.org/10.1128/JVI.78.20.11172-11186.2004 PMID: 15452237

71. De-Castro Arce J, Gockel-Krzikalla E, Rosl F. Silencing of multi-copy HPV16 by viral self-methylation and chromatin occlusion: a model for epigenetic virus-host interaction. Hum Mol Genet. 2012; 21 (6):1693–705. https://doi.org/10.1093/hmg/ddr604 PMID: 22210627

72. Fujii T, Brandsma JL, Peng X, Srimatkananda S, Li L, Canaan A, et al. High and low levels of cottontail rabbit papillomavirus E2 protein generate opposite effects on gene expression. J Biol Chem. 2001; 276 (2):867–74. https://doi.org/10.1074/jbc.M007120200 PMID: 11013251

73. Soeda E, Ferran MC, Baker CC, McBride AA. Repression of HPV16 early region transcription by the E2 protein. Virolology. 2006; 351(1):29–41. https://doi.org/10.1016/j.virol.2006.03.016 PMID: 16624362

74. Doeberitz MK, Vinokurova S. Host factors in HPV-related carcinogenesis: cellular mechanisms controlling HPV infections. Arch Med Res. 2009; 40(6):435–42. https://doi.org/10.1016/j.arcmed.2009.06.002 PMID: 19853183

75. Fernandez AF, Esteller M. Viral epigenomes in human tumorigenesis. Oncogene. 2010; 29(10):1405–20. https://doi.org/10.1038/onc.2009.517 PMID: 20101211

76. Albuquerque A. Cytology in Anal Cancer Screening: Practical Review for Clinicians. Acta Cytol. 2020; 64(4):281–7. https://doi.org/10.1155/2009/000502881 PMID: 31530994

77. Ruanpeng D, Chariyalertsak S, Kaewpoomwat Q, Supindham T, Settakorn J, Sukpan K, et al. Cytologic analysis of squamous intraepithelial lesions associated with anal high-risk human papillomavirus infections among men who have sex with men in Northern Thailand. PloS one. 2016; 11(5):e0156280. https://doi.org/10.1371/journal.pone.0156280 PMID: 27227684

78. Panther LA, Wagner K, Proper J, Fugelso DK, Chatis PA, Weeden W, et al. High resolution anoscopy findings for men who have sex with men: Inaccuracy of anal cytology as a predictor of histologic high-grade anal intraepithelial neoplasia and the impact of HIV serostatus. Clin Infect Dis. 2004; 38 (10):1490–2. https://doi.org/10.1086/383574 PMID: 15156490

79. Jin F, Gruilich AE, Poynten IM, Hillman RJ, Templeton DJ, Law CL, et al. The performance of anal cytology as a screening test for anal HSILs in homosexual men. Cancer cytopathol. 2012; 124(6):415–24. https://doi.org/10.1002/cncy.21702 PMID: 26915346

80. Gonçalves JCN, Macedo ACL, Madeira K, Bavaresco DV, Donossola ER, Grande AJ, et al. Accuracy of anal cytology for diagnostic of precursor lesions of anal cancer: Systematic Review and Meta-analysis. Dis Colon Rectum. 2019; 62(1):112–20. https://doi.org/10.1097/DCR.0000000000001231 PMID: 30451747

81. Betancourt EM, Wahbah MM, Been LC, Chiao EY, Citron DR, Laucirica R. Anal cytology as a predictor of anal intraepithelial neoplasia in HIV-positive men and women. Diagn Cytopathol. 2013; 41(6):697–702. https://doi.org/10.1002/dc.22941 PMID: 23288861

82. Clarke MA, Wentzensen N. Strategies for screening and early detection of anal cancers: A narrative and systematic review and meta-analysis of cytology, HPV testing, and other biomarkers. Cancer cytopathol. 2018; 126(7):447–60. https://doi.org/10.1002/cncy.22018 PMID: 29797691

83. Clarke MA, Wentzensen N, Mirabello L, Ghosh A, Wacholder S, Harari A, et al. Human papillomavirus DNA methylation as a potential biomarker for cervical cancer. Cancer Epidemiol Biomarkers Prev. 2012; 21(12):2125–37. https://doi.org/10.1158/1055-9965.EPI-12-0905 PMID: 23035178

84. Teixeira MF, Sabido M, Leturiondo AL, de Oliveira Ferreira C, Torres KL, Benzaken AS. High risk human papillomavirus prevalence and genotype distribution among women infected with HIV in Manaus, Amazonas. Virol J. 2018; 15(1):36. https://doi.org/10.1186/s12985-018-0942-6 PMID: 29454382

85. Muller K, Kazimiروف J, Fatahzadeh M, Smith RV, Wiltz M, Polanco J, et al. Oral Human Papillomavirus Infection and Oral Lesions in HIV-Positive and HIV-Negative Dental Patients. J Infect Dis. 2015; 212 (5):760–8. https://doi.org/10.1093/infdis/jiv080 PMID: 25681376

86. Hernández-Ramírez RU, Qin L, Lin H, Leyden W, Neugebauer RS, Althoff KN, et al. Association of Immunosuppression and Human Immunodeficiency Virus (HIV) Viremia With Anus Cancer Risk in Persons Living With HIV in the United States and Canada. Clin Infect Dis. 2020; 70(6):1176–85. https://doi.org/10.1093/cid/ciaa292 PMID: 31044245