Title: Potential biomarker of human papillomavirus 16 L1 methylation for prediction of anal intraepithelial neoplasia in men who have sex with men (MSM)

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Short title: Potential biomarker of HPV16 L1 methylation in MSM with anal intraepithelial neoplasia

Abstract

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

38 Introduction

39 Anal carcinoma is a rare disease found in men and women population globally with average
40 incidence of <1-2 per 100,000[1]. However, the incidence of anal cancer was high in HIV infected
41 women and HIV-infected men who have sex with men (MSM), accounting for 30/100,000 and
42 131/100,000, respectively [2]. There is association between human papillomavirus (HPV) infection
43 and anal carcinoma in which HPV DNA was found in men (68.7-91.2%) and women (90.4-90.9%)
44 with anal carcinoma worldwide [3]. Studies showed that HPV16 was predominantly detected in
45 men and women, accounting for 70-71.6% and 74-83.4%, respectively [2, 4, 5].

46 High prevalence of HPV infection was reported in anal cells collected particularly from HIV
47 infected MSM. Worldwide HPV prevalences in anal cells of HIV infected and HIV uninfected
48 MSM were 92.6% and 63.9%, respectively and HPV16 was found 35.4% and 12.5% in general
49 HIV infected and HIV uninfected MSM, respectively [6]. Recent study in China reported high
50 prevalence of HPV infection among HIV-infected MSM (82.69%) compared with HIV uninfected
51 MSM (62.81%)[7]. In Bangkok, Thailand, anal HPV infection was found 85% in HIV infected
52 MSM compared with 58.5% in HIV infected MSM in which HPV16 was detected 22.5% and 9.8%
53 in HIV infected and HIV uninfected MSM, respectively [8]. The prevalence of anal HPV infection
54 in northern Thailand was 80% among MSM, in which 100% and 70% were found in HIV infected
55 MSM and HIV uninfected MSM, respectively, HPV16 was the most common high risk types,
56 accounting for 40% in HIV infected MSM and 22% in HIV uninfected MSM [9]. It was reported
57 that HPV16 was the most persistent high risk HPV types [10] and less likely to have spontaneous
58 regression from cervical intraepithelial neoplasia (CIN)2-3 to normal when compared to other
59 HPV types [11, 12]. The study in HIV uninfected MSM revealed that HPV16 showed the longest
60 duration of infection with the lowest rate of viral clearance when compared to low risk HPV [13].

61 High risk HPV is considered to be the causative agent of cervical cancer and other HPV related
62 cancers such as vulva, head and neck cancer and anal. Viral oncoproteins E6 and E7 disrupt
63 normal function of host proteins involved in cell cycle regulation such as E6 causes p53

degradation and E7 inactivates retinoblastoma protein[14, 15]. However, the development of HPV related cancers takes more than 10-20 years, while the majority of HPV infected population has spontaneous regression [16, 17]. Up-regulation of viral E6 and E7 oncoproteins and down expression of viral proteins involved in viral particle assembly such as L1/L2 proteins are correlated with cancer progression[18, 19]. Epigenetic modification such as methylation of HPV genome is considered to be one factor to control the expression of viral genes during productive and transforming infections.

Differential methylation in HPV16 genome has been reported in cervical samples, during productive infection. HPV16 early promoter was found unmethylated in basal and intermediate cells at proximal E2 binding sites 2-4 (E2BS) but became highly methylated in superficial cells at the upper part of the epithelium. In latent HPV16 infection, viral long control region (LCR) including 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 [20]. One study showed that HPV16 p670 late promoter was highly methylated in cervical carcinoma cases [21]. The low expression of viral early gene and lack of capsid L1/L2 proteins expression in undifferentiated basal cells could prevent activation of immune response to viral infection [22].

The methylation pattern in early promoter especially HPV16 E2BS has been widely studied in cervical cells and the methylation patterns were reported either progressive hypomethylation [23, 24] and progressive hypermethylation [21, 25, 26]. One study showed that high methylation of E2BS was correlated with episomal form in high grade cervical lesions, cervical carcinoma and multiple copies of integrated HPV genome [27]. L1 gene hypermethylation was correlated with severe cervical lesions and cervical carcinoma [28-30]. We have previously reported the association between high methylation of HPV16 L1 gene especially at CpG site 5600 and 5609 and high grade cervical lesions and cervical carcinoma [31]. However, there are limited studies of HPV16 genome methylation in anal cells especially in Asian countries. This study aimed to detect methylation pattern of HPV16 in early promoter and L1 regions in anal cells obtained from Thai

MSM, analyzed by quantitative pyrosequencing assay. The pattern of methylation revealed the potential of using methylation at specific site as biomarker for screening of high-grade lesions of anal squamous intraepithelial cells.

Materials and methods

Clinical samples and cell lines

178 HPV16 positive DNA samples extracted from anal cells were collected from men who having sex with men (MSM) at the Thai Red Cross AIDS Research Centre (TRC-ARC) Bangkok, Thailand. There were 134 HIV positive cases and 44 HIV negative cases. Percentage of CD4+ results were obtained only from HIV positive men. DNA extracted from human cervical cancer cell lines containing of integrated HPV 16; CaSki, (CRL-1550 Lot No.3794357) containing approximately 500-600 copies per cell) and SiHa (HTB-35 Lot No.4031219) containing 1-2 copies per cell were used as positive control for amplification and pyrosequencing. This study has been approved (COA No. 053/2016) by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University.

Methylation analysis by Pyrosequencing

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

Table 1 The sequences of forward and reverse primers used in this study

Target gene	Nucleotide sequences	Size (bp)	Reference
Early promoter: CpG 31,37,43,52, 58	FW: 5'-TTGTAAAATTGTATATGGGTGTG-3' RV: Biotin-5'-AAATCCTAAAACATTA CAATTCTC-3' Sequencing primer 400S1: 5'-AATTTATGTATAAAATTAAGGG-3'	192	[32]
3'L1: CpG 7136 and 7145	FW: Biotin-5'-GGTTAAATTTAAATTTATATTAGGAAAA-3' RV: 5'-AAACATATACACAACAACAACACTAATTC-3' Sequencing primer 800: 5'-TACATACAATACTTACAAC-3'	140	[31]
5'L1: CpG 5600, 5606, 5609, 5615	FW Biotin 5'-TAATATATAATTATTGTTGATGTAGGTGAT -3' RV 5'-AACATAACCTCACTAAACAACCAAAA-3' Sequencing primer 5600: 5'-CCAAAAAACATCTAAAAAAAATATAATA-3'	130	[31]

PCR amplification protocol was as follows: 13.6 μ L DNase/RNase free water, 1X PCR buffer, 2.5mM $MgCl_2$, 250 μ M dNTP, 12.5pM of each forward and reverse primers, 1 Unit DNA polymerase (HotStart HiFidelity Polymerase, Affymetrix, USA). The PCR conditions were started with 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 of final extension at 72°C for 10 minutes. The PCR products were detected by 1.5% agarose gel electrophoresis. Prior to pyrosequencing, 20 μ L of amplified products labeled with biotin were mixed with beads, washed, denatured, mixed with 0.4 μ M of sequencing primers and loaded into the PyroMark™ Q96 machine (Qiagen, Germany).

Statistical analysis

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

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133 **Results**

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

135 Of 178 HPV16 positive samples including 134 HIV positive and 44 HIV negative samples, mean
 136 age was 31.23 years. Histology results were obtained from 123 samples classified as 16 normal, 67
 137 AIN1, 12 AIN2 and 28 AIN3. Methylation patterns in HPV16 early promoter and two regions
 138 within HPV16 L1 gene were analyzed. The methylation levels of early promoter comprising 5
 139 CpGs (31, 37, 43, 52 and 58) including proximal E2 binding sites (E2BSs) and Sp1 binding site of
 140 CaSki were 70%, 60.5%, 73.5%, 66.5%, 77% and of SiHa cells were 0-1% in all 5 CpGs,
 141 respectively. Methylation levels of L1 gene (5600, 5606, 5609, 5615, 7136 and 7145) were 84%,
 142 59%, 76%, 65%, 69% and 67% for CaSki and 95%, 96%, 80%, 80%, 69% and 76% for SiHa,
 143 respectively, as shown in Fig 1. High methylation of L1 gene was detected in both cervical cell
 144 lines regardless of copy number of integrated HPV16.

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Fig 1. A scatter plot of methylation percentage of 11 CpGs located within early promoter (positions 31, 37, 43, 52 and 58), 3'L1 (positions 7136 and 7145), and 5'L1 regions (positions 5600, 5606, 5609 and 5615) of Caski and SiHa cell lines. A and B were methylation levels obtained from SiHa and CaSki, respectively. C was methylation level of L1 gene obtained from both SiHa and Caski.

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

The methylation levels of early promoter in normal, AIN1 and AIN2-3 were shown in Fig 2. The methylation level was <10% in all normal anal cells, while intermediate methylation level (20-40%) was found in some of AIN1 and AIN2-3. The majority of AIN1 and AIN2-3 showed low methylation level in early promoter (<10%). For L1 gene, low methylation level was found in all detected normal anal cells (<20%). There was slightly increased in L1 gene methylation from normal to AIN2-3, especially at CpG5600 and 5609 which methylation levels were found in range 20-60% which were higher than other CpGs (5606, 5615, 7136 and 7145) (Fig 3). Intermediate to high methylation levels (20-60%) of CpG5600 was found in 0% (0/16) normal, 10.5% (7/67)

AIN2-3 was found in CpG5609. There was significant difference of samples number with methylation percentage >20% between normal/AIN1 (8.4%; 7/83) and AIN2-3 (27.5%; 11/40) in CpG5600 (Table 2).

Fig 2. A bar graph of methylation percentage of early promoter in anal samples stratified by histology as normal, AIN1, AIN2 and AIN3 (mean±SE).

Fig 3. A bar graph of methylation percentage of L1 gene in anal samples stratified by histology as normal, AIN1, AIN2 and AIN3 (mean±SE).

Table 2. Subject demographic and methylation status of HPV16 early promoter and L1 genes in anal cells with various grades of lesions. The *p*-values were calculated using Kruskal-Wallis test and Fisher Exact test. * indicated significant difference.

Group	Normal	AIN1	AIN2	AIN3	<i>p</i> -value
No. (123)	16	67	12	28	
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
% 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%	

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179 Correlation between CD4+ percentage with HPV16 gene methylation

180 There was no statistically differences in mean of HPV16 methylation percentage between
181 HIV negative and HIV positive cases (Table 3). Interestingly, HPV16 L1 gene high methylation
182 was moderately correlated with low percentage of CD4+ in AIN3 HIV positive cases, especially at
183 CpGs 5600, 5609, 5615 and 7145 (R=0.4692-0.5412) (Fig 4).

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Table 3. Mean of HPV16 gene methylation of each CpGs, HIV status and percentage of CD4+

Group	Normal (16)		AIN1(67)		AIN2(12)		AIN3(28)		p-value
HIV	negative	positive	negative	positive	negative	positive	negative	positive	
No. (123)	4	12	18	49	2	10	1	27	
Mean%CD4+	NA	19.5%	NA	16.83%	NA	19.2%	NA	16.93%	>0.05
Mean cell count		389		315		359		326	
% Methylation									
CpG 31	0%	0.43%	2.88%	1.11%	1.5%	0.5%	0%	3.12%	>0.05
CpG 37	0%	0.57%	2.0%	0.64%	2.0%	0.3%	0%	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

Fig 4. Correlation between percentage of HPV16 L1 gene methylation and number percentage of CD4+ in AIN3 cases.

Discussion

In the present study, the methylation pattern of HPV16 genome including early promoter region and L1 gene in anal cells was studied using pyrosequencing assay. The quantitative methylation analysis was first analyzed in CaSki and SiHa cell lines that methylation levels were consistent with previous reports [29, 32-35]. HPV16 and 18 L1 gene hypermethylation was reported previously in cervical carcinoma, vulva intraepithelial neoplasia (VIN), oral carcinoma and penile carcinoma [29, 30, 36-41]. Hypermethylation in the L1 gene was found to be correlated with integration form of HPV16 [38, 42]. The present study found high methylation of HPV16 L1 gene in some of AIN2/3 samples compared to normal anal cells. We also found that CpG sites 5600 and 5609 showed higher methylation (>20%) compared to the other CpG sites in L1 region (5606, 5615, 7136 and 7145). Previous reports in cervical cells showed that CpG sites 5600 and 5609 were the best sites for separation of normal cervical cells and high grade dysplasia [29, 31, 43]. It can be implicit that methylation patterns of HPV16 L1 gene in anal cells were similar to cervical cells and might be used for separation of normal cells and HPV related severely abnormal cells.

Methylation patterns of 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 [23, 24]. Other studies reported hypermethylation of early promoter in cervical carcinoma or progressive hypermethylation [21, 25, 26, 30, 44]. The physical state and copy number of integrated HPV16 genomes were the main reasons of these methylation differences as shown in CaSki and SiHa cells. Episomal form of HPV16 genome found in high grade cervical lesions and cervical carcinoma displayed high methylation levels at E2 binding sites in early promoter compared with single integrated HPV16 genome [27]. One study showed high methylation of early promoter in high grade anal cells [45]. We can speculate that anal samples with intermediate to high methylation levels (>20%) in the present study may be at risk to progress quickly compared to those with low methylation (<20%).

It has been reported that HIV infected patients were susceptible for HPV infection. One study reported that in HIV infected women with CD4+ less than 200 cells/mm³ have 59.3% of high risk-HPV infections and correlated with increasing severity of cervical lesions [46]. The study in oral samples reported that low CD4+ count (<200 cells/mm³) increased risk for oral HPV infection in HIV infected patients [47]. In the present study, we could not get anal carcinoma samples due to very low incidence of anal cancer. Thus, in order to employ methylation of specific CpG sites for screening of HPV related cancer, a large sample size including anal carcinoma samples should be further completed and evaluated.

Conclusions

The methylation patterns of HPV16 genome in anal intraepithelial neoplasia were similar to those of cervical abnormal cells. Hypermethylation in HPV16 L1 gene especially at CpG 5600 and 5609 found in AIN2/3 might be used for prediction of HPV related abnormal anal cells. Moreover, high HPV16 L1 gene methylation together with low CD4+ cell count in HIV infected patients may be used as the biomarker for rapid progression to more severely lesions than those with low methylation and high CD4+ cell count.

Acknowledgments

This work was supported by Research Grant for New Scholar Ratchadaphiseksomphot Endowment Fund, Chulalongkorn University.

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