Original article

Detection of human papillomavirus type 16 L1 gene methylation in leftover DNA samples obtained from Cobas 4800 HPV test

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Background: It has been known that persistent infections with the high-risk human papillomavirus (HR-HPVs) is the main risk factor for cervical cancer progression. However, a minor of HR-HPV infected women developed cancer. Therefore, it is necessary to look for additional test to particularly identify HR-HPV infected women who are at higher risk to progress to cervical cancer quickly.
Objective: We aimed to detect HPV16 L1 gene methylation levels in the leftover DNA samples of HPV16 positive samples with cytology diagnosed as atypical squamous cells of undetermined significance (ASCUS) obtained from Roche Cobas 4800 HPV assay.
Methods: Methylation analysis within HPV16 L1 gene at CpGs 5600, 5606, 5609 and 5615 was performed by using pyrosequencing assay in 79 HPV16 positive samples.
Results: Reliable HPV16 L1 methylation results were obtained from 29 of 79 (36.7%) HPV16 positive samples; 27.6% (8/29) samples showed methylation ≥ 10.0% at CpG5600, followed by CpG5609 that 20.7% (6/29) samples showed methylation ≥ 10.0%. There was only one sample (3.4%) showed methylation percentage ≥ 10.0% at CpG5606 and CpG5615. One sample showed high methylation (> 40.0%) at all four CpGs.
Conclusion: In all, 36.7% (29/79) of HPV16 positive samples) of DNA samples have adequate amount of DNA for further HPV16 L1 gene hypermethylation. HPV16 L1 gene hypermethylation was found at CpGs 5600 and 5609 in these samples. The study suggested that women with ASCUS cytology with high HPV16 L1 gene hypermethylation (≥ 10.0%) might be of concern and should be useful for clinician to manage HR-HPV infected women.

Keywords: Human papillomavirus 16, pyrosequencing, L1 gene methylation.

Cervical cancer is the fourth most frequently diagnosed cancer among women worldwide, with 569,847 new cases and 311,365 deaths in 2018.[1] It has been reported that high-risk human papillomavirus (HR-HPV) types were detected nearly 99.7% in women with cervical cancer.[2] HPV-16 was mostly detected in approximately 50.0%, followed by HPV18 that was detected in > 10.0% of all cervical cancer cases.[3 - 6] The other HR-HPV types reported in cervical cancer were 31, 33, 35, 45, 52 and 58.[5]

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Received: September 13, 2021
Revised: October 19, 2021
Accepted: November 30, 2021

Women persistently infected with HR-HPV types and overexpression of viral oncoproteins E6 and E7 are the main leading cause of cervical cancer progression and other HPV related cancer such as anal, head and neck cancer.[7 - 9] The molecular mechanisms of viral oncoproteins, E6 and E7, were E6 causes p53 degradation and E7 inactivates retinoblastoma proteins, these are host proteins involved in cell cycle regulation.[10 - 12]

Papanicolaou stained (Pap) smear has been used for cervical cancer screening for more than 50 years.[13] Atypical squamous cell of undetermined significance (ASCUS) detection rates ranged from 4.2% - 32.4% and HR-HPV infection rates in ASCUS ranged from 30.0 - 60.0%.[14 - 17] However, histology-confirmed CIN2+ of ASCUS cytology ranged from

5.0% -26.4%.[16 - 20] HPV16 and 18 were commonly identified genotypes in ASCUS women.[15 - 17, 21, 22] In Thailand, 26.4% of women with ASCUS cytology have histology diagnosed as high-grade cervical lesions.[18] One study reported 10.0% of normal and low-grade squamous intraepithelial lesion (LSIL) cytology showed histology diagnosed as CIN2 and 85.0% of high-grade squamous intraepithelial lesion (HSIL) cytology revealed histology results as CIN2+ and 15.0% of HSIL cytology had invasive cancer.[23] Normal cytology infected with HR-HPV types has lower absolute risk (1.0%) to develop CIN2/3 lesions.[24]

HR-HPV DNA testing has been employed as co-tests with cytology assay to screen women who are necessary for further colposcopy examination. However, after many years of persistent HR-HPV infections, only a minority of HR-HPV infected women developed cervical cancer.[25] Other molecular assays such as HPV16 L1 gene methylation has been widely studied and reported to be correlated with cervical cell transformation [26 - 31] and was recently reported to be correlated with abnormal anal cell.[32] A study in Thai women reported that HPV16 L1 gene hypermethylation (≥ 10.0%) was correlated with high grade cervical lesions and cancer cases, especially at CpGs 5600 and 5609.[26] This study aimed to investigate whether the leftover DNA samples obtained from Roche Cobas 4800 HPV assay could be used for the HPV16 L1 gene methylation analysis at CpGs 5600, 5606, 5609 and 5611 by using pyrosequencing assay.

Materials and methods

Clinical specimens

HPV 16 DNA samples were collected from leftover DNA specimens extracted from cervical cells using Roche Cobas 4800 HPV test. These DNA samples were collected from December 2016 to December 2017 at the Department of Microbiology, Faculty of medicine, Chulalongkorn University, Bangkok, Thailand. Two cervical cancer cell lines containing integrated HPV16 genome were used as positive control including CaSki (containing approximately 600 copies/cell, HPV16CRL-1550 Lot no. 3794357) and SiHa (containing 1 - 2 copies/cell, HTB-35 Lot no. 4031219). The study has been approved by the Institutional Review Board (IRB) of Faculty of Medicine, Chulalongkorn University, (COA no. 087/2016).

HPV16 L1 gene methylation analysis

DNA obtained from HPV16 positive samples were selected for bisulfite modification using the EZ kit Gold Bisulfite Conversion Kit (Zymo Research) following the manufacturer's instruction. The unmethylated cytosine was changed to uracil at the step of using sodium bisulfite to deaminate cytosine residues while methylated cytosine was not changed. The single cytosine adjacent to analyzed four CpGs was used as unmethylated control to investigate the complete bisulfite conversion from cytosine to uracil.

Methylation analysis by pyrosequencing

Previous study by our group revealed a strong association between high methylation within 5'L1 of HPV16 at CpG position 5600, 5606, 5609 and 5615 and CIN3+/cervical cancer samples.[26] Therefore, these four CpG sites were selected in the present study in samples with cytology as ASCUS. The sequences of the L1 forward and reverse primers for CpG positions 5600, 5606, 5609 and 5615 are as followed: FW biotin 5'-TAATATATAATTATTGTTGATGTAG GTGAT-3' and RV 52 AACAATAACCTCACTAAA CA ACCAAAA-32 (130 bps). Bisulfite modified HPV16 DNA positive samples were used for polymerase chain reaction (PCR) amplification as followed: DNase/RNase-free water, 1×PCR buffer, 2.5 mM MgCl2, 250 μM dNTP, 0.5 μM of each forward and reverse primers, 1 Unit Hot Start Taq DNA polymerase. The PCR conditions were 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. Next, pyrosequencing was performed using the PyroMark™ Q96 machine (Qiagen, Hilden, Germany). Sequencing primer was 5600 5'-CCAAAAAAACATCTAAAAAAAAT ATAATA-3'. The methylation percentage value of each CpG site was shown in the analyzed pyrogram. The reliable results can be obtained when the bisulfite conversion control that is single cytosine adjacent to analyzed CpG was completely converted to uracil, it was highlighted in yellow bar without a peak signal was found within the analyzed sequence. The gray bar in the pyrogram represents the analyzed four CpG sites within the L1 sequence. The percent methylation value of each CpG site shown on the top of the gray bar. High methylation showed peak signal at G (complementary to C) while low methylation showed peak signal at A (complementary to T) as shown in Figure 1.

Statistical analysis

Data analysis and graphics were performed using the SigmaPlot 12.0. Descriptive statistics were used to characterize the means values, median values, standard deviation (SD), standard error (SE) and interquartile range.

Results

Prevalence of HPV type 16 infection in cervical cells samples

In 2017, there were 1,231 cervical cells samples for HR-HPV types detection by Roche Cobas 4800 HPV test: HPV16 was detected 4.2% (79/1, 231): HPV18 was detected 1.9% (23/1, 231) and other 12 HR-HPV types was detected 17.3% (213/1, 231). 79 HPV16 positive samples were used for bisulfite modifications and PCR for pyrosequencing. Only 33 samples showed positive PCR results that could be used for pyrosequencing analysis; however, 29 samples showed reliable methylation results and can be used for further analysis.

Methylation levels of HPV16 L1 gene

Four CpGs within HPV16 L1 gene including CpGs 5600, 5606, 5609, 5615 were selected for methylation analysis. CaSki and SiHa cervical cancer cell lines containing L1 gene hypermethylation were used as positive controls in pyrosequencing assay. Pyrograms showing methylation levels of cervical cancer cell lines and HPV16 positive samples with low to high methylations are shown in Figure 1. Among 29 samples with cytology diagnosed as ASCUS, 8/29 (27.6%) samples showed methylation ≥ 10.0% at CpG5600, followed by 6/29 (20.7%) samples showed methylation ≥ 10.0% at CpG5609. There was only one sample (3.4%) showed methylation percentage ≥ 10.0% at CpG5606 and CpG5615 (Table 1). There were six samples showed methylation percentage ≥ 10.0% at both CpGs 5600 and 5609. Interestingly, one sample showed high methylation (> 40.0%) in all four CpGs (Table 2 and Figure 2).

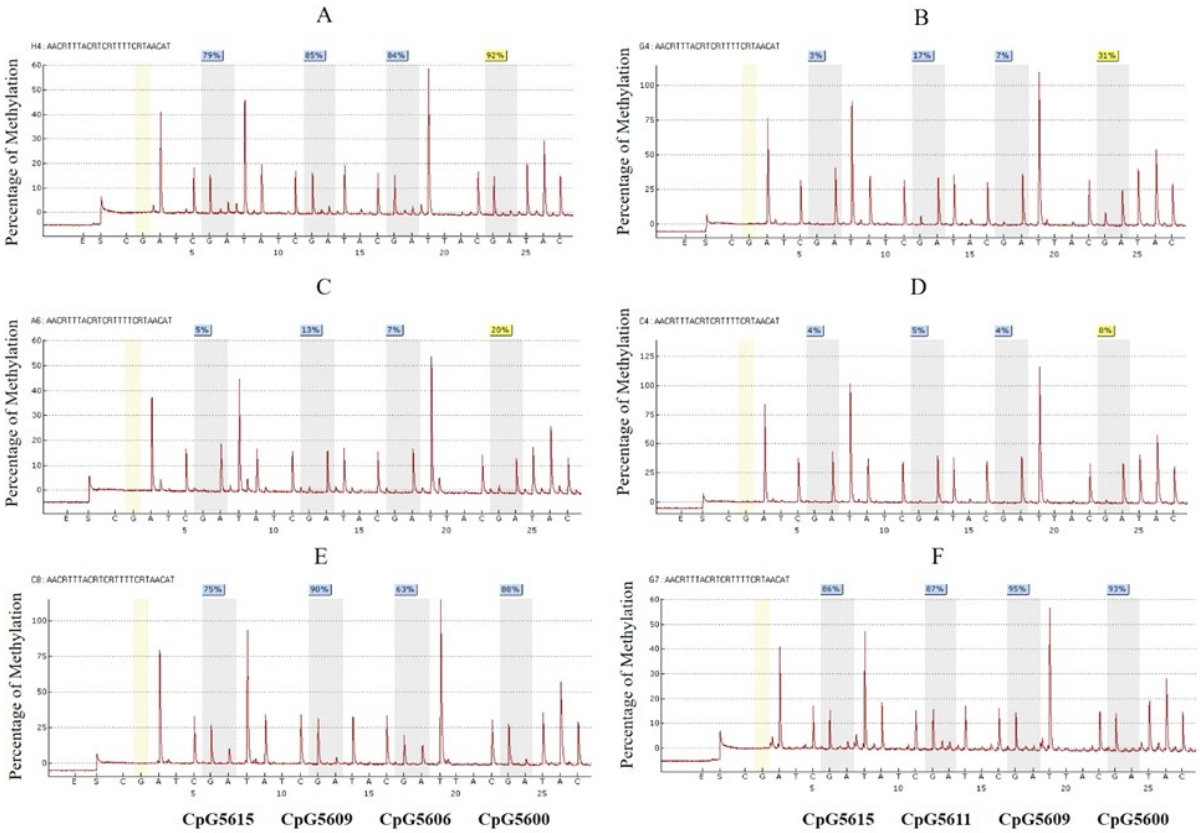

Figure 1. Pyrogram from quantification of HPV16 L1 gene methylation by Pyrosequencing. Y-axis represents percentage of methylation, X-axis represents CpG positions. The yellow bar represents the internal control in which no intensity signal was found due to unmethylated cytosine was completely converted to uracil within the analyzed sequence. The methylation percentage values of each CpG site were shown in the box on the top of the gray bar. (A) to (D) are pyrogram results obtained from four clinical samples, (E) and (F) were cervical cancer cell line, CaSki and SiHa, respectively.

Table 1. Descriptive statistics of methylation values obtained from 29 HPV16 positive samples and two cervical cancer cell lines including CaSki and SiHa.

	CpG positions in L1 gene			
	5600	**5606**	**5609**	**5615**
Mean (% methylation)	12.069	6.483	8.207	5.586
Median (% methylation)	7	4	4	3
Standard Deviation (SD)	16.716	14.999	15.333	14.204
Standard Error (SE)	3.104	2.785	2.847	2.638
Min (% methylation)	3	1	0	1
Max (% methylation)	92	84	85	79
25.0%	5	2.750	2	2
75.0%	13.750	5	9	3.250
No of samples with	8/29	1/29	6/29	1/29
≥ 10.0% methylation	(27.6%)	(3.4%)	(20.7%)	(3.4%)
CaSki (% methylation)	88	63	90	75
SiHa (% methylation)	93	95	87	86

Table 2. Methylation percentage of 29 HPV16 positive samples and two cervical cancer cell lines including CaSki and SiHa.

	Percentage of methylation					Percentage of methylation			
No.	**5600**	**5606**	**5609**	**5615**	**No.**	**5600**	**5606**	**5609**	**5615**
1	16	8	10	9	17	6	2	3	2
2	8	4	5	4	18	7	3	6	3
3	17	4	10	5	19	7	3	5	3
4	11	4	8	3	20	5	3	4	2
5	13	4	9	3	21	5	2	2	2
6	31	7	17	3	22	6	5	5	2
7	92	84	85	79	23	9	5	5	3
8	6	4	4	2	24	4	1	2	2
9	16	5	9	4	25	4	3	2	2
10	5	3	4	2	26	4	3	2	2
11	3	2	2	2	27	7	4	0	5
12	6	2	4	2	28	7	4	0	3
13	4	3	4	2	29	21	5	13	3
14	3	2	2	1	SiHa	93	95	87	86
15	7	2	3	2	CaSki	88	63	90	75
16	20	7	13	5					

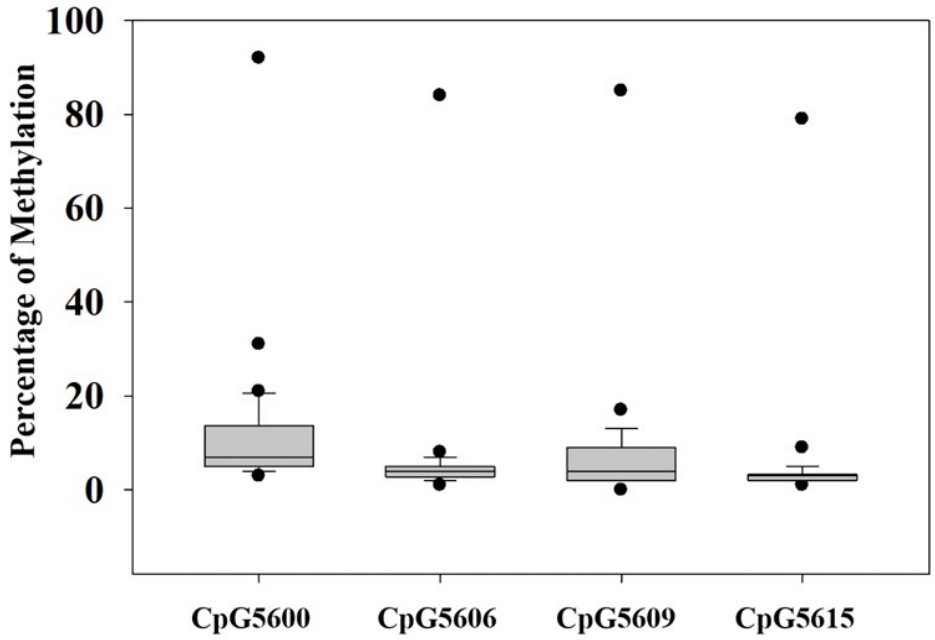

Figure 2. Box plot shows methylation levels of CpGs 5600, 5606, 5609 and 5615 of 29 HPV16 positive ASCUS samples. Low (0 – 20.0%), intermediate (20.0 – 40.0%) and high (> 40.0%) methylation values were shown.

Discussion

The present study revealed that HPV16, HPV18 and other 12 HR-HPV types detection rates found in Thai women with ASCUS cytology were lower than detected in US population (4.2% *vs* 9.2%, 1.9% *vs* 4.6% and 17.3 *vs* 44.4% for HPV16, HPV18 and other 12 HR-HPV types, respectively).[14] Combined cytology and HR-HPV types testing has been employed as a screening method for cervical cancer and could help to improve the detection rate of CIN2+ lesions.[19, 33 - 36] However, one study reported that 50.0% of HPV positive women referred to colposcopy showed normal cervical lesion.[37] Therefore, other molecular assays such as p16/Ki-67 dual staining, host gene methylation and HPV16 L1 gene methylation have been widely studied to specifically identify women who are at higher risk to develop cervical cancer quickly. [26, 38 - 48]

HPV16 L1 gene methylation especially at CpG5600 and 5609 was shown to be correlated with CIN3+.[26, 27, 49] It has been reported that during normal life cycle, the highly immunogenic L1/L2 proteins were not detected in undifferentiated basal cells, however, they were detected in differentiated superficial cells.[50] During viral life cycle, L1/L2 genes were highly methylated in undifferentiated basal cells but become unmethylated in differentiated cells leading to the expression of viral late proteins necessary for viral particle production in the upper part of the epithelium.[51] HPV late promoter was active and activated due to expression of cellular proteins such as cyclin dependent kinase 8 (CDK8) and CDK9 in differentiated cells.[52] However, in transforming HPV infected cells with dysregulated expression of E6 and E7 oncoproteins, it has been reported that HPV16 E7 protein binds and activates DNA methyltransferase 1, resulting in hypermethylation of tumor suppressor genes.[53, 54] It was hypothesized that viral self methylation by E7 protein may be the cause of late gene hypermethylation and closed chromatin was found in hypermethylated regions.[55] One study reported that methylation of CpG within open reading frame could repress gene expression.[56] Taken together, it is hypothesized that methylation at open reading frame of L1 gene leading to closed chromatin may interrupt the accessibility of transcription factors to late promoter region.

The present study detected HPV16 L1 gene methylation within four CpGs, there were 6/29 (20.0%) of ASCUS samples showed high methylation (≥ 10.0%) at both CpGs 5600 and 5609, these two CpG sites has previously reported to be associated with high grade cervical lesions or CIN2+. [26, 41, 57]

Pervious study in Thai women unveiled that methylation percentage ≥ 10.0% at both mentioned CpG sites were correlated well with CIN3+.[26] Therefore, some of ASCUS samples with ≥ 10.0% methylation at both CpG5600 and 5609 may have higher risk for abnormal cells progression. As mentioned above, methylation of L1 gene, resulting in L1 gene repression. Recent study detected L1 protein expression in cells collected from women with cytology diagnosed as ASCUS/LSIL, showed that approximately 80.0% of women with L1 protein negative have histology confirmed as CIN2+.[58]

The present study revealed that only 29/79 samples (36.7%) of leftover DNA from Cobas 4800 HPV test could be amplified by conventional HPV16 L1 methylation PCR after bisulfite treatment. It may be due to cervical cells that were send for HR-HPV typing were residual cervical cells from ThinPrep liquid based cytology preparation, leading to small amount of DNA extracted from residual cervical cells that is the limitation of the study. Real-time PCR was more sensitive to detect HPV DNA in residual material from ThinPrep.[59] DNA could be lost or fragmented during bisulfite treatment.[60, 61] In order to employ HPV16 L1 gene methylation as additional tests to HR-HPV typing, adequate cervical cell samples must be used.

Conclusion

HPV16 L1 gene hypermethylation was found at CpGs 5600 and 5609 in some of these ASCUS samples. The study suggested that women with ASCUS cytology with high HPV16 L1 gene hypermethylation (≥ 10.0%) at both CpG 5600 and 5609 might be of concern and should be useful for clinician to manage HR-HPV infected women. However, in order to employ hypermethylation status of mentioned CpG sites for screening of HPV16-related abnormal cervical lesions in women with ASCUS cytology, a large sample size with adequate DNA samples and follow-up biopsy should be further studied and evaluated.

Acknowledgements

This research is funded by Chulalongkorn University; Government Budget (grant number GB-A_60_010_30_05).

Conflict of interest

The authors, hereby, declare that we have no conflict of interest.

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