RESEARCH ARTICLE

CCNA1 Promoter Methylation: a Potential Marker for Grading Papanicolaou Smear Cervical Squamous Intraepithelial Lesions

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Abstract

Background: From our previous study, we established that cyclin A1 (CCNA1) promoter methylation is strongly correlated with multistep progression of HPV-associated cervical cancer, suggesting potential use as a diagnostic maker of disease. Objectives: The purpose of the present study was to assess the prevalence of *CCNA1* promoter methylation in residual cervical cells isolated from liquid-based cytology that underwent hrHPV DNA screening for cervical cancer, and then to evaluate this marker for diagnostic accuracy using parameters like sensitivity, specificity, predictive values and likelihood ratio. Methods: In this retrospective study, histopathology was used as the gold standard method with specimens separated into the following groups: negative (n=31), low-grade squamous intraepithelial lesions (LSIL, n=34) and high-grade squamous intraepithelial lesions or worse (HSIL+, n=32). The hrHPV was detected by Hybrid Capture 2 (HC2) and *CCNA1* promoter methylation was examined by CCNA1 duplex methylation specific PCR. Results: The results showed the frequencies of *CCNA1* promoter methylation were 0%, 5.88% and 83.33%, while the percentages of hrHPV were 66.67%, 82.35% and 100% in the negative, LSIL and HSIL+ groups, respectively. Although hrHPV infection showed high frequency in all three groups, it could not differentiate between the different groups and grades of precancerous lesions. In contrast, *CCNA1* promoter methylation clearly distinguished between negative/LSIL and HSIL+, with high levels of all statistic parameters. Conclusion: *CCNA1* promoter methylation is a potential marker for distinguishing between histologic negative/LSIL and HSIL+using cervical cytology samples.

Keywords: *CCNA1* promoter methylation - cervical cancer - HPV - duplex MS-PCR - Pap smear

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Introduction

Cervical cancer is the second most common type of cancer in women in less developed country. In South-east Asia regions, there was 51,000 newly diagnosed case and 24,000 deaths in the year 2012 (GLOBOCAN, 2012). Notably, cervical cancer has the pathological feature of long precancerous phase and spanning years. In both developed and developing countries, the cytology-based method (Pap test) using "The Bethesda reporting system" has been widely implemented as a method of choice for cervical precancer and cancer screening (O'Meara, 2002).

Fundamentally, the persistent infection with the high-risk HPV (hrHPV) is now known to be causal to cervical cancer pathogenesis and importantly, it is the presence of the virus in cytological specimens from routine screens that represents the single most significant risk factor in the etiology of this disease (Bulkmans et al., 2007; Rijkaart et al., 2012a). Therefore, women with positive HPV DNA test may be indicated for the presence of cervical precancerous and cancerous disease (de Sanjose et al., 2007). Currently, the hybrid capture II (HCII) assay is widely used in the detection of hrHPV DNA and this test is recommended to be a "co-test" together with cytology (Pap test) for cervical cancer screening in order to improve both sensitivity and specificity (Bhatla and Moda, 2009; Junyangdikul et al., 2013; Rai et al., 2014).

Although many studies supported the use of HPV DNA testing for primary screening largely due to a higher sensitivity, the data nevertheless, suggested that the specificity and positive predictive values were much lower when compared with cytology-based assays (Lui, 2013; Priebe, 2013). In this regard, women after primary screening who tested negative and positive, with the cytology-based and high-risk HPV assays, respectively, may only carry a risk of 3% to 7% for high-grade cervical intraepithelial neoplasia (CIN) (Ronco et al., 2006; Ronco et al., 2008). Furthermore, less than 10% of

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HPV-related low-grade squamous intraepithelial lesion (LSIL) progress to higher grade lesion or invasive cancer, inferring that HPV is not the sole determinant in disease progression (Mayrand et al., 2006; Snijders et al., 2006; Castle et al., 2009). Emerging from this is the question, "which test would be the best in triage for women who have positive HPV DNA test but negative cytological result for immediate referral for colposcopy?". HPV 16/18 genotyping, p16/Ki-67 dual immunocytochemistry staining, and HPV E6/E7 mRNA testing are currently available and acceptable markers for patient selection (Eide and Debaque, 2012; Wentzensen et al., 2012). Currently, better diagnostic biomarkers with higher specificity are being developed as additional tools for cervical cancer screening strategies.

Altered DNA methylation of promoter region of certain genes observed in cancer cells and not the normal counterpart, is now known to be causal to disease development and progression (Sarkar et al., 2013). To this end, hypermethylation of promoter-associated CpG islands is now a recognized as a frequent and an early event in many cancers, including cervical neoplasias (Lu et al., 2012). Recently, our group reported a strong association between *CCNA1* promoter methylation and histologic high-grade squamous intraepithelial lesion (HSIL) as well as invasive cervical carcinoma (Kitkumthorn et al., 2006).

In this study, we aim to evaluate whether *CCNA1* promoter methylation may be a complementary molecular marker for cervical precancer and cancer screening compared with HPV DNA testing (HC2). We expect that this marker can be a useful screening tool in the triage for colposcopic examination.

Materials and Methods

Sample procurement

All clinical samples, including cell pellets, were obtained from the Biospecimen bank of Department of Pathology, Faculty of Medicine, Chiang Mai University. All samples were collected with patient consent and approval. The ethics committee of Chiang Mai University, Chiang Mai, Thailand, approved this study (PAT-2556-02095). The cell pellets kept at -80ºC for DNA extraction were prepared from residual fluid from PreservCyt solution after the preparation of slide for cytologic interpretation and hrHPV DNA (HC2) test. The present study included samples from women who routinely participated in "cervical cancer screening" scheduled by the Ministry of Health between the months of May and September 2011 and were residents in three prefectures (Sankumpang, Mae-on, and Sarapee) of Chiang Mai. Each cervical specimen was collected for cytology and hrHPV test by a trained nurse, using a plastic spatula and a cytobrush. Cervical samples were then transferred from collecting devices into PreservCyt solution (Cytyc Corporation, Boxborough, MA, USA) for ThinPrep LBC preparation (Hologic, Marlborough, MA, USA) and for HC2 test (Qiagen, Hilden, Germany). According to the manufacturer's information, HC2 is designed to detect 13 high-risk HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68). Positive HC2 test is defined as one with a quantitative threshold of relative light unit/positive control (RLU/PC) ratio ≥1.0.

Women who had positive cytology (at least atypical squamous cells of undetermined significance (ASC-US) were referred for a colposcopic examination. All cytological results were classified for analysis as, a) negative, b) ASC-US/low-grade squamous intraepithelial lesion (LSIL), and c) atypical squamous cells, cannot exclude HSIL, or worse (ASC-H+). A final histologic diagnosis of all tissue specimen of each patient, obtained either by biopsy, conization, or hysterectomy was classified for analysis as; a) negative (no epithelial lesion identified), b) LSIL (or cervical intraepithelial neoplasia [CIN] 1) and c) high-grade squamous intraepithelial lesion or worse (HSIL+ or CIN2+). A final histologic diagnosis in this study referred to the worst histologic lesion detected in any tissue specimen of each patient, obtained either by biopsy, conization, or hysterectomy.

All women who have results of histopathologic examination and HC2 tests and banked cell pellets were included in this study. There were 30 negative (negative for epithelial lesion), 34 LSIL and 30 HSIL+ (22 HSIL and 8 SCC or squamous cell carcinoma) cases in total (n=94) that were available for analysis.

DNA extraction and sodium bisulfite modification

The frozen cell pellet of each case was thawed and the genomic DNA extracted using the DNeasy Blood & Tissue Kit (Qiagen, Cat no.69504), following the manufacturer's protocol. DNA qualification was done using the NanoDrop spectrophotometer (Thermo scientific™). Approximately 200 ng of DNA of each sample were subjected to sodium bisulfite treatment essentially following the guidelines provided (EZ DNA Methylation-Gold™ Kit, Zymo research corp, Orange, CA, USA).

Hybrid capture 2™ (HC2) test

We subjected all specimens to HC2 according to the manufacturer's protocol. HC2 is a sandwich-capture molecular hybridization assay that uses chemiluminescent detection to provide a semi-quantitative result. Briefly, HPV DNA from cell pellet was denatured, and then the single-stranded HPV DNA was hybridized with a mixture of single-stranded, full-genomic-length RNA probes specific for HPV. The RNA-DNA hybrids were then captured on the surface of an antibody-coated microtiter plate. Immobilized hybrids were detected by adding an alkaline phosphatase-conjugated antibody to the RNA-DNA hybrids, followed by the addition of a chemiluminescent substrate that is cleaved by the action of alkaline phosphatase to produce light. A luminometer semi-quantitatively measured the emission of light as relative-light-units (RLU). Measurements below the RLU cutoff of 1.0 were scored as negative. Positive and negative controls provided by the manufacturer were included in each run.

***CCNA1** promoter methylation*

Duplex MSPs were performed to identify the CCNA1 methylation status of all 94 samples as previously reported (Kitkumthorn et al., 2006). Briefly, the duplex

Table 1. HPV DNA and CCNA1 Methylation in Normal Cervix, LSIL and HSIL+

Colposcopic Biopsy	n	Age (Mean, Range)	HPV DNA test		*CCNA1* Promoter Methylation (HPV-Positive Sample)	
			Positive	Negative	Methylation	Unmethylation
Normal	30	46.07, 32-57	20	10	0 (0)	30 (20)
LSIL	34	43.32, 30-58	28	6	2 (2)	32 (28)
HSIL+	30	46.40, 33-57	30	0	25 (25)	5 (5)

PCR mixtures contained 10×PCR buffer (Qiagen, Chuo-ku,Tokyo), deoxynucleotide triphosphates (0.2 mM), primers CCNA1metF, CCNA1metR, CCNA1unmetF and CCNA1unmetR (final concentration 0.4 μM each per reaction), 1 U of HotStarTaq (Qiagen, Chuo-ku,Tokyo) and bisulfited DNA (80 ng). HeLa DNA was used as a positive control. The amplification reaction was carried out for 40 cycles. Then 10-μl aliquots of each PCR product was stained with cyber green, run on an 8% non-denaturing polyacrylamide gel. PCR band intensity was measured using a phosphoimager. Methylated and unmethylated bands were observed at 46 and 64 bp, respectively. Random CCNA1 methylation-positive bands were excised and processed for sequence verification.

Statistical analysis

We performed statistical analyses using STATA (version 10) for Windows. The frequency between HPV test and CCNA1 methylation were compared for sensitivity, specificity, negative predictive value (NPV) and positive predictive value (PPV), likelihood ratio positive (LR+) and likelihood ratio negative (LR-)

Results

HPV DNA testing

HC2 test was performed on all ninety-four samples constituting the 3 distinct histopathological groups. The results of each group are shown in Table 1 and Figure 1. The data shows that the frequencies of positive HPV DNA test increased along degree of epithelial lesions from 66.67% in negative group, 82.35% in LSIL and 100% in HSIL+. Notably, the test was still associated with a high rate of detection of hrHPV in the negative and the LSIL groups, suggesting low specificity of cervical cancer screening and highlighting the limitation of the test.

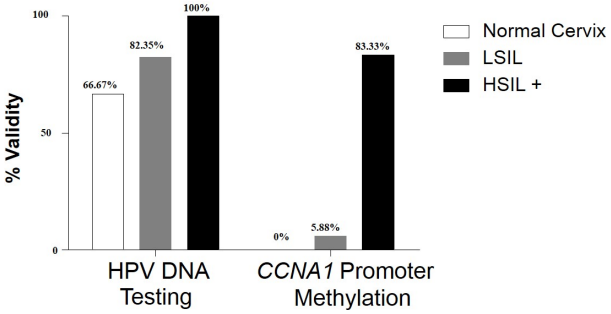

Figure 1. Percentage of Case Positive Compare between Hybrid Capture2™ HPV DNA Testing and *CCNA1* Promoter Methylation in Papanicolaou (Pap) Smear Samples of Three Patient Groups. The results showed HPV DNA were detect high percentage in all three groups. Interestingly, among three groups, CCNA1 methylation was significantly detected in HSIL+

Table 2. Comparing Sensitivity, Specificity, PPV, NPV and LR between HPV DNA and CCNA1 Methylation in HSIL+

	HSIL+	
	HPV DNA test	CCNA1 Methylation
Sensitivity	100.00%	83.33%
Specificity	21.88%	96.88%
Positive Predictive Value (PPV)	37.50%	92.59%
Negative Predictive Value (NPV)	100.00%	92.54%
Likelihood Ratio Positive (LR+)	1.28	26.67
Likelihood Ratio Negative (LR-)	0.00	0.17

***CCNA1* promoter methylation**

The frequencies of *CCNA1* promoter methylation were 0%, 5.88% and 83.33% in the negative, LSIL and HSIL+ groups, respectively. In HSIL+, all cases were hrHPV positive whereas 17 in 22 of HSIL and 7 in 8 of SCC were CCNA1 methytated. Interestingly, all samples that were assessed to be CCNA1 methylation positive by the duplex MSP test were also HPV positive, but a large number of samples in the negative and low grade groups that were detected for hrHPV were by contrast negative for *CCNA1* promoter methylation (Table 1, Figure 1).

*Sensitivity, specificity, PPV, NPV and LR between HPV DNA and **CCNA1** promoter methylation*

In a comparison between HPV DNA test and CCNA1 methylation, we used the frequency of the accuracy of each method to calculate the indicated values. Table 2 shows that the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), together with the likelihood ratio positive (LR+) and likelihood ratio negative (LR-) values of the two tests between the groups. Compared to the HSIL+ high grade group, although sensitivity of HPV DNA test (100%) was higher than CCNA1 methylation (83.33%), the specificity of HPV DNA test (21.88%) was calculated to be significantly less than CCNA1 methylation (96.88%). Importantly, for the statistical value of CCNA1 methylation, this marker showed that the efficiency was with 92.59% PPV and 92.545 NPV and further demonstrated a higher capacity than HPV DNA test alone (LR+, 26.67:1.28) to detect HSIL+.

Discussion

The triage model of cervical cancer prevention for primary HPV screening currently remains inconclusive (Rijkaart et al., 2012b). Cytology and hrHPV DNA testing are routinely recommended by ASCCP (American Society for Colposcopy and Cervical Pathology) before colposcopy (Saslow et al., 2012). In this study, hrHPV infection was observed in both LSIL and HSIL+ with high

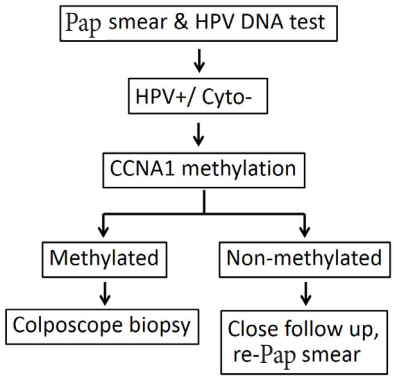

Figure 2. Proposed Model of Cervical Cancer Screening

sensitivity but low specificity. This means hrHPV infection alone was not enough to cervical cancer progression. Most importantly, our findings demonstrate that the methylation status of cyclin A1 promoter (CCNA1), a novel marker, can be successfully utilized for detection of cervical histological high grade HSIL+ in cervical cancer screening. This marker displays markably high level of sensitivity, specificity, NPV, PPV and LR+ with very low level of LR-. From several prior studies, isolated and combined promising methylation markers have been proposed to add in the triage of cervical cancer screening, for example; PAX1 (Wang, 2014), MAL/miR124a2 (Hesselink et al., 2014; Verhoef et al., 2014), combination of JAM3, EPB41L3, TERT and C13ORF18 (Hansel et al., 2014). Here, we propose to add CCNA1 methylation as a useful marker, especially in the event of HSIL+ detection. Moreover, there is a report in earlier published study with a rather similar strategy in European people (Yang et al., 2009). This will bring us to explore more details in Asian population. Fortunately, they come up with the same results that make a strong confirmation of CCNA1 methylation as a HSIL+ marker.

In our previous study, we demonstrated that HPV is strongly associated with CCNA1 methylation and reported that samples with integrated form of HPV had a higher percentage of CCNA1 methylation than the samples with episomal form of HPV (Kitkumthorn et al., 2006; Yanatatsaneejit et al., 2011). In general, HPV-E6 and E7 are up-regulated in the integrated form (Chung and Gillison, 2009). The proposed mechanism of increased CCNA1 methylation is thought to be occurred in the interaction between E6/E7 and DNA methyltransferase on the CCNA1 promoter, leading to promote methylation and gene silencing. To this end, the function of cyclin A1 is likely to be as a tumor suppressor and involvement in the DNA repair mechanism (Muller-Tidow et al., 2004; Tokumaru et al., 2004; Kitkumthorn et al., 2006; Yanatatsaneejit et al., 2008). Consequently, in contrast to HPV infection involved in the early stage cervical cancer, cyclin A1 could likely lose DNA repair function, which may be crucial in the development of high-grade epithelial lesions.

At present, cervical cancer screening tests essentially consists of two steps: screening by cytology (Paptest) with or without hrHPV DNA test followed by colposcopy. From our study, we propose to combine *CCNA1* promoter methylation and HPV DNA testing in cases of hrHPV+, especially hrHPV+/negative cytology, before colposcopy following the flowchart as detailed in Figure 2. Since, CCNA1 methylation detection is a molecular and a non-invasive technique, the strategy enables self- collected cervicovaginal specimens without biopsy (Snijders et al., 2013), for a quick and rapid analysis to help assist physician and pathologists to make informed decisions.

In conclusion, we demonstrate a potential molecular marker for the detection of high grade HSIL+ lesions of the cervix. However, due to the limitation of the sample size, we plan to evaluate this marker in a much larger sample cohort, multi-centered and prospective clinical trial setting.

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