DNA HYPERMETHYLATION PATTERNS OF *APC* GENE PROMOTER IN VIETNAMESE HIGH-RISK HPV INFECTED PATIENTS

Truong Kim Phuong[1], Lao Duc Thuan[2], Le Huyen Ai Thuy[3,*]

[1,2,3]Ho Chi Minh City Open University, Vietnam.

*Email: thuy.lha@ou.edu.vn

(Received: 28 /02/2016; Revised: 16 /03/2016; Accepted: 29/03/2016)

ABSTRACT

Cervical cancer is the leading cause of cancer death in women in Vietnam. Virtually, cervical cancers are associated with infection of HPV (Human papilloma virus). In addition, inactivation of tumor suppressor genes (TSGs), leading by aberrant hypermethylation, an epigenetic mechanism, has been observed in cervical cancer development. Screening for early detection of cervical cancer is importantly increasing from Vietnam, therefore, in current study, we analyzed the aberrant methylation status of APC (Adenomatous polyposis coli) gene, its product has an important role in cell cycle control and maintenance of genomic stability, as the pattern of potential biomarker for cervical cancer in Vietnamese population. The liquid-based Pap test samples which were identified whether HPV-infected or low-risk HPV infected or non-HPV-infected were enrolled and analyzed by MSP (Methylation specific PCR). As the results, the hypermethylation of APC was reached to 75%, 12.5% and 30% in high-risk HPV genotype infected group, in low-risk HPV genotype infected group, and non-HPV genotype infection, respectively. Especially, the characteristic of high-risk HPV infection was also associated with the hypermethylation of candidate gene ($p < 0.05$). Moreover, the odds ratio and relative risk were found in the high value, counting for 10.5 (95%CI, 2.3 – 47.2) and 3.37 (95%CI, 1.3 – 8.3), respectively. In conclusion, these outcomes suggested that the aberrant hypermethylation of APC gene, which accessed in non-invasive samples, led to the potential biomarker and application in early prognosis and diagnosis to cervical cancer in Vietnamese population.

Keywords: *APC gene; cervical cancer; hypermethylation; MSP; Vietnamese population.*

1. Introduction

The etiology of cervical cancer has been associated with several types of human papillomavirus (HPV). The common high-risk genotypes of HPV are HPV-16 and -18, which are identified as being key roles in the majority of cervical cancer, counting for approximately 70% (Burd, 2003; Castle and Maza, 2015; Ingles *et al*, 2015; Jenkins, 2008; zur Hausen, 1996). In Vietnam, the prevalence of high-risk HPV infection was ranged from 24.5% to 56.8%. Meanwhile, the prevalence of cervical infection with HPV type 16 and/or HPV type 18 was from 3.1% to 7.4% (Vu *et al*, 2013). Cervical cancer progression is a multi-steps process accumulating of genetic and epigenetic alterations in regulatory genes, leading to the inactivation or loss of expression of tumor suppressor genes (TSGs) or activation of oncogenes combined with the high-risk HPV infection and integration. In addition to the epigenetic alterations, in the past decades, abnormalities of DNA methylation have long been proved to be associated with cancer, both hypermethylation and hypomethylation. Observation on the lack of expression of several tumor suppressor genes due to the hypermethylation occurred on CpG islands in promoter regions is known to be an early

epigenetic event in driving carcinogenesis of many human cancers, including cancer of cervix (Alfonso *et al*, 2005; Baylin *et al*, 2001; Esteller *et al*, 2001; Lu *et al*, 2012; Truong *et al*, 2014; Truong *et al*, 2015).

Now, the presence of DNA in non-invasive specimens has been proved to be good at using as clinically resources for hypermethylation analysis in several human cancers (Kahn *et* al, 2008; Qureshi *et* al, 2010; Schwarzenbach and Pantel, 2015).

The Adenomatous Polyposis Coli (*APC*) tumor suppressor gene, maps on chromosome 5q21-22, has been investigated in several types of cancers. APC encodes a homodimeric protein that functions in the cytoplasm and nucleus of the cells and has an important role in cell cycle arrest and apoptosis (Aoki and Taketo, 2007). Genes encoding several key regulators of the oncogenic Wnt/β-catenin pathway, including APC, are frequently silenced via dense methylation of their promoter regions in cervical cancer (Erin *et al*, 2015; van der Meide *et al*, 2011). To access whether aberrant methylation in the promoter of CpG islands of *APC* gene from the cervical patients via analysis of various sample sources, such as serum, cervical tissue, formalin-fixed paraffin-embedded, etc by different methods, including MSP (Methylation specific PCR), has been published (Chen *et al*, 2013; Zarah *et al*, 2011; Yang *et al*, 2010; van der Meide *et al*, 2011; Wisman *et al*, 2006; Reesink-Peters *et al*, 2004).

The growing evidence that HPV infection, especially high-risk HPV types, plays as a major risk factor of cervical carcinogenesis and may serve as an important predictor, aberrant DNA hypermethylation on TSGs' promoter also is a hallmark in cancer of cervix. However, to date, almost none of the research was carried on to provide whether or not an association for patterns of DNA hypermethylation and high-risk HPV infection. A better understanding of those principles will provide the more favorable to the prognosis and diagnosis of cervical cancer.

Here, the aim at the present study is to evaluate the frequency of hypermethylation of CpG which are belonged to the promoter of *APC* gene, in Vietnamese population, as well as, to study about the association between the epigenetic event, hypermethylation, and high-risk HPV infection leading to the cancer of cervix. It was also noted here is the usage of liquid-based Pap's test specimens (PAP), non-invasive materials, in order to develop non-invasive method for prognosis and early diagnosis of cervical cancer in Vietnamese patients based on DNA methylation specific PCR.

2. Materials and methods

Sample collection

Total of 38 liquid-based Pap test samples were archived and admitted from the Au Lac Clinic, Vietnam. For input confirmed, the detection of HPV was carried out by using LightPoweriVA HPV genotype PCR-RDB Kit (Code: VA.A02-003E, Viet-A Corporation, Vietnam). As the results, all samples were divided into two groups: negative HPV infection group, which consisted of 10 samples; and positive HPV infection group, in which composed of 20 high-risk HPV (HPV genotype 16, 18 and other high-risk genotypes) infected samples and 8 low-risk HPV infected samples.

DNA isolation, bisulfite modification, MSP and BSP assay

Total of genomic DNA was isolated from PAP samples by phenol/chloroform method. Then, DNA concentration of DNA was quantified by the absorbance at OD_{260} and OD_{280}. The pure preparation of DNA with OD_{260}/OD_{280} ratio values of 1.8 to 2.0 was used to the bisulfite DNA modification assay. The bisulfite modification was carried out with approximately 2 µg genomic DNA of each sample by DNA modification Kit (Epitect Kit, Qiagen). The final precipitate were eluted in a volume of 20 µl for MSP assay.

MSP assay was carried out in a total of 15 µl containing 3 µl bisulfite-modified template DNA, 0.75 unit *iTaq* DNA polymerase (Biorad). MSP reaction was subjected to initial incubation at 95^{o}C for 5 mins, followed

by 40 cycles at 95^{o}C for 30s, X^{o}C for 30s, 72^{o}C for 30s and 72^{o}C for 6 mins for final incubation. (Note: X was the annealing temperature of each specific methylated or unmethylated primer to candidate gene). The sequences of primers and X^{o}C for each primer annealing were noted in Table 1. Each PCR product was directly loaded onto a 2.0% agarose gel, stained with ethidium bromide, and directly visualized under UV illumination. Then, MSP products were sequencing to confirm the specificity of primers, examine the efficiency of bisulfite modification and the hypermethylation status of target gene.

Table 1. Methylated and unmethylated of *APC* gene primer sequences

Primer name	Primer sequence (5' – 3')	X^{o}C	P (bp)
APC -M-F *APC* -M-R	**TATTGCGGAGTGCGGGTC** **TCGACGAACTCCCGACGA**	58^{o}C	98
APC -U-F *APC* -U-R	**GTGTTTTATTGTGGAGTGTGGGTT** **CCAATCAACAAACTCCCAACAA**	55^{o}C	108

*Note: CpG islands were bold and underlined; X^{o}C: primer annealing temperature.
M: methylated, U: Unmethylated; F: Forward; R: Reverse; P: product size.

Statistical analysis

Statistical analyses were performed using Medcalc® Version 12.7.0.0. that used the Chi-quare test of sample size. The correlation between methylation status and HPV infected status were examined by using the Chi-squared test. The differences in methylation frequencies of $p16^{INK4\alpha}$ among groups were considered statistically significant for $p \leq 0.05$. Moreover, the Odds ratio (OR), RR (Relative Risk) with 95% confidence intervals (CI) were also evaluated.

3. Results and Discussion

Hypermethylation status of *APC* promoter CpG Island

The methylation profile for *APC* promoter CpG Island was determined by using methylation specific PCR (MSP) and shown in figure 1 and table 2. According to table 2, in general, it indicated that, in the group of HPV-infection, the methylation frequency as significant higher than in two others groups. Moreover, in high-risk HPV infected samples, the methylation frequency was higher than unmethylation frequency. Conversely, the status of unmethylation in low-risk HPV or non-HPV infected group was higher than the methylation in both low-risk HPV infected group and non-HPV infected group. Especially, the characteristic of high-risk HPV infection was associated with the hypermethylation of candidate gene ($p < 0.05$).

Table 2. The methylation profile for *APC* gene

Samples	*APC* n (%)	
	M	U
High-risk HPV infected	15 (75)	5 (25)
Low-risk HPV infected	1 (12.5)	7 (87.5)
Non-HPV infected	3 (30)	7 (70)
p value	*0.05*	

As mentioned in the introduction, both the viral infection, especially high-risk HPV infection, and aberrant hypermethylation played key role in cervical carcinogenesis. In current study, HPV infection was considered as the input value of screening factor, especially high-risk HPV infection, which was proved as the majority of cervical cancer. The hypermethylation of *APC* gene's promoter was served as the candidate gene for the aims to evaluation the association for these two factors. *APC* gene, belonged to the cell cycle-related genes, has been studied in

cervical cancer, with the hypermethylation frequency up to over 60% (Chen *et al*, 2013; Zarah *et al*, 2011; Yang *et al*, 2010; van der Meide *et al*, 2011; Wisman *et al*, 2006; Reesink-Peters *et al*, 2004), which was also according to our study. The mechanism of *APC* gene's promoter methylation by high-risk HPV infection was unclear, in fact that it is more frequently methylated in advanced tumors. As the results, in the case of high-risk HPV infection, the methylation frequency of *APC* was 70%. In the contract, methylation frequency of low-risk HPV infection as well as non-HPV infection, almost samples were found as unmethylation status (counting for 12.5% and 30%, respectively). This suggested that the methylation of *APC* gene's promoter is significant phenomena of cervical cancer. Moreover, it could be inferred that in the case of high-risk HPV infection, *APC* gene's promoter was preferentially methylated. Statistically, we also found out the correlation between the high-risk HPV infection and the hypermethylation in candidate gene ($p < 0.05$). It could be highlighted that the combination of those two factors was led to the high rate of cervical carcinogenesis.

By using electrophoresis, the MSP product of *APC* was also observed in the band of 98 bps, 108 bps length in case of methylation and unmethylation, respectively, shown in Fig. 1.

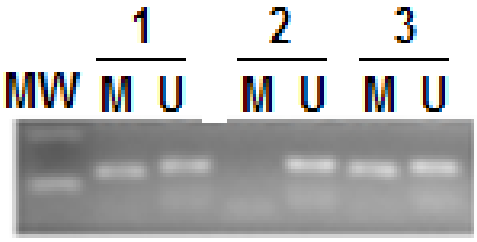

Figure 1. Methylated promoter of *APC* gene was analyzed on some representative samples by MSP. *(The MSP product was 98/108 bp in length. MW: 100 bp ladder.*

Then, MSP product was confirmed by Bisulfit-sequencing-PCR (BSP), according to Fig. 2, we successfully carried the bisulfite modification and MSP assay. By sequencing, making the comparison between the non-bisulfite modified (Fig. 2a) and bisulfite modified (Fig. 2b), all methylated Cytosines were unchanged, which were marked as green characters. Otherwise, all the unmethylated Cytosines were totally changed into Thymine in bisulfite sequence. Additionally, three methylated CpG sites were observed in methylated reverse primer, which were according to the primer designed. As shown in Fig 2c, the signal of peaks at MSP product sequencing was quite good for reading nucleotide sequencing. Therefore, for those reasons, it was concluded that the bisulfite modification was successfully carried out.

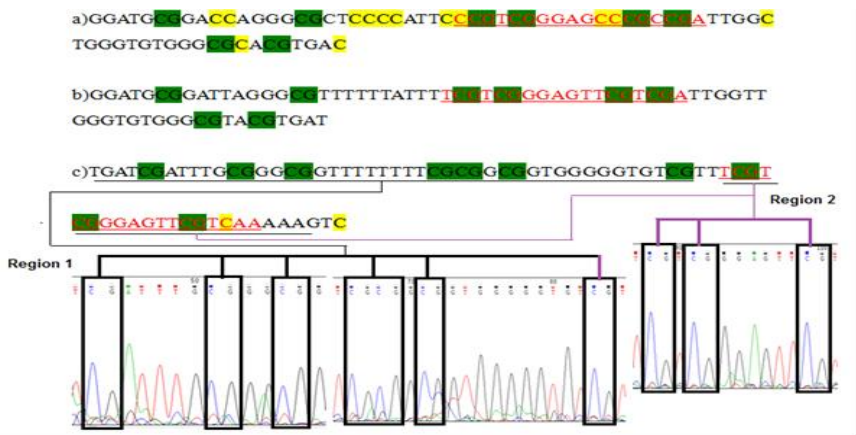

Figure 2. Sequencing profile of segment methylated of *APC*. CG sites were in the green highlight; Cytosine did not depend on the CpG site were in yellow. (a) DNA sequence was without bisulfite modified; (b) DNA sequence was bisulfite modified; (c) The *APC* sequencing by using the APC-M-R primer.

Calculation of odds ratio, relative risk

In this study, through the analysis of methylation or unmethylation status of *APC*, odds ratio, relative risk were also calculated. The odds ratio (OR) and relative risk (RR) were evaluation between high-risk HPV infection group and low-risk HPV group combined with non-HPV group, as shown in table 3.

Table 3. The result of odds ratio (OR) and relative risk (RR) calculation

	APC
OR	10.5
95% CI	2.3 – 47.2
p value	*< 0.01*
RR	3.37
95% CI	1.3 – 8.3
p value	*< 0.01*

According to table 3, the odds ratio was 10.5 (95%CI, 2.3 – 47.2) for *APC*. It meant that the odds for a positive hypermethylation of *APC* promoter of high-risk HPV infection was 10.5 times higher than in the case of cancer without methylation. The methylation status of above gene's promoter showed the significant correlation with the high-risk HPV infection, which leading to cancer of cervix. In addition, concerning to the RR, it indicated that the hypermethylation of *APC* promoter increasing the risk to cervical cancer up to 3.37 (95%CI, 1.3 – 8.3) in comparison with unmethylation. Therefore, from current study, it could be inferred that DNA methylation of *APC* gene's promoter in cervical cancer involving the status of HPV genotype infection, especially high-risk HPV infection, leading to cervical tumorgenesis.

Due to those results, the hypermethylation of *APC* was the characteristic of high-risk HPV infection, leading to the cervical cancer in Vietnamese population. In far, this characteristic was excessed by MSP method of non-invasive method will be the potential biomarker, especially combined with the HPV genotyping, for the clinical application in prognosis and early diagnosis of cervical cancer.

4. Conclusion

In summary, the frequency of *APC* was 70%, which was a specific phenomenon of high-risk HPV infection. The odds ratio and relative risk were found in the high value, counting for 10.5 (95%CI, 2.3 – 47.2) and 3.37 (95%CI, 1.3 – 8.3), respectively. The screening, which based on the combination of both high-risk HPV detection and *APC* gene's promoter methylation, will be an auspicious characteristic for early prognosis and diagnosis of cervical cancer. Moreover, these findings suggested that MSP assay done in candidate gene on the non-invasive samples (liquid-based PAP test) will provide the potential method, which was easily applied to the clinic, to prognosis and early diagnosis of cervical cancer in Vietnamese population. In further study, those methods will be continuously carried out on many potential genes in order to get the profile of methylated genes related to cancer of cervix.

REFERENCES

Alfonso Dueñas-González, Marcela L, Myrna C, et al. (2005). Epigenetics of cervical cancer. An overview and therapeutic perspectives. *Mol Cancer*, 4:38.

Aoki K., Taketo MM. (2007). *Adenomatous polyposis coli (APC): A multi-functional tumor suppressor gene. Journal of Cell Science,* 120(19): 3327-35.

Baylin SB, Esteller M, Rountree MR, et al. (2001). Aberrant patterns of DNA methylation, chromatin formation and gene expression in cancer. *Hum Mol Genet*, 10(7):687-92.

Burd EM. Human papillomavirus and cervical cancer. (2003). *Clin Microbiol Rev*, 16(1):1-17.

Castle PE, Maza M. (2015). Prophylactic HPV vaccination: past, present, and future. *Epidemiol Infect*, 2:1-20.

Chen Y., Zhang C-L., Yong-Zhen L., Yi L, Jing F. (2013). Promoter methylation of APC genes in cervical cancer: correlation with clinicopathologic characteristics. STMOPEN.net.

Esteller M, Corn PG, Baylin SB, et al. (2001). A gene hypermethylation profile of human cancer. *Cancer Res*, 61(8):3225-9.

Ingles DJ, Pierce Campbell CM, Messina JA, et al. (2015). Human papillomavirus virus (HPV) genotype - and age-specific analyses of external genital lesions among men in the HPV Infection in Men (HIM) Study. *J Infect Dis*, 211(7):1060-7.

Jenkins D. (2008). A review of cross-protection against oncogenic HPV by an HPV-16/18 AS04-adjuvanted cervical cancer vaccine: Importance of virological and clinical endpoints and implications for mass vaccination in cervical cancer prevention. *Gynecol Oncol*, 110:S18-25.

Kahn SL, Ronnett BM, Gravitt PE, et al. (2008). Quantitative methylation-specific PCR for the detection of aberrant DNA methylation in liquid-based Pap tests. *Cancer*, 114(1):57-64.

Lu Q, Ma D, Zhao S. (2012). DNA methylation changes in cervical cancers. *Methods Mol Biol*, 863:155-76.

Qureshi SA, Bashir MU, Yaqinuddin A. (2010). Utility of DNA methylation markers for diagnosing cancer. *Int J Surg*, 8(3):194-8.

Reesink-Peters N, Wisman GB, Jeronimo C, Tokumaru CY, Cohen Y, Dong SM, et al. (2004) Detecting cervical cancer by quantitative promoter hypermethylation assay on cervical scrapings: a feasibility study. *Mol Cancer Res*, 2: 289–295.

Schwarzenbach H, Pantel K. (2015). Circulating DNA as biomarker in breast cancer. *Breast Cancer Res*, 17:136.

Truong PK, Lao TD, Doan TP, Le TA.(2014). BRCA1 promoter hypermethylation signature for early detection of breast cancer in the Vietnamese population. *Asian Pac J Cancer Prev*, 15(22):9607-10.

Truong PK, Lao TD, Doan TP, Le TA. (2015). Loss of expression of cyclin d2 by aberrant DNA methylation: a potential biomarker in vietnamese breast cancer patients. *Asian Pac J Cancer Prev*, 16(6):2209-13.

Van der Meide WF, Snellenberg S, Meijer CJ, Baalbergen A, Helmerhorst TJ, van der Sluis WB, et al. (2011) Promoter methylation analysis of WNT/beta-catenin signaling pathway regulators to detect adenocarcinoma or its precursor lesion of the cervix. *Gynecol Oncol*, 123: 116–122.

Vu LT, Bui D, Le HT. (2013). Prevalence of cervical infection with HPV type 16 and 18 in Vietnam: implications for vaccine campaign. *BMC Cancer*, 13:53.

Wisman GB, Nijhuis ER, Hoque MO, Reesink-Peters N, Koning AJ, Volders HH, et al. (2006) Assessment of gene promoter hypermethylation for detection of cervical neoplasia. *International journal of cancer*, 119: 1908–1914.

Yang N, Nijhuis ER, Volders HH, Eijsink JJ, Lendvai A, Zhang B, et al. (2010) Gene promoter methylation patterns throughout the process of cervical carcinogenesis. *Cell Oncol*, 32: 131–143.

Zarah M., Wingren S., Nilsson T. K. (2011). Hypermethylation of promoter regions of the apc1a and p16ink4a genes in relation to prognosis and tumor characteristics in cervical cancer patients. *International journal of oncology*, 39: 683-688.