Original Article

DNA Methylation Assessment in Cervical Cancer Specimens

Wandee Udomchaiprasertkul MSc[1], Nuttavut Kantathavorn MD[2,3], Thaniya Sricharunrat MD[4], Kanyanee Laebua MD[5], Pornwaree Trirussapanich MD[5], Chakrit Bunyoo MSc[1], Kriangpol Wiriyaukaradecha MSc[1], Narongrit Sritana MSc[1], Sasikarn Chamchod MD[3,5], Chirayu Auewarakul MD, PhD[3]

[1] Molecular Biology and Genomic Research Laboratory, Division of Research and International Relations, HRH Princess Chulabhorn College of Medical Science, Chulabhorn Royal Academy, Bangkok, Thailand
[2] Gynecologic Oncology Unit, Woman Health Center, Chulabhorn Hospital, HRH Princess Chulabhorn College of Medical Science, Chulabhorn Royal Academy, Bangkok, Thailand
[3] Faculty of Medicine and Public Health, Chulabhorn Hospital, HRH Princess Chulabhorn College of Medical Science, Chulabhorn Royal Academy, Bangkok, Thailand
[4] Pathology Laboratory Unit, Chulabhorn Hospital, HRH Princess Chulabhorn College of Medical Science, Chulabhorn Royal Academy, Bangkok, Thailand
[5] Department of Radiation Oncology, Chulabhorn Hospital, HRH Princess Chulabhorn College of Medical Science, Chulabhorn Royal Academy, Bangkok, Thailand

Objective: To determine methylation levels of DAPK1, MGMT, FAS, TRAIL-R1 and p73 genes in formalin fixed, paraffin embedded [FFPE] tissues from patients with cervical cancer stage I-IV using high resolution melting [HRM].

Materials and Methods: DNA was extracted from FFPE of 63 cervical cancer specimens and 42 normal specimens. Methylation levels of DAPK1, MGMT, FAS, TRAIL-R1 and p73 genes were detected by HRM.

Results: The methylation levels of DAPK1, FAS, TRAIL-R1 and p73 genes showed statistically significant differences between the disease and the control cases ($p<0.005$). In contrast, the methylation of MGMT showed no significant difference between the disease and the control cases. DAPK1 methylation level was associated with squamous cell carcinoma patients.

Conclusion: DAPK1, FAS, TRAIL-R1 and p73 are aberrantly methylated in cervical cancer compared with normal tissue, while MGMT is less commonly methylated. HRM can be used for DNA methylation assessment of genes of interest in cervical cancer FFPE specimens.

Keywords: Cervical cancer, DNA methylation, High resolution melting [HRM], Epigenetics, Disease markers

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DNA methylation is an epigenetic modification mechanism that can result in either downregulation or complete abrogation of gene-associated expression. Several studies have indicated that DNA methylation of specific genes in cancer cells may be a potential applicable biomarker for clinical use. For example, in glioblastoma, MGMT methylation is a prognostic marker in patients treated with temozolomide[(1)]. Previous studies in cervical cancer patients have demonstrated a correlation between promoter methylation of a number of genes, including oncogenes and tumor suppressor genes, with cervical oncogenesis progression and treatment response[(2-4)]. Furthermore, methylation of genes encoding proteins that regulate

Correspondence to:
Udomchaiprasertkul W. Molecular Biology and Genomic Research Laboratory, Division of Research and International Relations, HRH Princess Chulabhorn College of Medical Science, Chulabhorn Royal Academy, 54 Kamphaengphet 6 Road, Talat Bangkhen, Laksi, Bangkok 10210, Thailand.
Phone: +66-2-5766389, **Fax:** +66-2-5766392
E-mail: wandee.udo@pccms.ac.th

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DNA damage-induced apoptosis, such as DAPK1 and FAS, have been demonstrated in cervical cancer patients who acquired therapeutic resistance to concurrent chemoradiothe rapy[2]. DNA methylation of pro-apoptotic genes that have been identified as tumor suppressor genes may cause resistance to apoptosis and development of cervical cancer[2,5,6].

The aim of this study was to estimate DNA methylation level of DAPK1, MGMT, FAS, TRAIL-R1 and p73 genes in patients with cervical cancer stage I-IV using high resolution melting [HRM], which is a rapid and sensitive method for DNA methylation assessment[7]. In addition, we investigated the correlations of DNA methylation with demographic data and treatment response. These results may provide useful information for further investigation of predictive markers for treatment of cervical cancer patients.

Materials and Methods

Patients

This study was approved by the Human research ethics commette Chulabhorn Research Institute (EC No. 15/2554). Formalin fixed, paraffin embedded tissues of 63 cervical cancer specimens were obtained from the pathology laboratory unit and 42 specimens obtained from normal cervix were used as controls. All samples were obtained at the time of diagnosis and before treatment. Informed written consent for molecular studies was obtained from all patients. After treatment for 3 months, pelvic examination was monitored to assess response to the therapy. Patients who showed no lesion or recurrence within 3 months were defined as therapeutic response; patients who lesions existed or progressed within 3 months were defined as therapeutic nonresponse.

DNA extraction and HRM analysis

Tumor sections were reviewed by a pathologist to confirm the diagnosis and define tumor-enriched areas for dissection. Laser microdissection or macrodissection was performed for samples with <70% malignant cells. DNA was extracted using the QIAamp DNA Micro kit (QIAGEN, Germany) and bisulfite converted using the EZ DNA methylation kit (Zymo Research, USA). The bisulfite-modified DNA was PCR amplified with the specific primers (Table 1) in a Precision Melt Supermix (BIO-RAD, USA). PCR amplification and HRM were performed using a CFX96 (BIO-RAD, USA). The methylation standard was constructed by diluting CpGenome universal methylated DNA (Merck Millipore, USA) with CpGenome universal unmethylated DNA (Merck Millipore, USA) to 100%, 75%, 50%, 25%, 10%, 5%, 2%, 1%, 0.5% and 0% methylation. The amplification protocol was 2 min at 95°C, then 45 cycles of 10 s at 95°C, 30 s at annealing temperature, 30 s at 95°C and a final step of 1 min at 60°C. HRM was performed with a ramping from 65°C to 95°C at an increase of 0.2°C/10 s. Melting curves were normalized using the Precision Melt Analysis software (BIO-RAD, USA). The methylation levels of each sample were assessed by comparison of the PCR product melting profiles between each sample and the methylation standards.

Statistical analysis

The differences between DNA methylation levels in the disease cases and the controls, as well as the association between DNA methylation level and demographic data of cervical cancer patients were determined by exact probability test. A p-value <0.05 was considered statistically significant.

Table 1. PCR primer sequences and annealing temperatures

Gene	Primer sequences (52-32)	Product size (bp)	Annealing temperature (°C)
DAPK1	F-CGGTATAAGTTGGGATTTTAGTATAT R-CGCCCCACCTATAACACATTA	101	56
MGMT	F-CGTTTCGGATATGTTGGGATAGT R-CGACCCAAACACTCACCAAA	108	60
FAS	F-CGCGTAGGTTAAGTTGTTGAATT R-CGCTTCCCTCACTCCCCAA	170	56
TRAIL-R1	F-CGTAAAAGTTTTTTAGAGGTTAGAT R-CGAAAATTAACCTCAACCTTTCTA	127	56
p73	F-CGGTTATATTTTTTGTTTTTTGGATTT R-CGAACTCCCTACTATCCCCCAAA	128	58

Results

A total of 63 tissue specimens of cervical cancer patients were included in this study. The methylations of DAPK1, MGMT, FAS, TRAIL-R1 and p73 genes were evaluated by HRM as described in Methods. The clinical data of the patients are shown in Table 2, and the methylation levels of the five genes are shown in Table 3. We categorized the methylation according to frequency: 0 to 5%, 5 to 10%, 10 to 25%, 25 to 50%, 50 to 75% and 75 to 100%. Most of the samples showed methylation level of DAPK1 between 0 to 5% to 50 to 75%. MGMT and TRAIL-R1 showed very low levels of methylation, at 0 to 5% and 5 to 10%. For FAS as well as p73, low levels of methylations were detected at 0 to 5%, 5 to 10% and 25 to 50%. One sample could not be evaluated for DAPK1, MGMT and FAS methylation due to failed amplification. Interestingly, this assay was able to detect FAS and MGMT methylation at 0.5% and 1% methylated DNA standard under the background of the unmethylated DNA standard (data not shown).

We also performed semi-quantitative analysis using the methylation level at 5 to 100% to define the gene as methylated. The results showed that DAPK1, MGMT, FAS, TRAIL-R1, and p73 genes were methylated in 37.1%, 1.6%, 14.5%, 65.1% and 11.1% cancer cases, respectively, and 0% in the controls (Table 4). The methylation levels of DAPK1, FAS, TRAIL-R1 and p73 gene were significantly different in the cervical cancer cases compared with the controls ($p<0.005$). In contrast, MGMT showed no significant difference in methylation levels between the disease cases and the controls.

We next examined the correlation between DNA methylation and demographic data of the cervical cancer patients (Table 5). The results show that there were no significant associations between the DNA methylation levels and the tumor staging. Regarding the histologic type, adenocarcinoma [ACC], squamous cell carcinoma [SCC], and adenosquamous cell carcinoma, the methylation of DAPK1 was significantly correlated with SCC ($p = 0.003$). We also examined the association between DNA methylation and therapeutic response at 3 months. There were 46 cases that responded to the therapy and 5 non-response cases. However, there was no significant association between individual gene methylation and treatment response at 3 months (data not shown).

Table 2. Clinical data of the cervical cancer patients (n = 63)

Clinical data	n (%)
Stage	
I	9 (14.3)
II	31 (49.2)
III	20 (31.7)
IV	3 (4.8)
Age*	
Min-max	26 to 78
(mean ± SD)	(55±11.9)
Histologic type*	
Adenocarcinoma	11 (20.8)
Squamous cell carcinoma	39 (73.6)
Adenosquamous cell carcinoma	3 (5.7)

* Data was missing in one case

Discussion

In the present study, we used HRM to determine DNA methylation in DNA from 63 FFPE tissues. However, we found that DNA derived from FFPE tissue was mostly degraded, with a limited amount of cervical tumor tissues in the specimens. Several

Table 3. Methylation levels of five genes in cervical cancer patients (n = 63)

Methylation levels	DAPK1	MGMT	FAS	TRAIL-R1	p73
0 to 5%	39 (61.9%)	61 (96.9%)	53 (84.1%)	22 (34.9%)	56 (88.9%)
5 to 10%	2 (31.2%)	1 (1.6%)	7 (11.1%)	41 (65.1%)	6 (9.5%)
10 to 25%	9 (14.3%)	0 (0%)	2 (3.2%)	0 (0%)	1 (1.6%)
25 to 50%	10 (15.9%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
50 to 75%	2 (3.2%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
75 to 100%	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
ND	1 (1.6%)	1 (1.6%)	1 (1.6%)	0 (0%)	0 (0%)

ND = not determined

Table 4. Distribution of DNA methylation in five genes in the cervical cancer cases and controls

	Methylation level	Cases (n = 63)	Controls (n = 42)	*p*-value
DAPK1*	0 to 5%	39 (62.9%)	42 (100%)	<0.001**
	5 to 100%	23 (37.1%)	0 (0%)	
MGMT*	0 to 5%	61 (98.4%)	42 (100%)	1.000
	5 to 100%	1 (1.6%)	0 (0%)	
FAS*	0 to 5%	53 (85.5%)	42 (100%)	0.010**
	5 to 100%	9 (14.5%)	0 (0%)	
TRAIL-R1	0 to 5%	22 (34.9%)	42 (100%)	<0.001**
	5 to 100%	41 (65.1%)	0 (0%)	
p73	0 to 5%	56 (88.9%)	42 (100%)	0.040**
	5 to 100%	7 (11.1%)	0 (0%)	

* Failed to amplify in one case

methods have previously been used to determine quantitative and semi-quantitative levels of DNA methylation.

The proteins encoded by DAPK1, MGMT, FAS, TRAIL-R1, and p73 are involved in the regulation of programmed cell death and inducing the apoptotic response to DNA damage. DAPK1, a stress-responsive serine/threonine kinase, acts as a positive mediator of apoptosis by inhibiting cell survival and prolifera tion[8,9]. MGMT is a DNA methyl transferase that is highly methylated in cancer tissues including cervical cancer, non-small cell lung cancer, gastric cancer and colorectal cancer[10]. Previous studies have revealed a strong association between DAPK1 methylation and cervical cancer[4,11]. Siegel et al measured DNA methylation using pyrosequencing and found DAPK1 methylation of about 50% in cervical tumors[11]. In addition, Sun et al showed DAPK1 and MGMT methylation in high-grade and low-grade squamous intraepithelial lesions[12]. Fas cell surface death receptor or FAS and tumor necrosis factor-related apoptosis-inducing ligand receptor-1 [TRAIL-R1] are pro-apoptotic genes that play roles in apoptosis initiation via the extrinsic pathway[2]. FAS hypermethylation was determined in colorectal cancer tissue and may involve cancer carcinogenesis[13]. Chaopatchayakul et al reported FAS and TRAIL-R1 methylations in more than 50% of cervical cancer tissues (n = 85). Moreover, combined methylation of DAPK1, FAS, and TRAIL-R1 was found in non-response to therapy[2]. p73 is a tumor-suppressor gene and functionally homologous to p53. Several studies have demonstrated aberrant promoter methylation of p73 in several types of cancer[14,15]. Liu et al reported that p73 hypermethy lation was detected in 40% of cervical cancer and was related to radiosensitivity of cervical cancer cells[14]. These studies and findings indicate that DAPK1, MGMT, FAS, TRAIL-R1, and p73 are important genes related to carcinogenesis, pathogenesis, and resistance to chemoradiotherapy of cervical cancer.

Our findings in this study revealed significant differences between the methylation levels of DAPK1, FAS, TRAIL-R1 and p73 genes in cervical cancer compared with control cases. In addition, we found that DAPK1 methylation was associated with SCC patients. These results are consistent with results from previous studies[2,11]. These results suggest that DAPK1, FAS, TRAIL-R1 and p73 methylation may be used as a potential diagnostic test for cervical cancer. However, this study demonstrated no association between DNA methylation and therapeutic response due to the low number of non-response cases, a relatively short follow-up period and loss to follow-up. Further research should be conducted to explore the correlation between DNA methylation and therapeutic response in a larger number of cases.

Conclusion

The authors confirmed that DAPK1, FAS, TRAIL-R1 and p73 are aberrantly methylated in cervical cancer compared with normal tissue, while MGMT is less commonly methylated. Furthermore, DAPK1 methylation was associated with SCC patients. Our results indicate that HRM can be used for DNA methylation assessment of genes of interest in cervical cancer formalin-fixed paraffin-embedded specimens. HRM may become an alternative method for DNA methylation assessment to provide useful information for clinical study and research of cervical cancer and other diseases.

Table. 5 Correlation between DNA methylation and demographic data of the cervical cancer patients

	DAPK1			MGMT			FAS			TRAIL-R1			p73		
	0 to 5%	5 to 100%	*p*-value	0 to 5%	5 to 100%	*p*-value	0 to 5%	5 to 100%	*p*-value	0 to 5%	5 to 100%	*p*-value	0 to 5%	5 to 100%	*p*-value
Stage			0.457			0.480			0.504			0.464			0.239
I	6 (20.7%)	1 (5.0%)		7 (14.3%)	0		5 2 (12.5%)	2 (20.0%)		3 (23.1%)	4 (10.8%)		6 (14.0%)	1 (14.3%)	
II	13 (44.8%)	12 (60.0%)		26 (53.1%)	0		21 (52.5%)	5 (50.0%)		5 (38.5%)	21 (56.8%)		24 (55.8%)	2 (28.6%)	
III	9 (31.0%)	6 (30.0%)		14 (28.6%)	1 (100%)		13 (32.5%)	2 (20.0%)		5 (38.5%)	10 (27.0%)		12 (27.9%)	3 (42.9%)	
IV	1 (3.5%)	1 (5.0%)		2 (4.1%)	0		1 (2.5%)	1 (10.0%)		0	2 (5.4%)		1 (2.3%)	1 (14.3%)	
Histologic type			0.003*			1.000			0.060			0.306			0.053
ACC	11 (35.5%)	0		11 (21.6%)	0		8 (19.1%)	3 (30.0%)		5 (33.3%)	6 (16.2%)		10 (22.2%)	1 (14.3%)	
SCC	19 (61.3%)	18 (90.0%)		37 (72.6%)	1 (100%)		33 (78.6%)	5 (50.0%)		10 (66.7%)	28 (75.7%)		34 (75.6%)	4 (57.1%)	
ACC and SCC	1 (3.2%)	2 (10.0%)		3 (5.9%)	0		1 (2.4%)	2 (20.0%)		0	3 (8.1%)		1 (2.2%)	2 (28.6%)	

Note: numbers may not add up to total due to missing data.
ACC = adenocarcinoma; SCC = squamous cell carcinoma; ACC and SCC = adenosquamous cell carcinoma

What is already known on this topic?

Methylation of DAPK1, MGMT, FAS, TRAIL-R1, and p73 genes has been explored in cervical cancer by using different quantitative and semi-quantitative methods. Methylation of these genes was frequently observed in cervical cancer.

What this study adds?

This work quantitated methylation levels of genes involved in apoptotic response using HRM, a rapid and sensitive method for DNA methylation assessment. Our findings were consistent with previous reports. In addition, we examined the correlation between treatment response and DNA methylation. However, there was no association between DNA methylation and therapeutic response due to a low number of non-response cases.

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Potential conflict of interest

The authors declare no conflict of interest.

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