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ORIGINAL ARTICLE

Promoter methylation of *DAPK1*, *FHIT*, *MGMT*, and *CDKN2A* genes in cervical carcinoma

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Abstract
Background and objectives Aberrant DNA methylation contributes to the malignant phenotype in virtually all types of human cancer. This study explored the relationship between promoter methylation and inactivation of the *DAPK1*, *FHIT*, *MGMT*, and *CDKN2A* genes in cervical cancer.
Methods The promoter methylation of *DAPK1*, *FHIT*, *MGMT*, and *CDKN2A* was investigated by using a methylation-specific polymerase chain reaction in 53 specimens of cervical cancer (42 squamous cell carcinoma, 11 adenocarcinoma), 22 specimens of intraepithelial neoplasia tissues, and 24 control normal cervical tissue specimens. The correlation of promoter methylation with the clinicopathological features of cervical cancer was analyzed. The expressions of *DAPK1*, *FHIT*, *MGMT*, and *CDKN2A* were detected by measuring relative mRNA levels.
Results The promoter methylation of *DAPK1*, *FHIT*, *MGMT*, and *CDKN2A* in cervical cancer vs. intraepithelial neoplasia vs. normal cervical tissue was 75.5 vs. 31.8 vs. 4.2 % ($p < 0.0001$), 66.0 vs. 59.1 vs. 25.0 % ($p = 0.0033$), 34.0 vs. 27.3 vs. 20.8 % ($p = 0.76$), and 17.0 vs. 31.8 vs. 8.3 % ($p = 0.11$), respectively. The methylation of the promoter region significantly decreased the expression of only *DAPK1* ($p = 0.03$). The methylation rate of the *DAPK1* gene promoter was significantly higher in cervical cancer tissues than in cervical intraepithelial neoplasia and normal cervical tissues.
Conclusion Promoter methylation may therefore lead to the inactivation of the *DAPK1* gene, and may be related to the progression of cervical oncogenesis.

Keywords Cervical cancer · Methylation · *DAPK1*

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Introduction

Cervical cancer is the fourth most common cancer in Japanese females, with an estimated 24,240 cases in 2003, including 8,674 cases of invasive cervical cancer and 6,955 cases of carcinoma in situ [1]. Multiple epidemiology and molecular biological studies indicate that human papillomavirus (HPV) is the major factor associated with the development of cervical cancer [2–5]. It is clear that other factors are involved in cervical carcinogenesis because the majority of patients with HPV-associated lesions, such as cervical intraepithelial neoplasia, do not progress to invasive cancer but remain stable or spontaneously regress over time [6]. Therefore, it is likely that host genetic and epigenetic events play an important role in cervical carcinogenesis. The CpG islands of a large number of genes that are unmethylated in normal tissues are methylated to various degrees in several types of human cancer and particularly in gynecological cancer [7–11]. The aberrant methylation of CpG islands within the promoter regions of several tumor suppressor genes has been reported in cervical cancer [11–23]. The extent of aberrant promoter hypermethylation and its association with loss of gene function in cancer suggests that CpG island methylation is an important mechanism in inactivating tumor suppressor genes. Many studies have previously reported the promoter hypermethylation of *DAPK1* (45 %), *FHIT* (11–50 %), *MGMT* (5–60 %), *CDKN2A* (7–57 %), and other genes during the progression

of cervical cancer [12, 13, 17, 18, 25, 30]. The purpose of the present study was to examine whether or not similar tendencies exist in Japanese patients. This study analyzed the relationship between the clinicopathological parameters and the methylation of the *DAPK1*, *FHIT*, *MGMT*, and *CDKN2A* genes in normal, premalignant, and malignant cervical samples to determine whether it is a useful molecular marker for monitoring cervical cancer.

Materials and methods

Clinical samples and DNA preparation

Samples were obtained from 53 primary cervical carcinoma patients, 22 cervical intraepithelial neoplasia and carcinomas in situ (CIN3-CIS), and 24 normal cases that were treated at Niigata University Medical and Dental Hospital, Ryukyu University Hospital, Niigata Cancer Center Hospital, and Nagaoka Red Cross Hospital, between 2004 and 2010. We extracted DNA from the tumor cells to assess the presence or absence of methylation, and performed a cytological examination of the lesion before or after surgery to confirm the presence of carcinoma in situ of the cervix in relation to cases of cervical dysplasia. All diagnoses were confirmed by pathological examination.

The clinicopathological characteristics of the samples are given in Tables 1 and 2. Thirty-seven of the tumors were stage 1, and 16 were stage 2–4. The stages of each cancer were established according to the 1995 staging system of the International Federation of Gynecology and Obstetrics (FIGO) criteria. 81 % (17/21) of CIN3 and 72 % (26/36) of cervical carcinoma patients were infected with HPV (Table 2).

Forty-two of 53 tumors were squamous cell carcinomas and 11 were adenocarcinomas. None of the patients had received radiotherapy or chemotherapy before the samples were obtained. The specimens were immediately processed in the laboratory for DNA extraction, HPV typing, and quantitative RT-PCR analysis.

Bisulfite modification and methylation-specific PCR

The bisulfite conversion of genomic DNA was performed using a CpGenome DNA modification kit (Chemicon International, Temecula, CA, USA). The promoter methylation of *DAPK1*, *FHIT*, *MGMT*, and *CDKN2A* was investigated by using a methylation-specific polymerase chain reaction (PCR).

Quantitative real-time PCR analysis

Total RNA was extracted from tissue samples using TRIzol reagent (Invitrogen). Total RNA (1 μg) from cervical cancer was used as a template in first-strand cDNA synthesis with the SuperScript III First-Strand Synthesis System (Invitrogen).

The cDNA was diluted 1:10 for subsequent real-time PCR, which was carried out using TaqMan Gene Expression

Table 1 Clinical characteristics in normal, CIN3, and cancer samples

	Normal	CIN3	Cancer	*p* value*
No.	24	22	53	
Age (mean ± SD)	48.1 ± 10.5	38.9 ± 13.5	49.3 ± 13.3	0.66
Smoking				
Yes	12	13	21	
No	12	9	32	0.27

CIN3 cervical intraepithelial neoplasia, grade 3

* Chi-squared test

Table 2 Clinical characteristics of CIN3 and cancer samples

	CIN3	Cancer
No.	22	53
HPV high-risk	17	26
Others	2	4
Negative	2	6
Unknown	1	17

HPV high-risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68)

Table 3 Frequency of promoter methylation in normal, CIN3, and cancer samples

Gene	Normal	CIN3	Cancer	*p* value*
CDKN2A	2/24 (8.3 %)	7/22 (31.8 %)	9/53 (17.0 %)	0.11
DAPK1	1/24 (4.2 %)	7/22 (31.8 %)	40/53 (75.5 %)	<0.0001
FHIT	6/24 (25.0 %)	13/22 (59.1 %)	35/53 (66.0 %)	0.0033
MGMT	5/24 (20.8 %)	6/22 (27.3 %)	18/53 (34.0 %)	0.76

Values are number of samples positive for hypermethylation out of the total number of samples tested that produced a valid result (percent)

* Chi-squared test

Table 4 Frequency of promoter methylation in SCC and AD samples

Gene	SCC	AD	*p* value*
CDKN2A	7/42 (2.4 %)	2/11 (18.2 %)	1
DAPK1	33/42 (78.6 %)	7/11 (63.4 %)	0.31
FHIT	28/42 (66.7 %)	7/11 (63.4 %)	1
MGMT	15/42 (35.7 %)	3/11 (27.3 %)	0.73

Values are number of samples positive for hypermethylation out of the total number of samples

tested that produced a valid result (percent)

SCC squamous cell carcinoma, *AD* adenocarcinoma

* Chi-squared test

Assays (Applied Biosystems) with the TaqMan Universal PCR Master Mix II (Applied Biosystems) on a 7900HT Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions.

The relative quantification method was used to measure the amounts of the respective genes in cervical cancer samples, which were normalized to *GAPDH*.

Table 5 The association between *DAPK1* methylation status and staging in squamous cell carcinoma

	Methylation (−)	Methylation (+)	Frequency of methylation (%)	*p* value*
Stage				
CIS ($n = 14$)	10	4	28.6	
I ($n = 29$)	7	22	75.9	
II, III, IV ($n = 13$)	2	11	84.6	0.0024

* Chi-squared test

Results

The promoter methylation of *DAPK1*, *FHIT*, *MGMT* and *CDKN2A* in cervical cancer vs. intraepithelial neoplasia vs. normal cervical tissue was 75.5 vs. 31.8 vs. 4.2 % ($p < 0.0001$), 66.0 vs. 59.1 vs. 25.0 % ($p = 0.0033$), 34.0 vs. 27.3 vs. 20.8 % ($p = 0.76$), and 17.0 vs. 31.8 vs. 8.3 % ($p = 0.11$), respectively (Table 3). There were no significant differences in the methylation rate of *DAPK1*, *FHIT*, *MGMT*, and *CDKN2A* between squamous cell carcinoma and adenocarcinoma (Table 4). There was a trend toward DAPK promoter hypermethylation among normal cervical tissues and cervical cancer (squamous cell carcinoma and adenocarcinoma). There was an increasing trend toward DAPK promoter methylation among carcinoma in situ, cervical cancer stage 1, and cervical carcinoma stage 2–4, with methylation rates of 28.6 % (4/14), 75.9 % (22/29), and 84.6 % (11/13), respectively ($p = 0.0024$; Table 5). The association between promoter methylation status and relative mRNA expression of *DAPK1*, *FHIT*, *MGMT*, and *CDKN2A* genes in cervical cancer is shown in Fig. 1. The comparison of the methylation of a promoter region and the

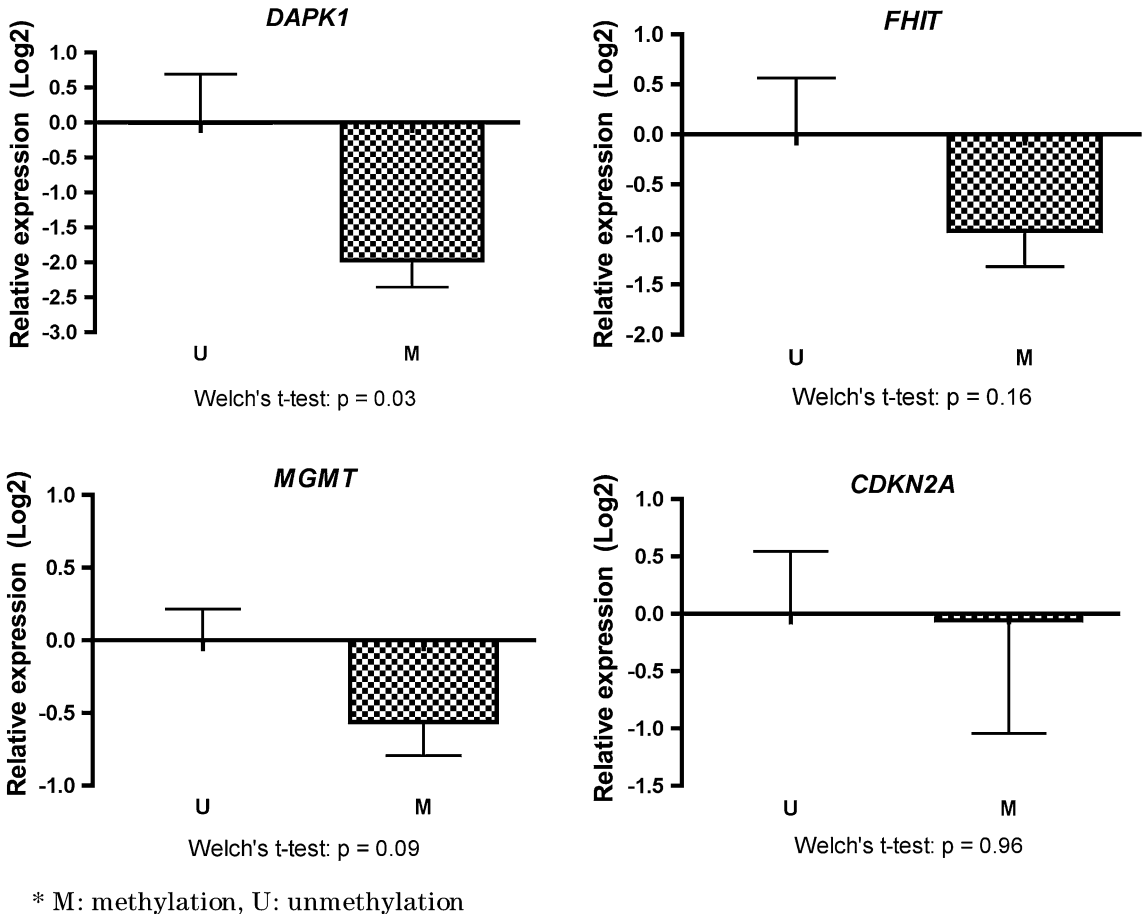

Fig. 1 The association between promoter methylation status and relative mRNA expression of 4 genes in cancer tissues

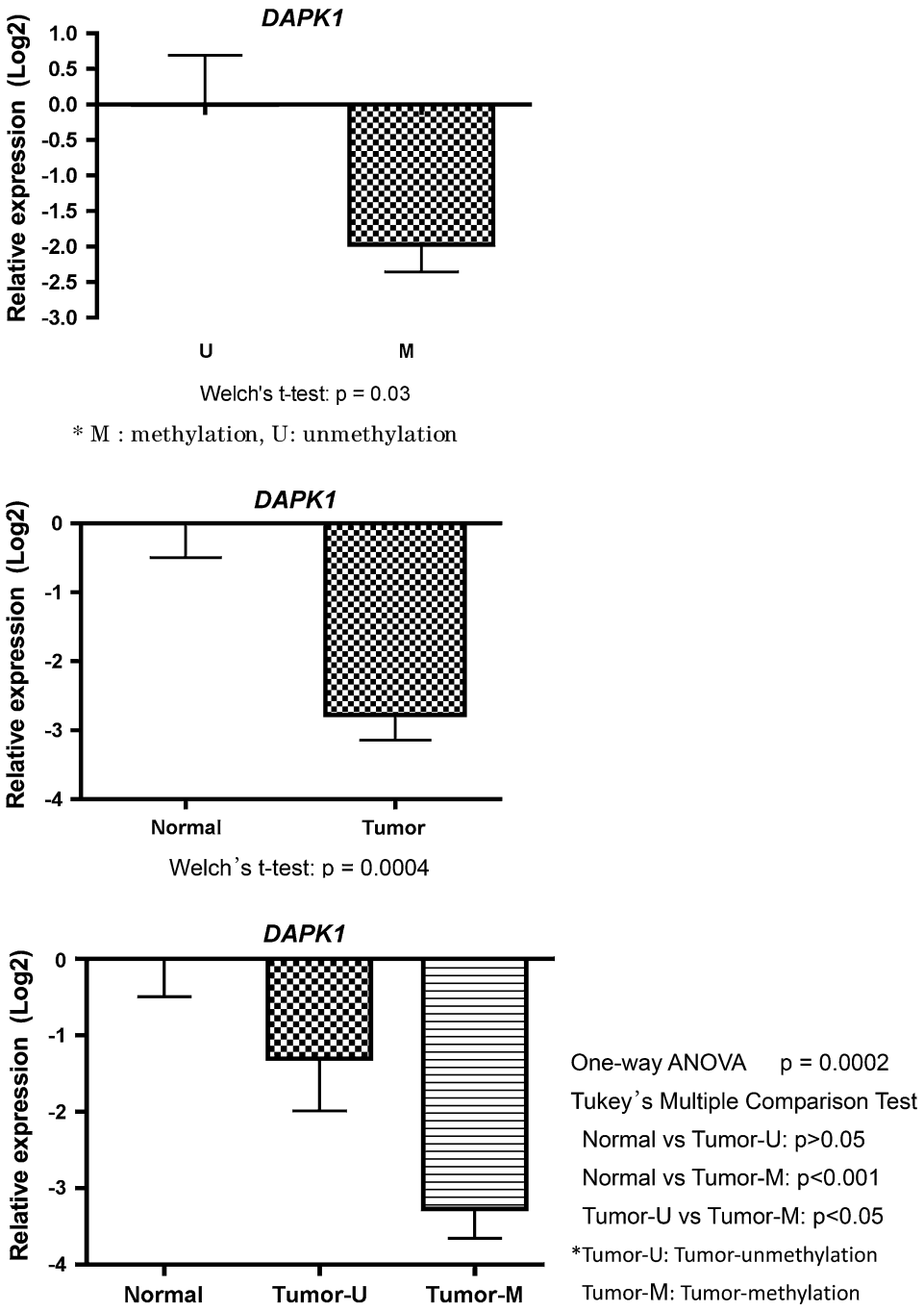

Fig. 2 The association between promoter methylation status and relative mRNA expression of DAPK in cancer tissues

relative level of mRNA between normal cervical tissue and cervical cancer revealed that only *DAPK1* showed a significant difference ($p = 0.03$). The association between promoter methylation status and relative mRNA expression of DAPK in cancer tissues and normal tissues showed decreased mRNA expression when *DAPK1* was methylated (Fig. 2).

Discussion

High-risk HPV infection is associated with cervical carcinoma [2–5]. In Japanese females, HPV infection was examined using restriction fragment length polymorphism (RFLP), and the rate of high-risk HPV infection was 94.8 % in CIN2/CIN3 cases and 93.4 % in patients with invasive cancer. In addition, in patients with cervical cancer, the rate of high-risk HPV infection was 90 % for patients in their twenties, 75.9 % for those in their 30s, 65.9 % for those in their 40s and 64.0 % for those in their 50s, indicating a decreasing trend in the rate of infection with age [29]. This study found that 81 % of CIN3 and 72 % of cervical carcinoma was associated with high-risk HPV infection. It was found that the average age of

patients with cervical cancer was 49 years, and this relatively older age may have been responsible for the low rate of HPV infection in our study. Other factors are also involved in cervical carcinoma. The inactivation of tumor suppressor genes has been investigated. Methylation plays an important role in tumorigenesis. The aberrant methylation of the normally unmethylated CpG islands of many tumor suppressor genes is associated with transcriptional inactivation and loss of expression [17, 26, 27]. Many studies have investigated the promoter hypermethylation of *DAPK1*, *FHIT*, *MGMT*, *CDKN2A*, and other genes during the progression of cervical oncogenesis [12, 13, 17, 18, 25, 30]. One study showed a correlation between tobacco use and methylation of *CDKN2A* [28].

The current study investigated the methylation profiles in the promoter region of *DAPK1*, *FHIT*, *MGMT*, and *CDKN2A* genes in normal, CIN3, and cervical carcinoma tissues.

No relationship was found between the methylation of *MGMT* and *CDKN2A* and cervical carcinoma in the current study.

FHIT has been identified as a candidate tumor suppressor gene, and re-expression of *FHIT* in a variety of human cell lines results in growth inhibition and induction of apoptosis. *FHIT* is hypermethylated in 40–50 % of cervical carcinomas [25]. The current study found that there is increased methylation of *FHIT* in cervical carcinoma tissue, but this was not associated with a significant decrease in the mRNA expression of FHIT.

DAPK1 is a pro-apoptotic serine/threonine protein kinase that is dysregulated in a wide variety of cancers. In addition, *DAPK1* is involved in the control of autophagy [24].

There was a significant decrease in the expression of mRNA associated with the methylation of the promoter region of *DAPK1*, thus suggesting that *DAPK1* was involved in the occurrence of cervical carcinoma. Although *DAPK1* is reported to show a higher level of methylation in squamous cell carcinoma than in adenocarcinoma [13], there was no significant difference in the current study. The increased methylation of *DAPK1* suggested the possibility that *DAPK1* was associated with the progression of the disease.

Conflict of interest The authors do not have any conflict of interests.

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