Clinico-epigenetic combination including quantitative methylation value of DKK3 *augments survival prediction of the patient with cervical cancer*

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ORIGINAL PAPER

Clinico-epigenetic combination including quantitative methylation value of *DKK3* augments survival prediction of the patient with cervical cancer

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Abstract
Purpose *DKK3* is a target of methylation in various cancers and has been studied by a non-quantitative method. We assessed the quantitative methylation levels of *DKK3* in cervical carcinoma, determined the potential clinical correlations, and tested whether the combination of clinical and epigenetic factors augmented the prediction power of prognosis.
Methods Sixty-two patients with cervical squamous cell carcinoma were included in this study. Quantitative methylation levels were evaluated by pyrosequencing. Clinical and pathologic findings were obtained from medical records. Survival data were analyzed using Kaplan–Meier estimates and compared with the log-rank test. The best clinico-epigenetic combinations were found using the Cox proportional hazard model.
Results Four of five CpG positions of *DKK3* were strongly methylated in cervical carcinoma compared to normal controls ($p = 0.0048$). The methylation in positions 1 and/or 2 were stronger in patients with higher serum levels of the SCC tumor marker and/or larger tumors ($p = 0.01$). The patients with a methylation level $\geq$26.3 % at position 1 had a lower survival rate than the patients with methylation levels at position 1 that were <26.3 % ($p = 0.03$). The combination of methylation level of position 1, position 3, age, parametrial invasion, and lymphovascular space invasion (LVSI) have a significant correlation with survival ($p = 0.0006$). Recurrence was significantly related to the combination of methylation level of position 2, position 3, age, parametrium, and LVSI ($p = 0.0041$).
Conclusions *DKK3* methylation is unfavorable to prognosis. This study defined a threshold level of methylation associated with recurrence-free survival and, furthermore, identified novel clinico-epigenetic combinations predicting disease survival or recurrence.

Woong-Sun Kang, Sung Bum Cho contributed equally to this paper.

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Keywords *DKK3* · Quantitative evaluation · DNA Methylation · Uterine cervical cancer · Prognosis

Introduction

Uterine cervical cancer is the second most common cancer in women worldwide and the fifth leading cause of cancer deaths. There are >470,000 cervical cancer cases diagnosed annually and 233,400 deaths (Parkin et al. 2005). While the incidence of cervical cancer has declined significantly, cervical cancer continues to be a significant burden for Korean women, in whom approximately 4,500 new cases of invasive cervical cancer and 3,000 cases of carcinoma in situ were diagnosed in 2002 (Chung et al. 2006; Shin et al. 2007). Despite recent advances in cancer treatment, the disease recurs in approximately 30 % of cases with cervical cancer (Cannistra and Niloff 1996). Therefore, the identification of biomarkers for the accurate prediction of early recurrence of cervical cancer might improve patient outcome.

There have been many reports describing the association between hypermethylation of individual genes and overall clinical outcome for various types of cancer (Graziano et al. 2004; Toyooka et al. 2004; Roman-Gomez et al. 2004; Brock et al. 2003; Gerdes et al. 2002; Esteller et al. 2000). For example, hypermethylation of the O6-methylguanine methyltransferase (*MGMT*) promoter has been associated with increased survival in patients with glioma treated using alkylating agents (Esteller et al. 2000). In addition, a panel of 35 methylation markers revealed an association of DNA methylation profiles with the hormone receptor status and response to tamoxifen treatment in 148 patients with breast cancer (Widschwendter et al. 2004). Although all of these examples require confirmation, there is little doubt that aberrant methylation of tumor-derived DNA may serve as potential markers for neoplastic disease.

Recently, we found that *DKK3* was frequently downregulated by promoter methylation in cervical cancer tissues (Lee et al. 2009). Likewise, several published reports showed that *DKK3* is a frequent target for methylation (Roman-Gomez et al. 2004; Kobayashi et al. 2002; Lodygin et al. 2005; Urakami et al. 2006; Bafico et al. 2001; Mao et al. 2001). However, the majority of previous methylation studies used non-quantitative methods such as a methylation-specific PCR (MSP) or modified MSP and was not conducted with the aim of identifying clinical correlations.

The non-quantitative methods yield the risk of false-positive or false-negative results. Pyrosequencing technology is a simple technique for accurate and quantitative analysis of DNA sequences. When the three quantitative techniques are combined bisulfite restriction analysis (COBRA), SNuPE ion pair-reverse phase high-performance liquid chromatography, and pyrosequencing, and they are compared, the pyrosequencing assay provides the most accurate and most robust analytic tool (Mikeska et al. 2007). An attempt to define novel and stringent criteria by the quantification of *DKK3* methylation might enable more precise stratification of patients with cervical cancer into risk groups.

In the present study, we therefore performed pyrosequencing to assess the quantitative levels of the promoter region of *DKK3* in human cervical squamous cell carcinoma and determine potential clinical correlations. Furthermore, we tested whether the combination of clinical and epigenetic factors based on a statistical approach improved the prediction accuracy of disease survival or disease recurrence.

Materials and methods

Patients

Sixty-two patients with histopathologically proven primary cervical squamous cell carcinoma and from whom frozen tissues were available were included in this study. None of the patients received preoperative chemotherapy or radiotherapy. Patients with other types of cancers or a secondary malignancy were excluded. We reviewed medical records and pathologic findings. All patients underwent surgery, which consisted of a type III radical hysterectomy with or without salphingo-oophorectomy and pelvic lymph node dissection, as primary treatment between 1998 and 2004 in the Department of Gynecologic Oncology of Chung-Ang University Hospital in Seoul, Korea. The stage of disease was assigned according to the Fédération Internationale de Gynécologie et d'Obstétrique (FIGO) staging system. Pathologic classification of the resected specimens was performed according to the International Union against Cancer TNM classification of malignant tumors (Sobin and Wittekind 2002). Histologic typing was performed according to the criteria of the World Health Organization International Histological Classification of Tumors (Scully et al. 1994).

Clinical specimens

Sixty-two fresh frozen tissues were used. Immediately after removal, the samples were snap-frozen in liquid nitrogen and kept at −70 °C for further study. We selected samples at least 1 cm in size to minimize contamination from normal tissues. The histology and cellular composition of the tissues were confirmed before DNA extraction. Tumor samples were selected that contained more than 80 %

tumor cells as estimated by haematoxylin and eosin staining of frozen sections. Thirteen patients who underwent hysterectomy due to benign uterine leiomyomas were included as normal controls; the pathologic findings showed no atypical lesion in all 13 cervical tissues. The cervical epithelial layer was thinly stripped to avoid contamination of stromal tissues and was confirmed to have more than 80 % epithelial cells by haematoxylin and eosin staining of frozen sections. The study protocol was reviewed and approved by the Institutional Review Board.

Genomic DNA extraction, bisulfite treatment, and pyrosequencing methylation analysis

Genomic DNA was extracted by standard methods with a commercially available kit (QIAamp DNA Mini Kit; Qiagen, CA, USA) according to the manufacturer's instructions. The extracted DNA was quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). DNA (500 ng) was modified by sodium bisulfite treatment using an EZ DNA Methylation Kit™ (Zymo Research, Orange, CA, USA) according to the manufacturer's protocol. The final elution was performed with 10 µl of 0.1× TE buffer. Forward (5′-GATTAA GGTGGGATTGAGGAATAT-3′) and reverse primers (5′-CCCAAAACAACCATCTCCTT-3′) yielding a 134-bp product were designed by Pyrosequencing Assay Design Software (Pyrosequencing AB) (Online resource). The reverse primers were biotin-labeled and used to purify the final PCR product using Sepharose beads. For the pyrosequencing analysis, the bisulfite-treated DNA was first amplified by PCR to generate amplicons that included five CpG sites in the target region. Blank reactions (for PCR and pyrosequencing) were included in each assay to exclude cross-contamination. PCR was carried out with EF *Tag* polymerase (Solgent, Daejoen, Korea) using 4 µl of bisulfite-treated DNA. Mixtures were denatured for 7 min at 95 °C, then thermal-cycled for 30 s at 95 °C, 30 s at 54 °C, and 30 s at 72 °C, repeating the cycle 50 times to ensure complete exhaustion of the primers. Next, 20 µl of the biotinylated PCR product was bound to Streptavidin Sepharose HP (Amersham Biosciences, Uppsala, Sweden), and the Sepharose beads containing the immobilized PCR product were purified, washed, and denatured using a 0.2 M NaOH solution and washed again using the Pyrosequencing Vacuum Prep Tool (Pyrosequencing, Westborough, MA, USA), as recommended by the manufacturer. Then, 15 pmol of the respective sequencing primer (5′-GTTGTAAGTTTGGAGGTTA G-3′) were annealed to the purified single-stranded PCR product. Pyrosequencing was carried out using the PSQ96MA System (Pyrosequencing AB) according to the manufacturer's protocol. Methylation quantification was performed using the provided software. Every sample was tested two times per marker to confirm the reproducibility of the results. The average of the duplicates was used for the statistical analysis.

Statistical analysis

The median values were analyzed using the Mann–Whitney *U* test. Dichotomous results were analyzed using the χ^2 and Fisher's exact test as appropriate. Disease-free survival (DFS) was defined as the length of time (months) from the last treatment to the diagnosis of the first recurrence. Disease-specific survival (DSS) was defined as the length of time from surgery to the report of a cancer-related death. DFS and DSS curve were estimated using the Kaplan–Meier method and were compared by nonparametric survival analysis (log-rank test) where indicated. Multivariate analysis was performed using the Cox regression method. For the five methylation sites, the Cox proportional hazard model was used to determine an association between the methylation level and DFS length. All p values reported are two-sided, and statistical significance was defined as $p < 0.05$. All statistical analyses were performed using the statistical software R (www.cran.r-project.org/) and SPSS for Windows (version 15.0; SPSS Inc., Chicago, IL, USA).

To determine the potential clinical correlations of the quantitative methylation level of five CpG sites, we used two approaches. The first approach was to explore the relationship between the quantitative methylation level at each site and a single clinical factor. For this purpose, correlation or t test analysis was applied. If the clinical factors were continuous variables, correlation analysis between the clinical factors and methylation level was used. For the categorical clinical factors, the average methylation values were compared among different clinical factors using the t test. The second approach was to apply survival analysis. For single variable-wise survival analysis, we used log-rank test and Kaplan–Meier plot. In the single variable analysis, we bisected the methylation values as follows. First, all data values at each position were used as threshold value. Then, the discrete values for each threshold were used in log-rank tests. For each position, the threshold values that showed the most significant p values were used for threshold for bisection of the methylation signal.

In the survival analysis, to test the association of combinations of available variables with DFS length and disease recurrence, a multivariate Cox proportional hazard model was applied. The variables that were significant in the Kaplan–Meier survival analysis were used. In addition to the clinical variables, the quantitative methylation levels of five CpG sites were included and clinico-epigenetic factors related to disease prognosis were explored. We

applied an exhaustive search strategy to identify related factors. The variables were the quantitative methylation level of five CpG sites and clinical factors including age, height, weight, body mass index, menopausal status, FIGO stage, histology type, lymphovascular space invasion (LVSI) status, a parametrium (PM) involvement, resection margin involvement, and lymph node involvement. After generating all possible combinations of the 16 variables, we tested the association between DFS or disease recurrence and all combinations of the variables ($n = 65{,}535$) using the Cox proportional hazard model.

Results

Characteristics of the 62 patients and survival analysis with clinical variables

Tables 1 and 2 list the characteristics of the patients. The patients were followed for 1–117 months (median, 53 months) or until death. Two patients were lost to follow-up. Forty-seven patients (75.8 %) were without evidence of disease. Fifteen (24.2 %) patients had persistent ($n = 2$) or recurrent disease ($n = 13$), among whom 7 patients died of the disease. DFS and DSS were unfavorable in patients with advanced stage disease and tumor involvement of the parametrial tissues (Table 2). The 5-year DFS rate was 94.4 % for women with stage 1 cervical carcinoma and 77.6 % for women with stage 2 cervical carcinoma (Fig. 1a, $p = 0.024$). The 5-year DFS rate was 62.5 % for women with involvement of the PM compared to 93.8 % for the patients without PM involvement (Fig. 1b, $p = 0.002$). The 5-year DSS was also shorter in women with PM involvement (mean DSS, 49.28 months) compared to the women without PM involvement (mean DSS, 110.96 months); that difference was statistically significant (Fig. 1d, $p = 0.001$). Multivariate analysis showed that the clinical FIGO stage and PM involvement were independent prognostic factors for DFS ($p = 0.039$; hazard ratio = 1.94; 95 % confidence interval = 1.02–3.73) and DSS ($p = 0.021$; hazard ratio = 6.56; 95 % confidence interval = 1.21–35.53), respectively. Women with PM involvement had an approximately 6.56-fold increased risk of death compared

Table 1 Patient characteristics

Characteristics	No (%)
Age (years)	
Mean	47
Range	23–68
FIGO stage	
Ib1	29 (46.8)
Ib2	9 (14.5)
IIa	22 (35.5)
IIb	2 (3.2)
Type of treatment	
Surgery only	18 (29.0)
Surgery and adjuvant radiation	16 (25.8)
Surgery and concurrent chemoradiation	28 (45.2)
Recurrence	
No	47 (75.8)
Yes	13 (21)
Persistent disease	2 (3.2)

Table 2 Survival analysis according to clinicopathologic factors ($N = 62$)

	N (%)	Univariate analysis (*p* value)	
		DFS	DSS
SCC tumor marker			
<1.5 ng/mL	24 (38.7)	*NS*	*NS*
≥1.5 ng/mL	32 (51.6)		
Unknown	6 (9.7)		
FIGO stage			
I	38 (61.3)	0.024	0.02
II	24 (38.7)		
PM[a] involve			
No	54 (87.1)	0.002	0.001
Yes	8 (12.9)		
Lymph node metastasis			
Negative	41 (66.1)	*NS*	*NS*
Positive	21 (33.9)		
Resection margin involved			
No	59 (95.2)	*NS*	*NS*
Yes	3 (4.8)		
Tumor size			
<4 cm	38 (61.3)	*NS*	*NS*
≥4 cm	24 (38.7)		
Invasion depth			
<1/2	16 (25.8)	*NS*	*NS*
≥1/2	46 (74.2)		
LVSI[b]			
Negative	46 (74.2)	*NS*	*NS*
Positive	16 (25.8)		
HPV[c] infection			
Positive	51 (82.3)	*NS*	*NS*
Negative	9 (14.5)		
Unknown	2 (3.2)		

[a] Parametrium

[b] Lymphovascular space invasion

[c] Human papilloma virus

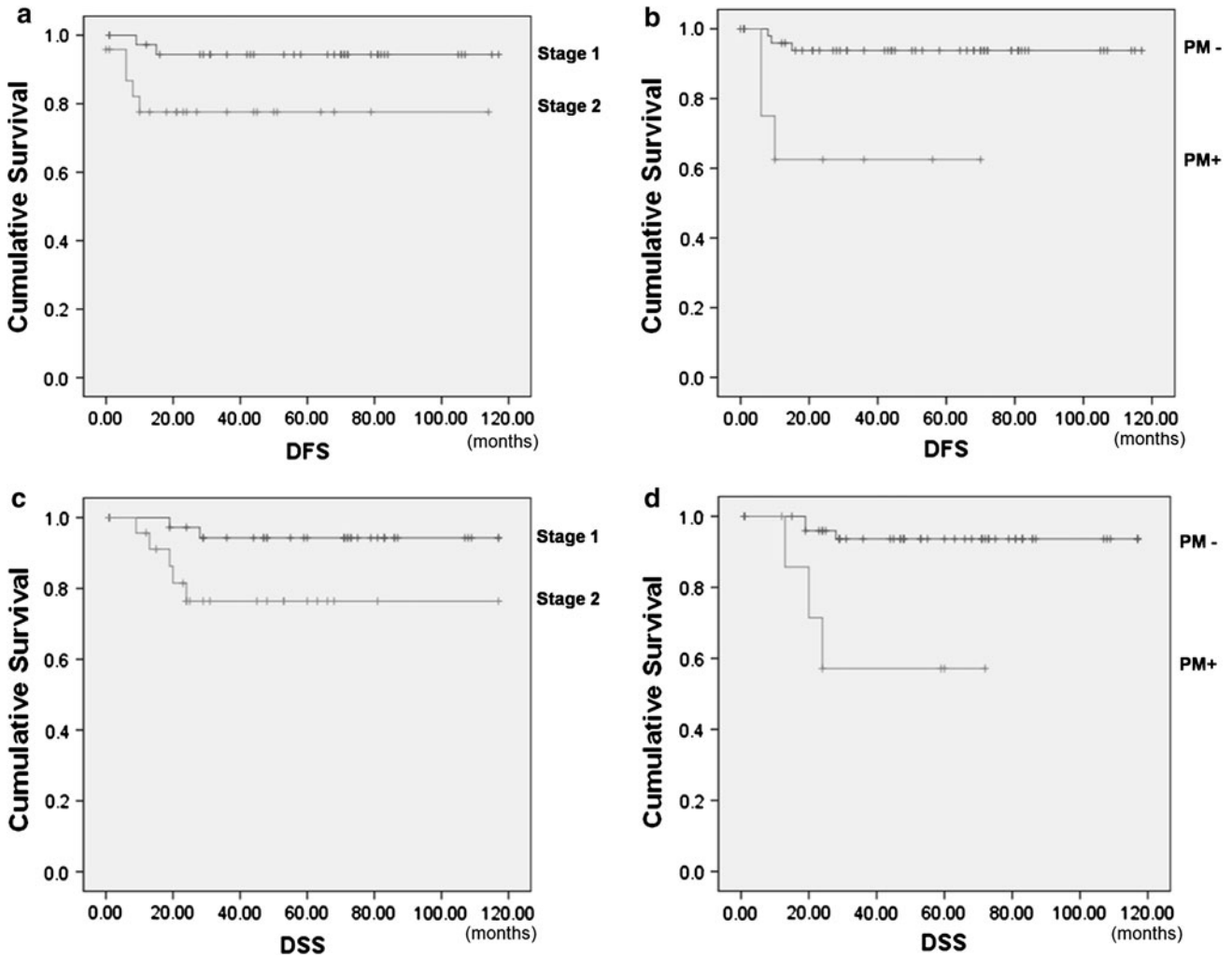

Fig. 1 Kaplan–Meier actuarial survival curves for patients with uterine cervical carcinoma. Statistically significant differences in DFS are observed for women with different surgical stages (**a**, $p = 0.024$ [log-rank]) and for women with tumor involvement in PM versus women with clear PM (**b**, $p = 0.002$ [log-rank]). The overall cumulative DSS of women with uterine cervical carcinoma was stratified by clinical staging and tumor involvement in the PM. The probabilities of survival differed among women with different stages (**c**, $p = 0.03$ [log-rank]). Women in whom tumors involved PM tissue had a lower cumulative survival rate than women in whom tumors did not involve PM tissue (**d**, $p = 0.001$ [log-rank])

to women without PM involvement, and this clinical finding was associated with a poor survival rate.

The promoter region of *DKK3* was hypermethylated in the cervical cancer tissues compared to the normal tissue samples

DNA methylation analysis was performed by comparative quantitative methylation analysis including bisulfite treatment, PCR, and pyrosequencing. We selected CpG-rich stretches on CpG islands that included five CpG sites. We used the non-CpG cytosine as a built-in control for bisulfite conversion efficiency and confirmed successful bisulfite conversion by complete conversion of that cytosine. As shown in Fig. 2, that methylation profiling revealed that the promoter region of *DKK3* had extensive methylation of the cervical cancer tissues but not the normal cervical tissues. The site-specific percent methylation of each of the five CpG cytosines was measured (Fig. 2c). Quantitatively, the methylation levels in positions 1–4 of the CpG sites were significantly higher in the malignant cervical tissues than in the normal cervical tissues.

Methylation at positions 1 and 2 was associated with higher serum levels of the SCC tumor marker and larger tumors

The site-specific percent methylation at five CpG sites of *DKK3* was evaluated for the association with clinical factors including the serum SCC antigen level, FIGO stage, tumor size, LVSI, PM involvement, lymph node metastasis, and invasion depth. With the exception of the serum

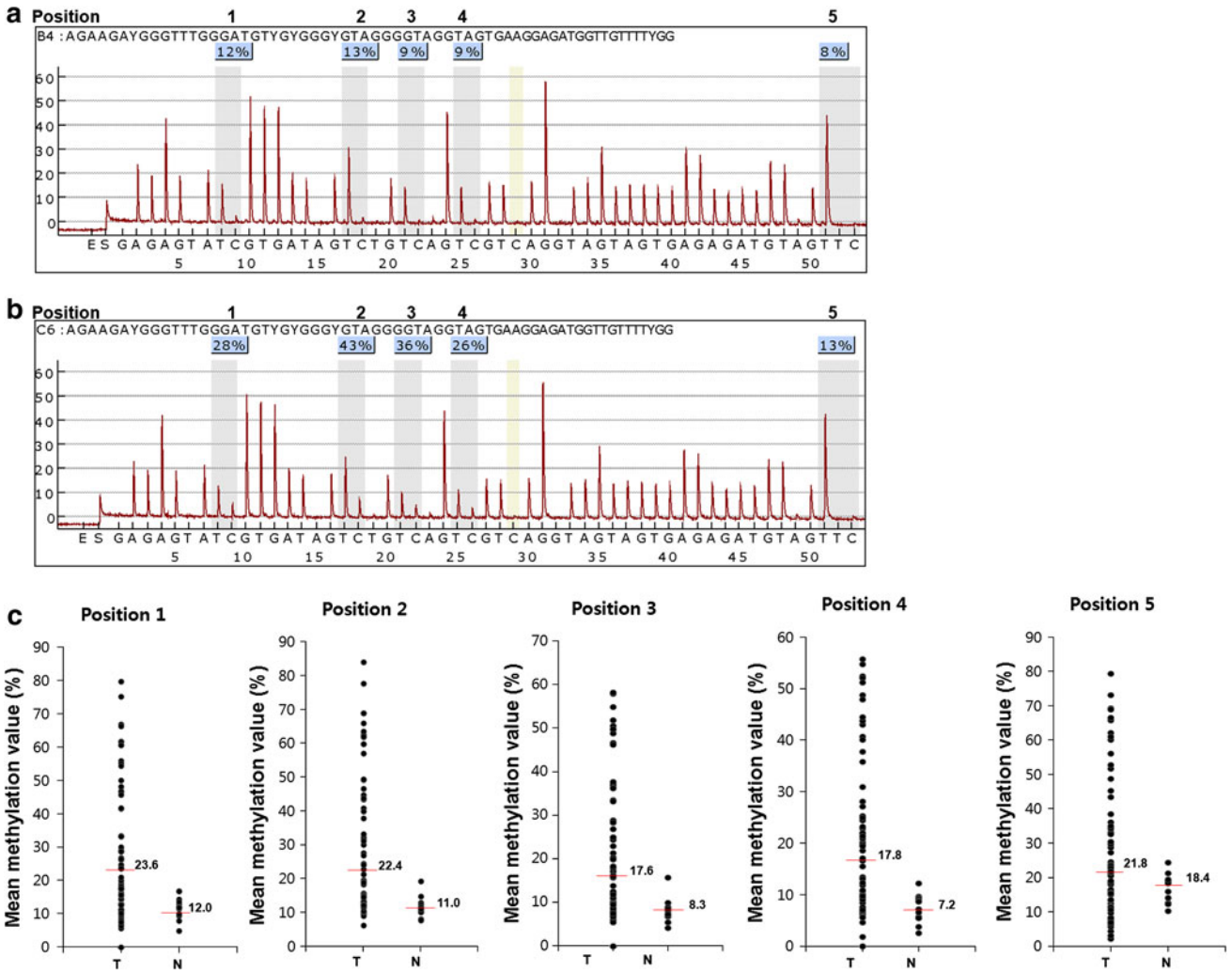

Fig. 2 Representative programs and dot plot of methylation levels of five CpG positions of *DKK3*. **a** and **b** The five targeted CpG sites are enclosed in *gray columns*. The percentage (%) numbers (in *blue shade*) are proportions of C and T at each CpG site after bisulfite conversion, and the methylation level of each CpG site is estimated by the proportion of C (%). The control, non-CpG cytosine showing complete conversion of cytosine to uracil by bisulfite treatment is shown in the *yellow column*. Cancer tissue (**b**) demonstrates a significantly high level of methylation at five target bases compared to normal tissue (**a**). *Y* axis, signal peaks proportional to the number of nucleotides incorporated; *X* axis, the nucleotides incorporated. *Dot* plot of methylation levels of five CpG positions of *DKK3* in tumorous (T) and normal (N) cervical tissues. Each *dot* represents one sample. Tumor showed a significant increase in *DKK3* methylation levels compared to normal controls. *Y* axis represents percent of methylation

SCC antigen level and tumor size, the five clinical factors each had two categorical values. Therefore, the *t* test was used to assess whether the mean methylation value of each site was different based on the categorical levels. For the serum SCC antigen level and tumor size, a correlation analysis was used to test for the association with each methylation site. There was no correlation between the methylation level and clinical parameters, including FIGO stage, LVSI, PM involvement, lymph node metastasis, and invasion depth. However, the methylation level at positions 1 and 2, but not positions 3, 4, and 5, were significantly correlated with the serum SCC antigen level ($p = 0.01$ for positions 1 and 2). The CpG at positions 1 and 2 had greater methylation in the patients with higher serum levels of the SCC tumor marker. Moreover, the methylation level at positions 1 and 2 had a significant correlation with the tumor size ($p = 0.01$ for positions 1 and 2); that finding indicated that cancer cells from more bulky tumors had a higher methylation level at positions 1 and 2.

A higher methylation level at position 1 was associated with a shorter recurrence-free survival in cervical cancer patients

Using the categorized methylation levels, we performed survival analysis. First, we bisected the methylation values such that maximize significance of survival analysis. The threshold values for dichotomization were 26.3 % for

position 1, 16.3 % for position 2, 86.0 % for position 3, 43.7 % for position 4, and 13.3 % for position 5. The univariate analysis showed that the methylation levels at position 1 above the thresholds were strongly associated with a shorter DFS rate (Fig. 3, p values of position 1 = 0.03). However, the multivariate analysis adjusted for stage, PM involvement, and defined threshold value of methylation at position 1 failed to show a statistical significance. The analysis with DSS length showed no significance between the survival length and the discrete methylation values.

Clinico-epigenetic combinations have a significant correlation with prognosis

Using the 16 variables of five CpG sites and clinical factors, we generated 65,535 combinations. The combinations of the variables were used as covariates in Cox proportional hazard model analysis. In this analysis, we used continuous methylation values. When the combinations of the clinico-epigenetic variables were tested with the DFS, p values from log-likelihood ratio test (LRT) of the fitted model were used for sorting the results, and the combination of the position 2, position 3, age, LVSI, and PM involvement showed the lowest p value (Table 3, $p = 0.0041$). When variables were tested with the disease recurrence, a combination composed of the quantitative methylation level of position 1, position 3, age, LVSI, and PM involvement showed the lowest p value in the likelihood-ratio test of the fitted Cox proportional model (Table 3, $p = 0.0006$).

Discussion

The risk factors predicting outcome in patients with cervical cancer have included FIGO clinical stage, PM involvement, resection margin status, pelvic lymph node metastasis, LVSI, bulky tumor size, and deep stromal invasion (Holtz and Dunton 2002). Accordingly, the current multivariate analysis showed that the FIGO clinical staging and PM involvement are independent prognostic factors in patients with cervical squamous cell carcinoma. The 5-year DFS according to disease stage in the present study (94.4 % for stage 1 and 77.6 % for stage 2) is comparable to a previous report (Komaki et al. 1995), although the patients with stage Ia were excluded in this study because the patients with a >1 cm visible mass were selected to diminish contamination of normal tissue during tissue sampling.

We recently showed aberrant hypermethylation of the *DKK3* promoter in cervical cancer tissue samples by the combination of methylation-sensitive restriction enzyme and PCR, a non-quantitative method (Lee et al. 2009). That result prompted us to assess whether the quantitative methylation level of *DKK3* could be correlated with clinical and pathologic characteristics of cervical cancer. Consistent with our previous data, among a total of five CpG sites tested, four CpG sites were distinctly different between the cervical cancer tissues and the normal cervical tissues based on the methylation levels. Moreover, the methylation levels at positions 1 and/or 2 were higher in patients with higher serum levels of the SCC tumor marker and/or with larger tumors. Both the pre-treatment SCC level and tumor size were associated with disease recurrence (Chou et al. 1994), and, in particular, larger tumor size is currently used as an indicator of postoperative adjuvant treatment. Therefore, the high methylation level of *DKK3* reflects the high-risk clinicopathologic factors and could predict the clinical outcome of cervical cancer patients.

We then determined the values that best discriminate the patients according to survival outcome and found that recurrence-free survival of patients with a methylation level ≥26.3 % at position 1 was significantly shorter than for patients with methylation levels <26.3 % at position 1. That could be novel criteria for the stratification of cervical cancer patients into risk groups and facilitate the optimization of therapies. Our finding is consistent with dichotomous data that reported the prognostic impact of *DKK3* methylation of patients with acute lymphocytic leukemia (Roman-Gomez et al. 2004). Specifically, the 10-year DFS rate was significantly higher in patients without methylation compared to patients with hypermethylation. Taken together, *DKK3* methylation could be associated with an unfavorable clinical outcome for some cancers.

In this study, we sought the best prognostic model from combinations of clinico-pathologic parameters and epigenetic markers. With the exhaustive search, we identified novel combinations of clinico-epigenetic variables that were highly associated with survival outcome. It seemed that epigenetic information played a role in augmenting the information of the clinical variables. For example, if we tested the Cox model with age, LVI, PM, and disease relapse length, we found that the p value was comparably significant in LRT ($p = 0.0068$). However, when the epigenetic information about the methylation level of position 1 and 3 was added, the p value was more significant ($p = 0.0006$). When the disease survival length was tested with age, LVI, and PM, the p value of the LRT ($p = 0.03$) was less significant than that of the model having information about the methylation level of position 2 and position 3 ($p = 0.0041$). That combination will be further tested in a large set of patients to validate the reliability.

The establishment of normal distributions by age is an essential step in validating biomarkers (Mayeux 2004).

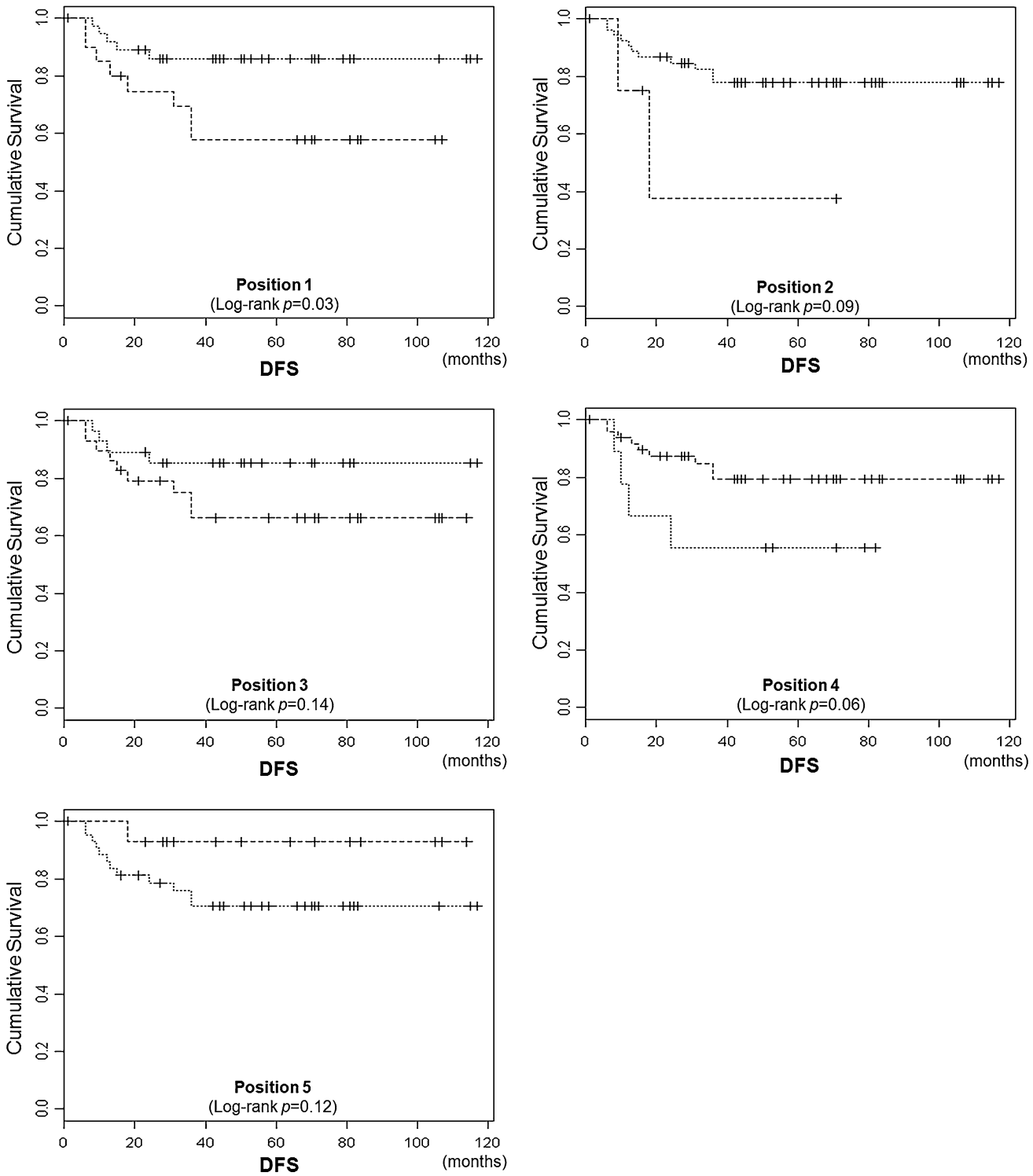

Fig. 3 Kaplan–Meier recurrence-free survival estimates in patients according to the methylation thresholds

Moreover, anomalous epigenetic signaling has a crucial role in tumorigenesis (Jones and Baylin 2002), but it can also be a determinant of cellular senescence and aging (Fraga and Esteller 2002). Thus, it is important to determine the relationship between DNA methylation and increased age. *DKK3* was originally discovered to be a reduced expressed gene in immortalized cells, which escape from cellular senescence (Tsuji et al. 2000), and promoter methylation is supposed to be the major mechanism of down-regulation (Lee et al. 2009). That fact

Table 3 The most significant clinico-epigenetic combinations for disease survival and disease recurrence

	Variables	Coefficient	*Z* value	*p* value
Disease survival	Position 2	−0.531	−2.525	0.012
	Position 3	0.608	2.493	0.013
	Age	0.139	2.088	0.037
	LVSI[a]	−1.797	−1.447	0.148
	PM[b] invasion	3.080	3.003	0.003
Disease recurrence	Position 1	−0.108	−2.330	0.020
	Position 3	0.080	1.781	0.075
	Age	0.061	1.760	0.078
	LVSI	−1.294	−1.492	0.136
	PM invasion	3.131	3.658	0.000

[a] Lymphovascular space invasion

[b] Parametrium

suggests that *DKK3* may be one of the genes in which the methylation of the promoter region increases with age; however, present study failed to prove this notion (data not shown). The promoter methylation level of *DKK3* had no correlation with age. Therefore, the methylation level of *DKK3* could meet at least one of the requirements for biomarkers and is able to progress to the validation step as a possible biomarker to predict the prognosis of cervical cancer patients.

In conclusion, the results of this study showed comparative quantitative methylation profiling using a pyrosequencing methylation analysis technique and defined a threshold value of the methylation level at position 1 of the promoter region of *DKK3* associated with the DFS rate in patients with cervical squamous cell carcinoma. Furthermore, we identified novel clinico-epigenetic combinations that have the high prediction power of disease survival length and recurrence. To clarify the full clinical potential of this type of analysis, additional studies are needed.

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Conflict of interest The authors declare that no conflict of interest exists.

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