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Original Article

Expression of DNA methylation-related proteins in breast phyllodes tumor

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Abstract: The purpose of this study is to research the expression of DNA methylation-related proteins in phyllodes tumors of the breast and to study the implication on patient outcomes. We generated tissue microarrays (TMAs) of 196 phyllodes tumors (PT) and performed immunohistochemical staining for 5-meC and the DNA methylation-related proteins DNMT1 and ISL-1. The staining results were analyzed and compared with clinicopathologic parameters. A total of 196 cases were included in this study, of which 153 were benign, 27 were borderline, and 16 were malignant. The levels of DNMT1, 5 meC, and ISL-1 in the stromal component of tumors increased with increasing grade (P<0.001). Especially, high stromal positivity of DNMT1 and ISL-1 were associated with increased distant metastasis (P=0.001, and P=0.013, respectively). Univariate analysis for factors associated with decreased disease free survival and overall survival identified DNMT1 high positivity (P=0.002 and P<0.001, respectively) and stromal ISL-1 high positivity (P<0.001 and P<0.001, respectively). Among borderline phyllodes tumors, stromal DNMT1 high positivity was associated with decreased OS (P=0.015). In conclusion, DNA methylation and expression of methylation-related proteins in the stromal component increased with increasing histologic grade in phyllodes tumors. In addition, overexpression stromal expression of DNMT1 and ISL-1 was associated with poor prognosis.

Keywords: Breast, methylation, phyllodes tumor

Introduction

Phyllodes tumors are a relatively rare tumor type, accounting for 0.3-1.5% all breast tumors, and have histological features similar to fibroadenoma, a fibroepithelial tumor. Establishing an accurate differential diagnosis including phyllodes tumors is difficult due to their heterogeneous histological appearance [1, 2]. Clinically, phyllodes tumors are known to be malignant and recurrent and can metastasize to a number of different sites [3]. Although there are differing opinions among researchers regarding histologic classification of phyllodes tumors, the WHO classifies the histologic types of phyllodes tumor as benign, borderline, and malignant [2]. According to this classification scheme, the frequency of phyllodes tumor recurrence and distance metastasis increase with increasing histologic grade and are thus reflective of aggressiveness.

Insensitivity to growth inhibitory signals is an important difference between normal cells and cancer cells and is meditated primarily by tumor suppressor genes [4]. DNA hypermethylation is an important mechanism of inhibition of tumor suppressor genes and is catalyzed by DNA methyltransferases (DNMTs) [5]. *DNMT1*, *DNMT2*, *DNMT3A*, and *DNMT3B* have all been identified as DNA methyltransferase genes. Among these genes, DNMT1 is both the most common and most important gene involved in maintenance of methyltransferase activity in humans. The DNA residue 5-methylcytosine (5-MeC) is generated by DNMT1 via the addition of a methyl group to the 5' position of the cytosine ring in CpG dinucleotides and is a marker of DNMT1 activity. In addition, a previous breast cancer study reported that insulin gene enhancer binding protein-1 (ISL-1) is a direct target of DNMT1 [6].

A number of previous studies have studied the methylation status of genes in phyllodes tumors [7-9], where DNA promoter methylation appears to be more frequent compared to follicular adenomas [8]. In addition, one study reported that

Table 1. Source, clone, and dilution of the antibodies used

Antibody	Company	Clone	Dilution
DNA methylation-related proteins			
DNMT1	Abcam, Cambridge, UK	2B5	1:200
5-meC	Abcam, Cambridge, UK	33D3	1:200
ISL-1	Abcam, Cambridge, UK	Polyclonal	1:200

the frequency of methylation in phyllodes tumors increases with increasing grade [9]. Given these data, we hypothesized that the expression of DNA methylation-related proteins will increase in phyllodes tumors. Thus, the purpose of this study was to investigate the expression and implications of DNA methylation-related proteins in phyllodes tumor of the breast.

Materials and methods

Patient selection

We obtained tissue samples from patients who were diagnosed with phyllodes tumor and who underwent surgery (from 2000 to 2010) in the Department of Pathology in Severance Hospital. The study was approved by the Institutional Review Board of Yonsei University Severance Hospital. All tissues were fixed in 10% buffered formalin and embedded in paraffin. All archival hematoxylin and eosin (H&E)-stained slides for each case were reviewed by two pathologists (JS Koo and W Jung). The histologic grade of phyllodes tumors was evaluated using H&E-stained slides according to the WHO blue book criteria [2]. We also obtained associated clinical characteristics such as patient age, tumor recurrence, distant metastasis, and survival.

Tissue microarray

A representative area of each H&E-stained tumor slide was selected, and the corresponding location on the surface of the paraffin block was marked. The selected area was then punched out, and a 5 mm tissue core was placed in a 5×6 recipient block using a biopsy needle. Two tissue cores were obtained to minimize extraction bias. Each separate tissue core was given a unique tissue microarray location number that was linked to a database containing the associated clinical pathology data.

Immunohistochemistry

The antibodies used for immunohistochemistry in this study are shown in **Table 1**. All immunostaining was performed using tissue sections that were formalin-fixed and paraffin-embedded. A microtome was used to obtained 5-μm-thick sections that were transferred to adhesive slides and dried at 62°C for 30 minutes. After incubation with primary antibodies, immunodetection was performed with biotinylated anti-mouse immunoglobulin and visualized with peroxidase-labeled streptavidin using a labeled streptavidin biotin kit with 3,3'-diaminobenzidine chromogen as the substrate. As a negative control, the primary antibody incubation step was omitted. Slides were counterstained with Harris hematoxylin. All immunohistochemical markers were analyzed by light microscopy.

Immunohistochemical staining data were evaluated by multiplying the scores assigned for proportion of stained cells and immunostaining intensity. Specifically, the proportion of stained cells was scored as 0: negative, 1: less than 30% of cells positive, and 2: more than 30% of cells positive. Likewise, immunostaining intensity was scored as 0: negative, 1: weak, 2: moderate, and 3: strong. A multiplied intensity and proportion score of 0-1 was deemed as a negative staining result, while a score of 2-6 was considered positive [10]. Stromal component positivity was divided into low positive (2-3) and high positive (4-6) categories.

Statistical analysis

Data were analyzed using SPSS for Windows, Version 12.0 (SPSS Inc., Chicago, IL, USA). Student's *t* and Fisher's exact tests were used to determine statistical significance for continuous and categorical variables, respectively. The threshold for statistical significance was set as $P<0.05$. Kaplan-Meier survival curves and log-rank statistics were used to evaluate time to tumor recurrence. Multivariate regression analysis was performed using Cox proportional hazards model.

Results

Basal characteristics of phyllodes tumor

The basal characteristics of the 196 phyllodes tumor patients studied in this research are shown in **Table 2**. Among all cases, 153 were identified as benign, 27 were borderline, and

Table 2. Clincopathological characteristics of phyllodes tumor patients

Parameters	Total N=196 (100%)	PT, Benign N=153 (100%)	PT, Borderline N=27 (100%)	PT, Malignant N=16 (100%)	*P*-value
Age (years, mean ± SD)	40.1±12.3	38.9±12.2	42.3±11.5	47.6±12.9	0.017
Tumor size (cm, mean ± SD)	4.0±2.6	6.7±4.6	4.3±2.5	3.6±2.1	<0.001
Stromal cellularity					<0.001
Mild	121 (61.7)	120 (78.4)	1 (3.7)	0 (0.0)	
Moderate	63 (32.1)	33 (21.6)	23 (85.2)	7 (43.8)	
Marked	12 (6.1)	0 (0.0)	3 (11.1)	9 (56.2)	
Stromal atypia					<0.001
Mild	156 (79.6)	151 (98.7)	5 (18.5)	0 (0.0)	
Moderate	30 (15.3)	2 (1.3)	20 (74.1)	8 (50.0)	
Marked	10 (5.1)	0 (0.0)	2 (7.4)	8 (50.0)	
Stromal mitosis					<0.001
0-4/10 HPFs	154 (78.6)	153 (100.0)	1 (3.7)	0 (0.0)	
5-9/10 HPFs	31 (15.8)	0 (0.0)	26 (96.3)	5 (31.2)	
≥ 10/10 HPFs	11 (5.6)	0 (0.0)	0 (0.0)	11 (68.8)	
Stromal overgrowth					<0.001
Absent	179 (91.3)	153 (100.0)	24 (88.9)	2 (12.5)	
Present	17 (8.7)	0 (0.0)	3 (11.1)	14 (87.5)	
Tumor margin					<0.001
Circumscribed	176 (89.8)	150 (98.0)	20 (74.1)	6 (37.5)	
Infiltrative	20 (10.2)	3 (2.0)	7 (25.9)	10 (62.5)	
Tumor recurrence	18 (9.2)	5 (3.3)	6 (22.2)	7 (43.8)	<0.001
Distant metastasis	8 (4.1)	0 (0.0)	1 (3.7)	7 (43.8)	<0.001

PT, phyllodes tumor; HPFs, high-power fields.

Table 3. DNA methylation and expression of proteins related to methylation according to phyllodes tumor grade

Parameters	Total N=196 (100%)	PT, Benign N=153 (100%)	PT, Borderline N=27 (100%)	PT, Malignant N=16 (100%)	*P*-value
DNMT1 (E)*					0.473
Negative	129 (72.1)	107 (70.4)	19 (82.6)	3 (75.0)	
Positive	50 (27.9)	45 (29.6)	4 (17.4)	1 (25.0)	
DNMT1 (S)					<0.001
Low	183 (93.4)	151 (98.7)	23 (85.2)	9 (56.2)	
High	13 (6.6)	2 (1.3)	4 (14.8)	7 (43.8)	
5 meC (E)*					n/a
Negative	179 (100.0)	152 (100.0)	23 (100.0)	4 (100.0)	
Positive	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	
5 meC (S)					<0.001
Low	103 (52.6)	94 (61.4)	6 (22.2)	3 (18.8)	
High	93 (47.4)	59 (38.6)	21 (77.8)	13 (81.2)	
ISL-1 (E)*					0.194
Negative	149 (83.2)	128 (84.2)	19 (82.6)	2 (50.0)	
Positive	30 (16.8)	24 (15.8)	4 (17.4)	2 (50.0)	
ISL-1 (S)					<0.001
Low	182 (92.6)	150 (98.0)	22 (81.5)	10 (62.5)	
High	14 (7.1)	3 (2.0)	5 (18.5)	6 (37.5)	

*Seventeen tumors without an epithelial component were excluded. E, epithelial; S, stromal.

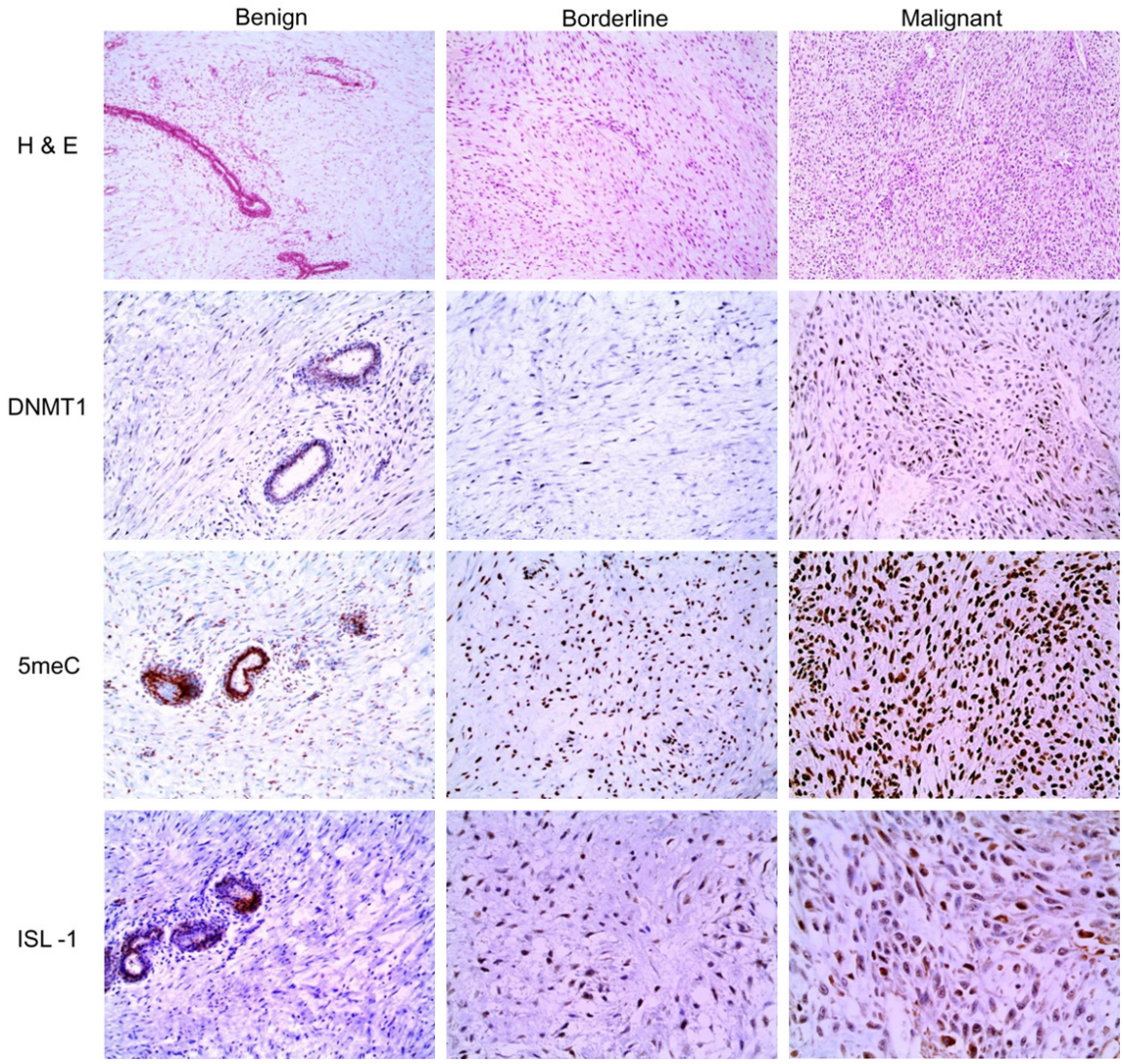

Figure 1. DNA methylation and expression of methylation-related proteins according to histologic grade of phyllodes tumor. Levels of DNMT1, 5 meC, and ISL-1 staining in the stromal component increased with increasing histologic grade.

16 were malignant. Age and tumor size increased with increasing histological grade (P=0.017 and P=0.001, respectively). Tumor recurrence and distance metastasis also increased with increasing histological grade (P<0.001). The eight examples of distance metastasis were all lung metastases.

Methylation and expression of methylation-related proteins according to phyllodes tumor grade

In analyzing the methylation status and expression of methylation-related proteins according to histologic type of phyllodes tumor, the expression of methylation-related proteins in the epithelial component did not appear to be associated with histologic grade. On the other hand, DNMT1, 5 meC, and ISL-1 positivity in the stromal component exhibited significant differences according to histologic grade (**Table 3** and **Figure 1**). Specifically, as histologic grade increased, the ratios of DNMT1, 5 meC, and ISL-1 positivity increased (P<0.001).

Correlations of methylation and the expression of methylation-related proteins with pathologic parameters

Comparison of methylation and expression of methylation-related proteins and pathologic parameters revealed that stromal DNMT1 high

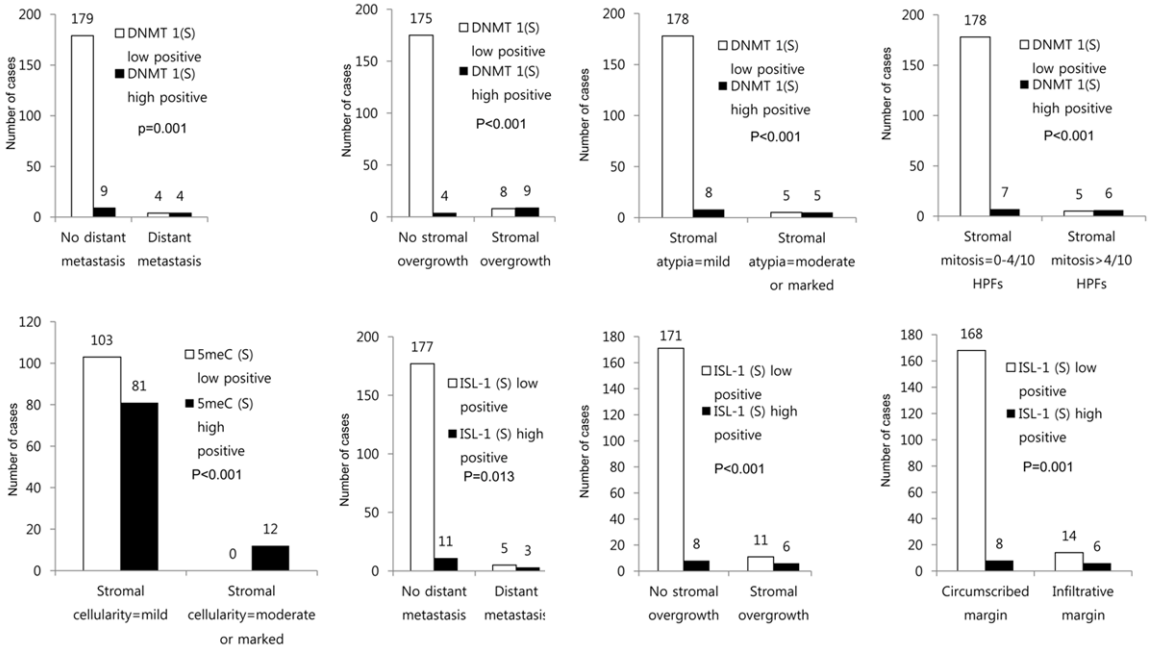

Figure 2. Correlation of DNA methylation and expression of methylation-related proteins in phyllodes tumor according to pathologic parameters.

Table 4. Univariate analysis of methylation and methylation-related proteins according to patient prognosis using the log-rank test

Parameters	No. of patients Total/recurrence/ metastasis	Disease-free survival		Overall survival	
		Median survival (95% CI) months	*P*-value	Median survival (95% CI) months	*P*-value
DNMT1 (E)*			0.242		n/a
Negative	129/6/1	174 (167-181)		n/a	
Positive	50/5/0	134 (123-145)		n/a	
DNMT1 (S)			0.002		<0.001
Low	183/14/4	169 (162-176)		179 (175-182)	
High	13/4/4	96 (65-127)		92 (58-125)	
5meC (S)			0.067		n/a
Low	103/6/0	172 (164-180)		n/a	
High	93/12/8	155 (143-167)		n/a	
ISL-1 (E)*			0.633		n/a
Negative	149/9/1	168 (162-175)		n/a	
Positive	30/2/0	171 (155-186)		n/a	
ISL-1 (S)			<0.001		<0.001
Low	182/13/5	170 (163-176)		178 (173-182)	
High	14/5/3	82 (46-119)		106 (78-135)	

*Seventeen tumors without an epithelial component were excluded. E, epithelial; S, stromal.

positivity was associated with distant metastasis (P=0.001), stromal overgrowth (P<0.001), increased stromal atypia (P<0.001), and increased stromal mitosis (P<0.001). Likewise, high stromal 5 meC positivity was associated with increased stromal cellularity (P<0.001). Lastly, high stromal ISL-1 positivity was associated with distant metastasis (P=0.013), stromal overgrowth (P<0.001), and infiltrative margin (P=0.001, **Figure 2**).

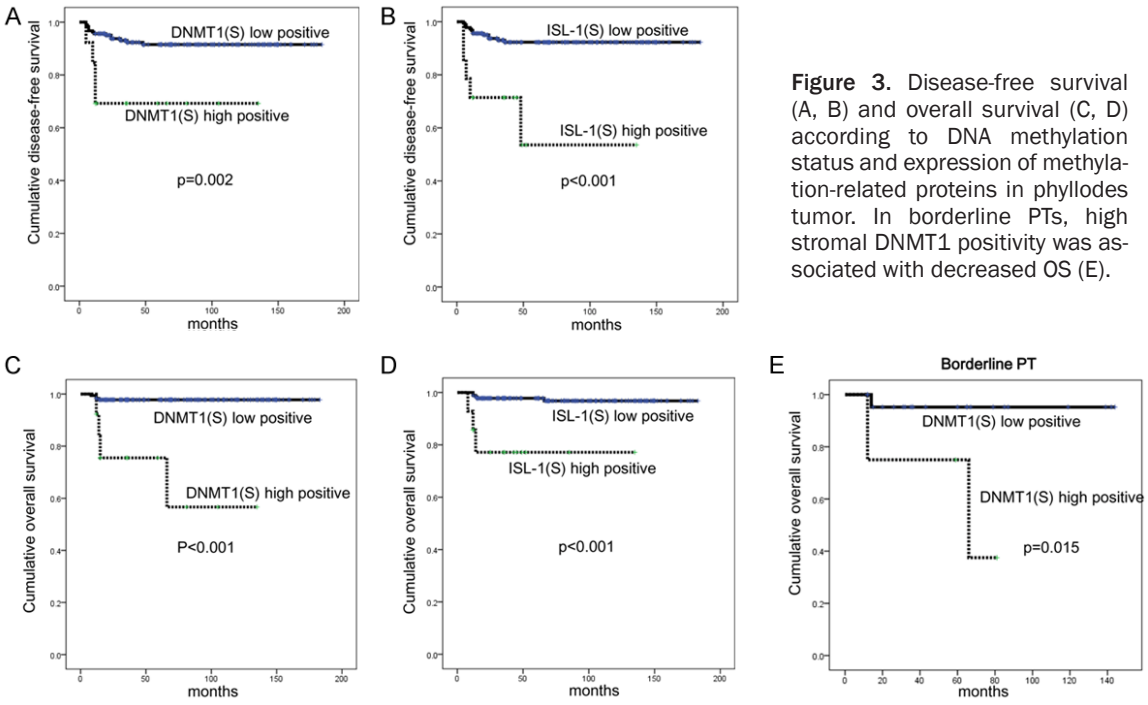

Figure 3. Disease-free survival (A, B) and overall survival (C, D) according to DNA methylation status and expression of methylation-related proteins in phyllodes tumor. In borderline PTs, high stromal DNMT1 positivity was associated with decreased OS (E).

Impact of methylation and expression of methylation-related proteins on patient prognosis

Univariate analysis to evaluate methylation status and expression of methylation-related proteins on phyllodes tumor patient prognosis showed that decreased DFS and OS were associated with stromal DNMT1 high positivity (P=0.002 and P<0.001, respectively) and stromal ISL-1 high positivity (P<0.001 and P<0.001, respectively, **Table 4** and **Figure 3**). Univariate analysis of phyllodes tumor according to grade showed that high stromal DNMT1 positivity was associated with decreased OS (P=0.015) in borderline phyllodes tumors. According to multivariate Cox analysis, independent factors associated with decreased DFS consisted of histologic grade (hazard ratio: 7.087, 95% CI: 1.857-27.05, P=0.004) and stromal overgrowth (hazard ratio: 10.78, 95% CI: 1.859-62.59, P=0.008), while stromal overgrowth was identified as an independent factor associated with decreased OS (hazard ratio: 59.47, 95% CI: 3.258-1085, P=0.006, **Table 5**).

Discussion

This research analyzed the relationships between patient outcomes and the methylation and expression of DNA methylation-related proteins in phyllodes tumors of the breast. The results showed that 5 meC staining and the expression of DNMT1 and ISL-1 were increased in the stromal component of phyllodes tumors with increased histologic grade. Consistent with these results, previous studies have reported that TWIST1 [7-9] and RASSF1A [8, 9] are also significantly methylated in phyllodes tumors. Likewise, the methylation frequency of five genes (GSTP1, HIN-1, RAR-beta, RASSF1A, and Twist) has been showed to be increased in phyllodes tumors according to histologic grade [9]. The same study reported that, while the methylation profiles of benign and borderline/malignant phyllodes tumors are distinguishable, those of borderline and malignant tumors are not [9]. In contrast to this result, we found that the expression of DNMT1 and ISL-1 was significantly higher in the stromal component of malignant phyllodes tumors compared to borderline tumors. A possible explanation for this discrepancy is the use of different experimental methods. Specifically, while previous methylation studies targeting phyllodes tumor evaluated the specific methylation status of genes of interest, we focused on the expression of proteins involved in DNA methylation (DNMT1) and DNA-methylation target genes (ISL-1). Thus, the increased expression of DNA methylation-related proteins according to histologic grade that was observed in this study may have been due

Table 5. Multivariate analysis of disease-free survival and overall-survival in phyllodes tumor patients

Included factor	Disease-free survival			Overall survival		
	Hazard ratio	95% CI	*P*-value	Hazard ratio	95% CI	*P*-value
Histologic grade			0.004			n/a
Benign vs. borderline or malignant	7.087	1.857-27.05		n/a	n/a	
Stromal cellularity			0.317			0.475
Mild vs. moderate or marked	0.336	0.040-2.847		2.805	0.165-47.59	
Stromal atypia			0.671			0.866
Mild vs. moderate or marked	0.671	0.106-4.246		0.812	0.072-9.118	
Stromal mitosis			0.673			0.426
0-4/10 HPFs vs. >4/10 HPFs	1.874	0.102-34.58		0.209	0.004-9.898	
Stromal overgrowth			0.008			0.006
Absent vs. Present	10.78	1.859-62.59		59.47	3.258-1085	
Tumor margin			0.158			0.176
Circumscribed vs. Infiltrative	0.395	0.109-1.436		0.333	0.068-1.638	
DNMT1 (S)			0.191			0.687
Low vs. High	0.374	0.086-1.631		0.701	0.125-3.934	
ISL-1 (S)			0.075			0.432
Low vs. High	2.901	0.899-9.369		1.976	0.362-10.78	

to carcinogenesis and/or tumor progression following epigenetic silencing of various tumor-related genes by DNMT1, especially tumor suppressor genes [4]. Thus, as histologic grade of phyllodes tumor increases, the number of genes that undergo methylation-induced silencing increases, which would be consistent with our observation that 5-MeC positivity and expression of DNMT1 were increased in higher grade tumors.

In the present study, expression of DNMT1 and ISL-1 in the stromal component of phyllodes tumors was associated with increased distant metastasis. Consistent with this observation, previous studies have shown that DNMT1 expression is associated with metastasis in both esophageal cancer [11] and stomach cancer [12], while ISL-1 expression is associated with metastasis in stomach cancer [13]. Increased metastasis following epigenetic silencing by DNA methylation of metastasis-inhibiting genes has been reported [11, 14]. Such metastasis-related genes include protocadherin 17, RASSF1A, and DAPK, which were mentioned previously. Among these, RASSF1A has been reported to undergo DNA methylation in phyllodes tumor [8, 9] and should be investigated in similar future studies.

Our results showed that high levels of expression of DNMT1 and ISL-1 in the stromal compartment of phyllodes tumors were associated with poor prognosis. Consistently, high DNMT1 expression is associated with poor prognosis in malignant lymphoma [15], renal cell carcinoma [16], pancreatic cancer [17], and bladder cancer [18], while high ISL-1 expression is associated with poor prognosis in gastric cancer [13]. Especially in borderline phyllodes tumors, high stromal DNMT1 expression was significantly associated with decreased OS. Establishing an accurate clinical prognosis for borderline phyllodes tumor is difficult because borderline high-grade tumors can be hard to distinguish from benign or malignant tumors [2]. An effective prognostic factor is therefore needed to overcome this problem. Importantly, our results suggest that stromal DNMT1 expression could serve as a prognostic marker of borderline phyllodes tumors, a possibility that will require further study.

A clinically significant outcome of this study is the identification of the epigenetic methylation-related protein DNMT1 as a target in malignant phyllodes tumors. Specifically, by identifying an association between DNMT1 overexpression and increased tumor progression, our study supports the possibility of inhibiting tumorigenesis by re-expressing tumor suppressor genes that have been inhibited through selective DNMT1 inhibition [19]. Consistent with this possibility, numerous studies have already

attempted to target DNMT1 in various types of cancer [19-22].

In conclusion, the expression of DNA methylation-related proteins in the stromal component of phyllodes tumors of the breast was found to increase with increasing histologic grade, and stromal DNMT1 and ISL-1 overexpression was associated with a poor prognosis.

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Disclosure of conflict of interest

None.

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