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Expression of DNA methylation-related proteins in metastatic breast cancer

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We aimed to investigate the expression of methylation-related proteins (5-meC and DNMT1) in the metastatic breast cancers of variable sites and its association with clinicopathologic factors. A total of 126 metastatic breast cancers (31 bone metastases, 36 brain metastases, 11 liver metastases, 48 lung metastases) were made into tissue microarray and immunohistochemical staining of ER, PR, HER-2, Ki-67, 5-meC, and DNMT1 were performed. Molecular classification was made on the basis of immunohistochemical staining result of ER, PR, HER-2, Ki-67; luminal A, luminal B, HER-2, triple negative breast cancer (TNBC). Methylation-related proteins were differentially expressed based on the metastatic sites. Tumoral and stromal 5-meC showed the lowest expression in the bone metastasis ($P < 0.001$), tumoral DNMT1 showed the least expression in bone metastasis and the highest expression in the brain metastasis ($P < 0.001$). Expression of DNMT1 was correlated with ER negativity ($P = 0.004$), PR negativity ($P = 0.011$), HER-2 positivity ($P = 0.016$), higher Ki-67 labeling indices ($P = 0.016$), and non-luminal A type ($P = 0.017$). DNMT1 positivity was associated with shorter overall survival in bone metastasis ($P = 0.017$) and lung metastasis ($P = 0.028$) by univariate analysis. In conclusion, methylation-related proteins differentially expressed according to the metastatic sites in metastatic breast cancer. Tumoral and stromal 5-meC showed the lowest expression in the bone metastasis. Tumoral DNMT1 expression was low in bone metastasis and highest in brain metastasis.

Key words: breast cancer, DNA methylation, DNMT1, 5-meC, metastasis

Cancer cells are different from normal cells in insensitivity to growth inhibitory signals by inhibition of tumor suppressor genes [1]. DNA hypermethylation is one of the key mechanisms of tumor suppressor gene inhibitions. DNA methylation is enhanced by enzymes named DNA methyltransferases (DNMTs) [2], encoded by DNA methyltransferase genes including *DNMT1*, *DNMT2*, *DNMT3A*, and *DNMT3B*. DNMT1 is the most common and important key maintenance methyltransferase. 5-methylcytosine (5-meC) is a molecule associated with DNMT1, which is a product of DNA methylation, since a methyl group is attached to the 5' position of the cytosine ring. In previous study of epigenetic methylation-related protein in breast cancer showed overexpression of DNMT1 and DNMT3a in tumor and its association of poor prognosis [3].

Distant metastasis in breast cancer is one of the causes of high morbidity and mortality. Common metastatic site of breast cancer includes the lung, brain, liver, and bone [4, 5], and the brain and bone metastases have been thoroughly investigated [6-11]. Tumor metastasis occurs via interaction of tumor cells and host tissue, by the processes of adhesion, proteolysis, invasion, and angiogenesis [5, 12]. However, not every tumor showed similar metastatic pattern, the seed and soil hypothesis has been suggested. It explained that specific tumor (seed) can survive in the specific visceral organ (soil) [13]. Metastatic breast cancer also showed characteristic features based on the metastatic sites. Brain metastasis has been reported to associate with young age, estrogen receptor (ER) negativity, prior lung metastasis, HER-2 overexpression, EGFR overexpression, and basal subtype [8-10], and bone metastasis has been reported to associate with lower histologic grade, ER positivity, ER positivity/progesterone receptor (PR) negativity, strand growth pattern, and presence of fibrotic foci in invasive ductal carcinoma [7, 14, 15]. Therefore, metastatic breast cancers of different metastatic sites are expected to have different characteristics, including expression of epigenetic methylation-related proteins, which is not surveyed yet. We aimed to investigate the expression of methylation-related proteins (5-meC and DNMT1) in the metastatic breast cancers of variable sites and its association with clinicopathologic factors.

Patients and methods

Patient selection. Invasive primary breast cancer and metastatic breast cancer to distant organs (liver, lung, brain, and bone) were retrieved from data files of the Department of Pathology of Severance Hospital, Seoul, South Korea. Only patients with a diagnosis of invasive ductal carcinoma were included. A total of 126 cases were included, and 27 cases consisted of paired primary and metastasis carcinomas. All slides were reviewed again and pathologic diagnoses were approved by two pathologists (JSK and WJ). The histological grade was assessed using the Nottingham grading system [16]. This study was approved by the Institutional Review Board of Severance Hospital.

Tissue microarray. On H&E-stained slides of tumors, a representative area was selected and a corresponding spot was marked on the surface of the paraffin block. Using a biopsy needle, the selected area was punched out and a 3-mm tissue core was placed into a 6 x 5 recipient block. Tissue of invasive tumor was extracted. More than 2 tissue cores were extracted to minimize extraction bias. Each tissue core was assigned with a unique tissue microarray location number that was linked to a database containing other clinicopathologic data.

Immunohistochemistry. The antibodies used for immunohistochemistry in this study are shown in Table 1. Three-micrometer paraffin sections were deparaffinized and rehydrated by xylene and alcohol solution. Immunohistochemistry was performed using the Ventana Discovery XT automated stainer (Ventana Medical System, Tucson, AZ, USA). Antigen retrieval was performed using Cell Conditioning 1 (CC1; citrate buffer pH 6.0, Ventana Medical System). Appropriate positive and negative controls for immunohistochemistry were included.

Interpretation of immunohistochemical results. A cut-off value of 1% or more positively stained nuclei was used to define ER and AR positivity [17]. HER-2 staining was analyzed according to the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guidelines using the following categories: 0 = no immunostaining; 1+ = weak incomplete membranous staining, less than 10% of tumor cells; 2+ = complete membranous staining, either uniform or weak in at least 10% of tumor cells; and 3+ = uniform intense membranous staining in at least 30% of tumor cells [18]. HER-2 immunostaining was considered positive when strong (3+) membranous staining was observed whereas cases with 0 to 1+ were regarded as negative.

Immunohistochemical staining for 5-meC, DNMT1 was assessed by light microscope by semiquantitative manner. Staining results in cancer cells and stromal cells were assessed as 0, negative or weak immunostaining in <1% of the tumor/stroma; 1, focal expression in 1-10% of tumor/stroma; 2, positive in 11-50% of tumor/stroma; and 3, positive in 51-100% of tumor/stroma. This evaluation was applied to all areas of the tumor in all samples; grade 0, 1 were negative and grades 2, 3 were positive [19]. Ki-67 labeling indices (LI) were scored by counting the number of positively stained nuclei and expressed as a percentage of total tumor cells.

Tumor phenotype classification. In this study, we classified breast cancer phenotypes according to the immunohistochemistry results for ER, PR, HER-2 and Ki-67 and FISH results for HER-2 as follows [20]; *Luminal A type*: ER or/and PR positive and HER-2 negative and Ki-67 LI <14%, *Luminal B type*: (HER-2 negative) ER or/and PR positive and HER-2 negative and Ki-67 LI ≥14% , (HER-2 positive) ER or/and PR positive and HER-2 overexpressed or/and amplified, *HER-2 type*: ER and PR negative and HER-2 overexpressed or/and amplified, *TNBC type*: ER, PR, and HER-2 negative.

Statistical analysis. Data were statistically processed using SPSS for Windows, version 12.0 (SPSS Inc., Chicago, IL, USA). Correlation analysis of immunostaining results between primary breast cancer and metastatic breast cancer were calculated by McNemar test. Student's *t* and Fisher's exact tests were used to examine any differences in continuous and categorical variables, respectively. A corrected *p*-value and the Bonferroni method were used for multiple comparisons. Statistical significance was assumed when $P < 0.05$. Kaplan-Meier survival curves and log-rank statistics were employed to evaluate time to tumor metastasis and time to survival. Multivariate regression analysis was performed using Cox proportional hazards model.

Results

Baseline characteristics of patients. A total of 126 cases were composed of 31 bone metastases (24.6%), 36 brain me-

Table 1. Source, clone, and dilution of antibodies

Antibody	Company	Clone	Dilution
DNA methylation-related proteins			
DNMT1	Abcam, Cambridge, UK	2B5	1:200
5-meC	Abcam, Cambridge, UK	33D3	1:200
Molecular subtype-related proteins			
ER	Thermo Scientific, San Diego, CA, USA	SP1	1:100
PR	DAKO, Glostrup, Denmark	PgR	1:50
HER-2	DAKO, Glostrup, Denmark	Polyclonal	1:1500
Ki-67	Abcam, Cambridge, UK	MIB	1:1000

tastases, 11liver metastases (28.6%), and 48 lung metastases (38.1%) Bone metastases and liver metastases showed higher rates of ER and PR positivity ($P < 0.001$). Brain metastases showed higher rate of HER-2 positivity and higher Ki-67 LI ($P = 0.032$ and $P = 0.008$, respectively). Luminal A type was common among bone metastases and liver metastases, and TNBC was common among brain metastases and lung metastases ($P < 0.001$, Table 2).

Expression of methylation-related proteins in breast cancer metastasis according to metastatic site. In metastatic site, 5-meC expressed in both tumor and stromal cells whereas DNMT1 expressed only in tumor cells (Figure 1). Tumoral 5-meC and stromal 5-meC showed lowest expression in bone metastasis ($P < 0.001$), and tumor DNMT1 showed the least expression in bone meatstases and the highest expression in the brain metastases ($P < 0.001$, Figure 2) (Table 3). Normal tissue of all metastatic sites – brain, bone, liver and lung – showed lower DNMT1 expression than tumor cells (Figure 3).

Correlation of expression of methylation-related proteins between primary and metastatic breast cancer according to metastatic site. In 27 paired primary and metastatic cancers, expression of methylation-related proteins was not different between the primary cancer and metastatic cancer. In bone metastases, primary cancers with 5-meC positive in both tumor and stroma were all negative for 5-meC (Table 4).

Table 2. Basal clinicopathologic characteristics of breast cancer metastasis according to the metastatic sites

Parameters	Total, *N*= 126 (%)	Bone metastasis *N* = 31 (%)	Brain metastasis *N* = 36 (%)	Liver metastasis *N* = 11 (%)	Lung metastasis *N*= 48 (%)	*P* – value
Age (years)						**0.605**
≤50	65 (51.6)	17 (54.8)	17 (47.2)	4 (36.4)	27 (56.2)	
>50	61 (48.4)	14 (45.2)	19 (52.8)	7 (63.6)	21 (43.8)	
ER						**<0.001**
Negative	59 (46.8)	6 (19.4)	25(69.4)	2 (18.2)	26 (54.2)	
Positive	67 (53.2)	25 (80.6)	11 (30.6)	9 (81.8)	22 (45.8)	
PR						**<0.001**
Negative	86 (68.3)	16 (51.6)	35 (97.2)	3 (27.3)	32 (66.7)	
Positive	40 (31.7)	15 (48.4)	1 (2.8)	8 (72.7)	16 (33.3)	
HER-2						**0.032**
Negative	86 (68.3)	25 (80.6)	18 (50.0)	9 (81.8)	34 (70.8)	
Positive	40 (31.7)	6 (19.4)	18 (50.0)	2 (18.2)	14 (29.2)	
Ki-67 LI						**0.008**
≤14	84 (66.7)	27 (87.1)	18 (50.0)	9 (81.8)	30 (62.5)	
>14	42 (33.3)	4 (12.9)	18 (50.0)	2 (18.2)	18 (37.5)	
Molecular subtypes						**<0.001**
Luminal A	44 (34.9)	21 (67.7)	3 (8.3)	6 (54.5)	14 (29.2)	
Luminal B	24 (19.0)	5 (16.1)	8 (22.2)	3 (27.3)	8 (16.7)	
HER-2	25 (19.8)	3 (9.7)	12 (33.3)	1 (9.1)	9 (18.8)	
TNBC	33 (26.2)	2 (6.5)	13 (36.1)	1 (9.1)	17 (35.4)	
Patients death	41 (32.5)	16 (51.6)	11 (30.6)	4 (36.4)	10 (20.8)	**0.041**

Table 3. Expression of methylation-related proteins in tumor cell compartment of breast cancer metastasis according to the metastatic sites

Parameters	Total *N*= 126 (%)	Bone metastasis *N* = 31 (%)	Brain metastasis *N* = 36 (%)	Liver metastasis *N* = 11 (%)	Lung metastasis *N*= 48 (%)	*P* – value
5-meC (T)						**<0.001**
Negative	19 (15.1)	16 (51.6)	1 (2.8)	2 (18.2)	0 (0.0)	
Positive	107 (84.9)	15 (48.4)	35 (97.2)	9 (81.8)	48 (100.0)	
5-meC (S)						**<0.001**
Negative	18 (14.3)	15 (48.4)	1 (2.8)	2 (18.2)	0 (0.0)	
Positive	108 (85.7)	16 (51.6)	35 (97.2)	9 (81.8)	48 (100.0)	
DNMT1 (T)						**<0.001**
Negative	64 (50.8)	22 (71.0)	5 (13.9)	11 (100.0)	26 (54.2)	
Positive	62 (49.2)	9 (29.0)	31 (86.1)	0 (0.0)	22 (45.8)	

T, tumor cell, S, stromal cell

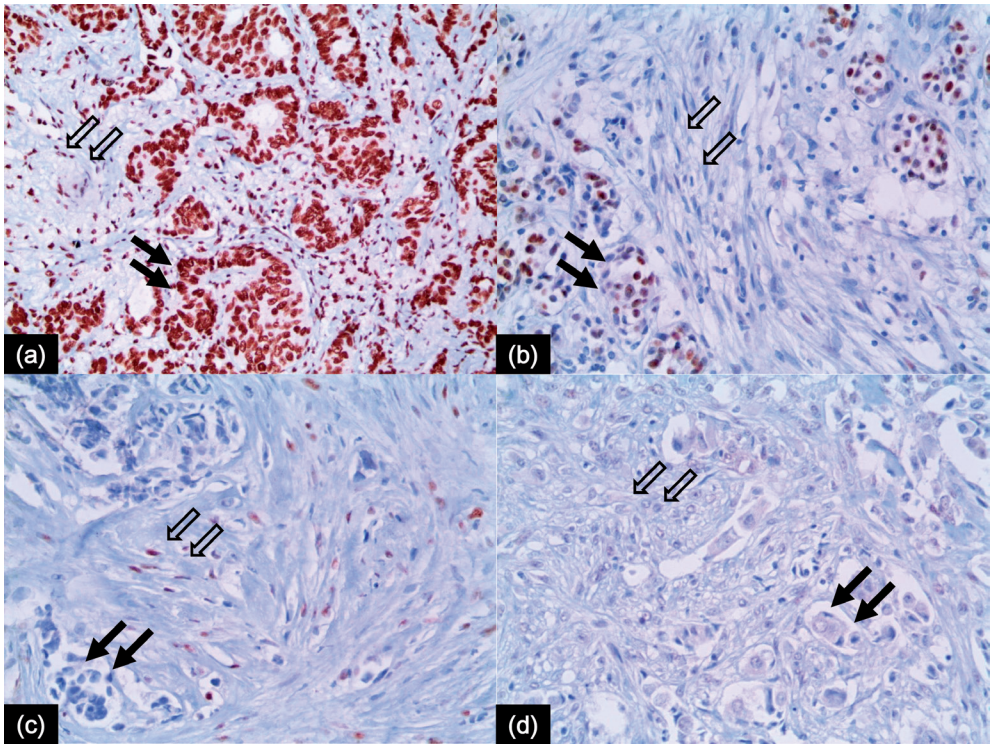

Figure 1. Expression of 5-meC in tumor cells (black arrow) and stromal cells (blank arrow) of metastatic breast cancer. 5-meC is expressed in both tumor and stromal cell (a), only cancer cells (b), only stromal cells (c), or not expressed in both cancer cell and stromal cell (d).

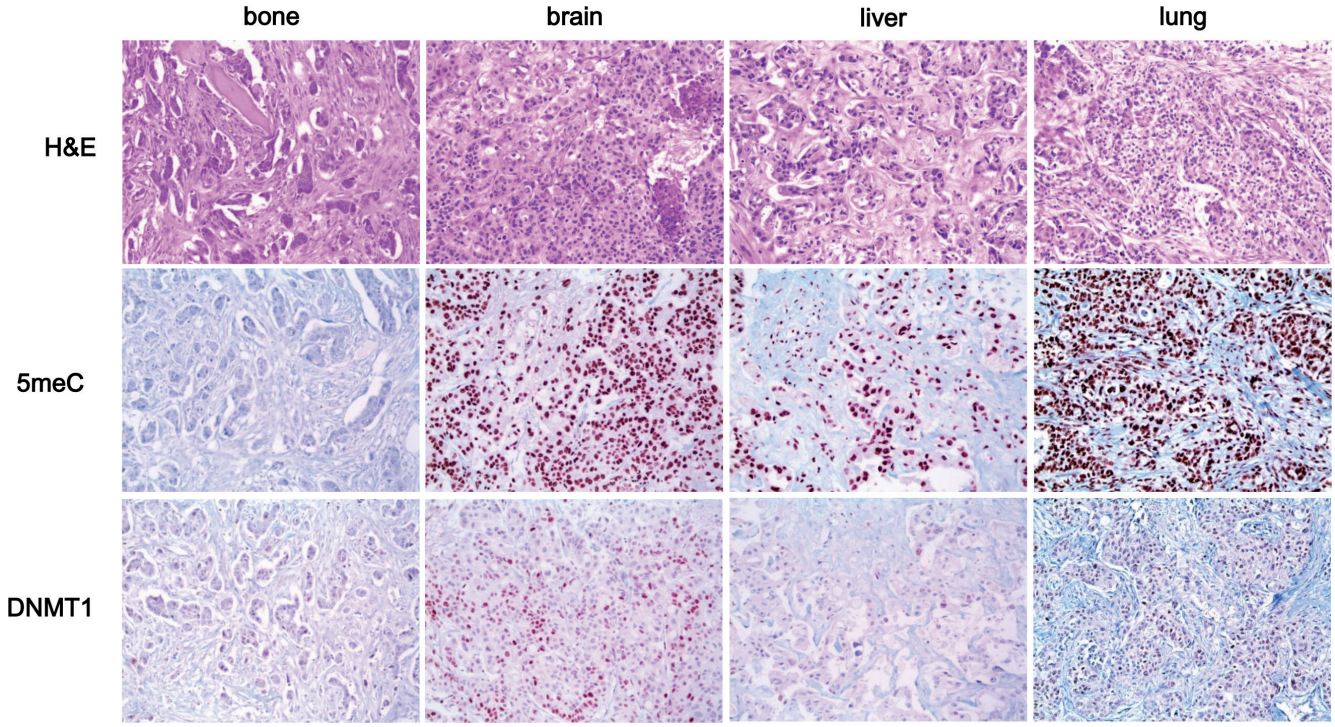

Figure 2. Expression of methylation-related proteins in metastatic breast cancer according to metastatic site. Tumoral 5-meC and stromal 5-meC show the lowest expression in bone metastases, and tumoral DNMT1 is reduced in bone metastases and overexpressed in brain metastasis.

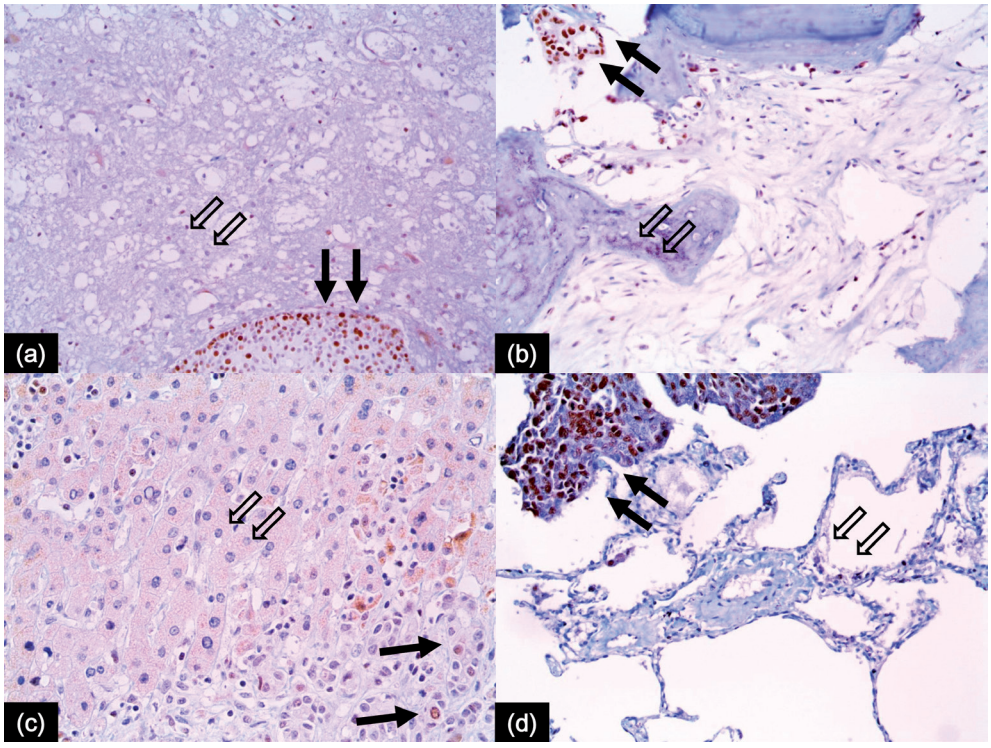

Figure 3. Expression of DNMT1 in normal tissue of brain, bone, liver, and lung. DNMT1 in normal tissue (blank arrows) of brain (a), bone (b), liver (c), and lung (d) shows lower expression than adjacent metastatic tumor cells (black arrows).

Correlation between pathologic factors and expression of methylation-related proteins. DNMT1 expression of metastatic breast cancer was correlated with ER negativity ($P = 0.004$), PR negativity ($P = 0.011$), HER-2 positivity ($P = 0.016$), higher Ki-67 LI ($P = 0.016$), and molecular subtype of non-luminal A type ($P = 0.017$) (Figure 4).

The impact of the expression of methylation-related proteins on patient prognosis. On univariate analysis of total

Table 4. Correlation of expression of methylation-related proteins between primary and metastatic breast cancer according to the metastatic sites

Parameters	Total $N = 27$ (%)	Bone metastasis $N = 5$ (%)	Brain metastasis $N = 5$ (%)	Liver metastasis $N = 1$ (%)	Lung metastasis $N = 16$ (%)	P – value
5-meC (T)						0.063
(+) → (+)	22 (81.5)	0 (0.0)	5 (100.0)	1 (100.0)	16 (100.0)	
(+) → (-)	5 (18.5)	5 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	
(-) → (+)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	
(-) → (-)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	
5-meC (S)						0.063
(+) → (+)	22 (81.5)	0 (0.0)	5 (100.0)	1 (100.0)	16 (100.0)	
(+) → (-)	5 (18.5)	5 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	
(-) → (+)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	
(-) → (-)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	
DNMT1 (T)						1.000
(+) → (+)	6 (22.2)	0 (0.0)	4 (80.0)	1 (100.0)	2 (12.5)	
(+) → (-)	2 (7.4)	0 (0.0)	1 (20.0)	0 (0.0)	1 (6.2)	
(-) → (+)	1 (3.7)	0 (0.0)	0 (0.0)	0 (0.0)	1 (6.2)	
(-) → (-)	18 (66.7)	5 (100.0)	0 (0.0)	0 (0.0)	12 (75.0)	

T, tumor cell, S, stromal cell

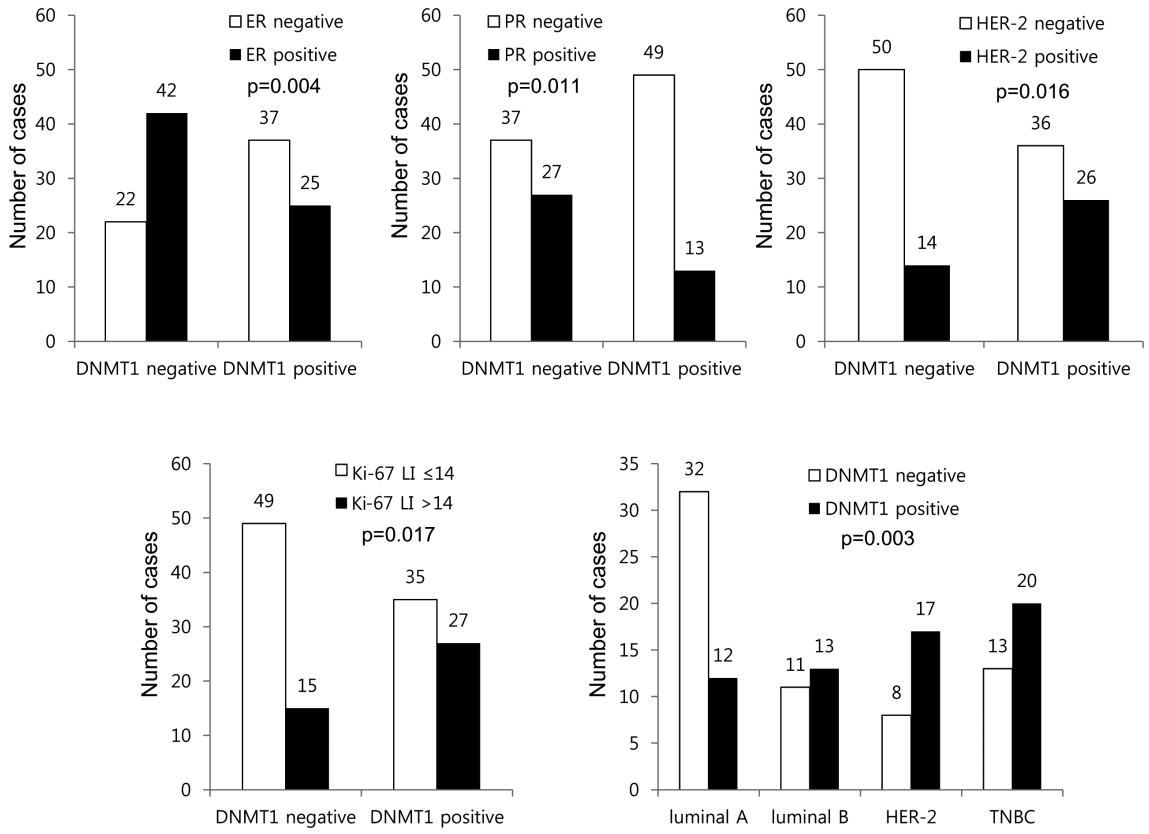

Figure 4. Correlation between pathologic factors and expression of methylation-related proteins in metastatic breast cancer

patients, expression of methylation-related proteins showed no association with shorter overall survival (OS) (Table 5). However, according to the metastatic sites, DMMT1 positivity was associated with shorter OS in bone metastases ($P = 0.017$), and lung metastases ($P = 0.028$) (Figure 5).

Discussion

In present study, we evaluated the expression of methylation-related proteins in metastatic breast cancers. Expression of both 5-meC and DNMT1 was low in bone metastases.

Table 5. Univariate analysis of the impact of expression of methylation-related proteins in metastatic breast cancers on overall survival by the log-rank test.

Parameters	Total *N*= 126 (%)		Bone metastasis *N* = 31 (%)		Brain metastasis *N* = 36 (%)		Liver metastasis *N* = 11 (%)		Lung metastasis *N*= 48 (%)	
	Mean survival months (95% CI)	*P* -value	Mean survival months (95% CI)	*P* – value	Mean survival months (95% CI)	*P* – value	Mean survival months (95% CI)	*P* – value	Mean survival months (95% CI)	*P* – value
5-meC (T)		0.523		0.050		n/a		n/a		n/a
Negative	105 (70-139)		107 (70-143)		n/a		n/a		n/a	
Positive	117 (100-133)		48 (32-63)		n/a		n/a		n/a	
5-meC (S)		0.558		0.222		n/a		n/a		n/a
Negative	87 (64-111)		89 (64-114)		n/a		n/a		n/a	
Positive	116 (100-132)		64 (31-97)		n/a		n/a		n/a	
DNMT1 (T)		0.111		**0.017**		0.195		n/a		**0.028**
Negative	124 (104-144)		104 (73-136)		54 (10-98)		n/a		153 (127-179)	
Positive	93 (71-116)		41 (22-61)		107 (83-131)		n/a		89 (38-140)	

T, tumor cell, S, stromal cell

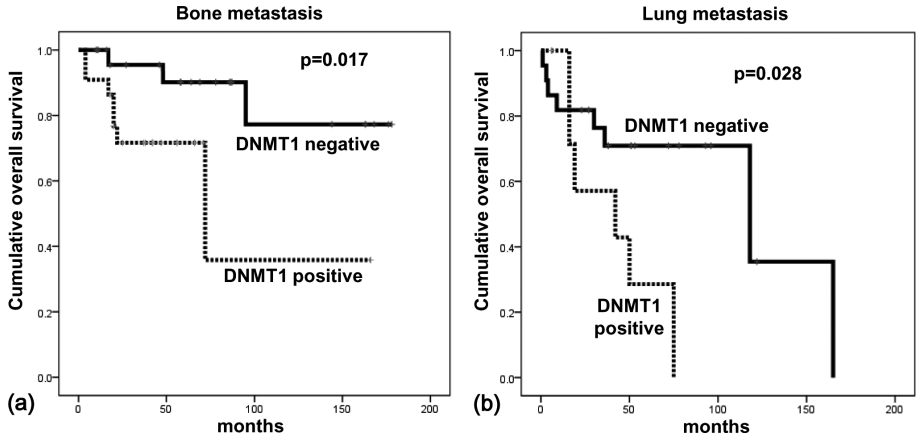

Figure 5. The impact of methylation-related proteins on patient prognosis in bone metastasis (a), and lung metastasis (b)

One of the scenarios for this result would be the association with ER. Yu et al. reported that protein and mRNA levels of DNMT1 were reduced in normal breast specimens and ERα-positive breast cancer specimens compared to the ERα-negative breast cancer specimens [3]. In present study, ER status was different according to the metastatic sites, showed the highest positive rate in liver metastases (81.8%) followed by bone (80.6%), lung (45.8%), and brain (30.6%) metastases. DNMT1 positive rate was highest in brain metastases (86.1%) followed by lung (45.8%), bone (29.0%), and liver (0.0%) metastases, displaying the opposite order with ER positive rate, that was corroborated by results of previous study. Expression of DNMT1 induces promotor methylation of ERα and results reduction of ER expression [21]. DNMT1 methylates variable genes and suppresses them, which could influence over cancer characteristics. In metastatic breast cancers, BRMS1 (Breast cancer metastasis suppressor 1) [22] and CREB3L1 (cAMP-responsive element-binding protein 3-like protein 1) [22] are methylated and silenced, which could impact on the process of metastasis. Differential expression of methylation-related proteins based on the metastatic sites would be resulted from different tumor microenvironment. Tumor microenvironment (TME) refers the non-tumor, non-transformed elements which are exists in the territory of tumor cells; immune system element (such as macrophages and lymphocytes), blood vessel cells, fibroblast, myofibroblast, mesenchymal stem cells, adipocytes and extracellular matrix (ECM). Most key element of TME is cancer-associated fibroblasts (CAFs) [23]. Previous study demonstrated that IL-6 increases nuclear translocation of DNMT1 by phosphorylation of nuclear localization sequence [24]. Secretion of IL-6 by tumor infiltrating lymphocyte [25] and tumor associated macrophage [26] indicated that tumor stroma would affect the expression of methylation-related proteins. In primary breast cancer, expression of methylation-related proteins differed according to the phenotype of tumor stroma [27], as well as expression of CAF-related proteins according to the metastatic breast cancers [28], which imply that different phenotype of TME metastatic site may be related with different methylation status and should be clarified on further study. Moreover, intrinsic properties of metastatic sites could result different DNMT1 expression specific to the metastatic sites. In the traumatically injured [29] or hypoxically damaged [30] brain tissue have altered expression of DNMT1. Thus, DNMT1 expression could also be affected by brain metastasis state.

We identified the expression of 5-meC in tumor stroma as well as in tumor cells. Previous studies of epigenetic alteration in CAF reported different specific methylation status between tumor-associated stroma and non-tumor stroma in prostate cancer by methylation pattern analysis [31, 32], and global DNA hypomethylation in CAFs has been demonstrated in lung cancer [33]. In present study, 5-meC-negative spindle-shaped stromal cells were found in 14.3% of total cases, and these 5-meC-negative spindle cells could be considered as hypomethylated CAFs. Hypomethylated CAFs were most frequently observed in bone metastases (48.4%), and global hypomethylation of stromal cells of bone marrow under pathologic condition has been reported [34], thus stromal cell hypomethylation may have association with bone metastases, and further study is required. We observed DNMT1 negativity in tumor stroma, which was compatible with previous study addressed the low expression of DNMT not only in tumor stroma but also in non-tumor stroma [35].

Tumoral DNMT1 expression was associated with shorter OS in present study. This result agreed with previous data that high expression of DNMT1 was correlated with poor prognosis in breast cancer [27], malignant lymphoma [19], renal cell carcinoma [36], pancreatic cancer [37], bladder cancer [38]. MUC1-C oncoprotein could be the link between DNMT1 expression and poor prognosis. DNMT1 overexpression in human breast cancer cells can be induced by MUC1-C oncoprotein [39], and CDH1 gene is one of the downregulated genes by MUC1-C induced DNA methylation [39, 40]. Higher expression of DNMT1 in tumor cells

could enhance hypermethylation of CDH gene, which plays a significant role in cell adhesion. Reduction of cell adhesion would offer tumor cells increased metastatic potential and lead to poor prognosis.

Clinical implication of present study was assessment of epigenetic methylation-related proteins like DNMT1 as a potential therapeutic target for cancer therapy. Endeavors to target DNMT1 in multiple cancers have been explored [41-44], and metastatic breast cancer would be the possible candidate for DNMT1 inhibition. Investigation of treatment response to DNMT1 targeted therapy in metastatic breast cancer is necessary considering the different expression levels of DNMT1 in specific metastasis sites and of which stroma.

In conclusion, methylation-related proteins revealed different expression levels in metastatic breast cancer based on the metastatic sites. 5-meC showed low expression in tumor and stroma of bone metastases, and tumoral DNMT1 showed reduced expression in bone metastases and overexpression in brain metastases.

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