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From Cell Biology to Tissue Engineering

Expression of DNA methylation-related proteins in invasive lobular carcinoma of breast: comparison to invasive ductal carcinoma

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Summary. Purpose: We aimed to compare the expression of DNA methylation-related proteins in invasive lobular carcinoma (ILC) of breast with those of invasive ductal carcinoma (IDC) of breast and to assess its potential clinical application.

Methods: Immunohistochemical staining of DNA methylation-related proteins (5-meC, DNMT1, DNMT3B and ISL-1) was applied to tissue microarrays generated from 108 ILCs and 203 IDCs. Protein expression and its correlation with clinicopatholgic variables were statistically analyzed.

Results: ISL-1 and DNMT3B were highly expressed in ILC ($p<0.001$) and tumoral 5-meC was highly expressed in IDC ($p=0.006$). DNMT1 ($p<0.001$) showed higher expression rate in luminal A type ILC. ISL-1 and DNMT3B showed higher expression rate in both luminal A type and luminal B type of ILC ($p<0.05$). In IDC, tumoral 5-meC commonly showed high positivity ($p=0.039$). On univariate analysis, shorter disease-free survival of ILC was associated with DNMT1 high positivity ($p=0.001$) and ISL-1 positivity ($p=0.018$).

Conclusion: DNA methylation-related proteins are differentially expressed in ILC and IDC, and DNMT1, DNMT3B and ISL-1 show high expression rate in ILC.

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Key words: Breast cancer, Lobular cancer, DNA methylation, DNMT1

Introduction

Breast cancer is the most common malignancy in females, and is composed of variable histologic subtypes which are roughly divided into invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC) (Tavassoli et al., 2003). ILC comprises approximately 5-15% of invasive carcinoma (Li et al., 2003, 2005), and recently the incidence has been rapidly increasing compared to IDC, derived from hormone replacement therapy and increased alcohol consumption (Reeves et al., 2006; Li et al., 2010). In contrast to IDC, ILC shows frequent multiplicity and bilateral presentation clinically (Lesser et al., 1982; Silverstein et al., 1994), and is composed of non-cohesive cancer cells histologically, which lack e-cadherin expression (De Leeuw et al., 1997). Common metastatic sites of ILC include bone, gastrointestinal tract, uterus, meninges, ovary and serosa that have a different metastatic pattern from IDC (Lamovec and Bracko, 1991; Silverstein et al., 1994; Sastre-Garau et al., 1996).

Cancer cells have insensitivity to growth inhibitory signals that is a result of inhibition of tumor suppressor genes, and DNA hypermethylation is one of the mechanisms of inhibition of tumor suppressor genes (Jones, 2002). DNA methyltransferases (DNMTs) enhance the DNA methylation (Siedlecki and Zielenkiewicz, 2006), encoded by *DNMT1, DNMT2, DNMT3A,* and *DNMT3B,* and *DNMT1* is the most

common and important in human, and plays a role as a key maintenance methyltransferase. 5-methylcytosine (5-meC) and insulin gene enhancer binding protein-1 (ISL-1) are molecules associated with DNMT1. 5-meC is the product of DNA methylation, as DNA methylation follows the binding of methyl group to 5' position of the cytosine ring, located in CpG dinucleotides and generation of 5-meC. ISL-1 appeared to be a direct target of DNMT1 in breast cancer (Pathania et al., 2015). Thus, it is expected that DNMT-1 expression induces DNA methylation and subsequent expression of 5-meC and ISL-1. Pioneering research regarding DNA methylation-related protein demonstrated that DNMT1 expression was dysregulated in breast cancer, and protein expression evaluated by immunohistochemistry was higher in IDC than in ILC (Agoston et al., 2005). The same group further identified that the retinoblastoma pathway may be the underlying cause of DNMT1 dysregulation (Agoston et al., 2007). Since ILC and IDC show clinical, histological, and molecular differences, it is expected that methylation status of both tumors would be also different from each other, but only few studies have been conducted on this issue (Fackler et al., 2003; Roessler et al., 2015; Schrijver et al., 2015; Ali et al., 2016). In the present study, we evaluated the expression of DNA methylation-related proteins in ILC, and aimed to find its implication.

Material and methods

Patient selection and clinicopathologic evaluation

This study was approved by the Institutional Review Board of Severance Hospital. Between January 2000 and December 2012, 108 patients received surgical resection for ILC in Severance Hospital. 203 IDC patients who received surgical resection in 2006 in Severance Hospital were included for comparison. Patients who received preoperative neoadjuvant chemotherapy were excluded. Hematoxylin and eosin (H&E)-stained slides of all cases were reviewed by breast pathologist (Koo JS) retrospectively. The histological grade was assessed using the Nottingham grading system (Elston and Ellis, 1991). Tumor staging was based on the 7th American Joint Committee on Cancer criteria. Disease-free survival (DFS) was calculated from the date of the first curative surgery to the date of the first loco-regional or systemic relapse, or death without any type of relapse. Overall survival (OS) was estimated from the date of the first curative operation to the date of the last follow-up or death from any cause. The following clinico-pathologic parameters were evaluated; age at initial diagnosis, lymph node metastasis, tumor recurrence, distant metastasis, and patient's survival.

Tissue microarray

The most appropriate tumor area was selected in H&E–stained slide and matched formalin-fixed paraffin-embedded (FFPE) tumor tissue sample was punched out from paraffin block. Every 2 tissue cores were extracted in each patient with a 3 mm punch, and made into a 6x5 tissue microarray.

Immunohistochemistry

The antibodies used for immunohistochemistry (IHC) in this study are shown in Table 1. IHC were applied on 3 μm-thickness tissue sections from FFPE tissue. After being deparaffinized and rehydrated with xylene and alcohol solution respectively, IHC was performed on Ventana Discovery XT automated stainer (Ventana Medical System, Tucson, AZ, USA). CC1 buffer (Cell Conditioning 1; citrate buffer Ph 6.0, Ventana Medical System) was used for antigen retrieval. Appropriate positive and negative controls were included.

Interpretation of immunohistochemical results

A cut-off value of 1% or more nuclear staining was considered for ER and PR positivity (Hammond et al., 2010). HER-2 staining was interpreted based on 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 (Wolff et al., 2007). Only strong (3+) HER-2 expression was considered positive. 0 and 1+ HER-2 staining were regarded as negative. Cases showing equivocal HER-2 expression (2+) were further evaluated for HER-2 gene amplification by fluorescent *in situ* hybridization (FISH).

IHC for 5-meC, DNMT1, and ISL-1 was assessed by light microscopy in a semiquantitative manner (Zhao et al., 2015). All stained areas of available tumor and stromal cells were scored as follows: 0, negative or weak immunostaining (<1% of the tumor/stroma); 1, focal expression (1-10% of tumor/stroma); 2, positive (11%-50% of tumor/stroma); 3, positive (51%-100% of

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
DNMT3B	Abcam, Cambridge, UK	Polyclonal	1:200
5-meC	Abcam, Cambridge, UK	33D3	1:200
ISL-1	Abcam, Cambridge, UK	Polyclonal	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

tumor/stroma). Score 0 was considered negative, and score 1 or more were considered positive. Positive scores were further divided into low (score 1 and 2) and high (score 3).

Tumor phenotype classification

Breast cancer phenotypes were subcategorized according to the IHC results of ER, PR, HER-2, and Ki-67 labeling indices (LI) and FISH results for HER-2 as follows (Goldhirsch et al., 2011): *luminal A type:* ER and/or PR positive, HER-2 negative, and Ki-67 LI <14%; *luminal B type:* (HER-2 negative) ER and/or PR positive, HER-2 negative, and Ki-67 LI≥14% and (HER-2 positive) ER and/or PR positive and HER-2 overexpressed and/or amplified; *HER-2 type:* ER and PR negative and HER-2 overexpressed and/or amplified; Triple negative brest cancer (*TNBC*) *type:* ER, PR, and HER-2 negative.

Table 2. Clinicopathologic characteristics of invasive lobular carcinoma.

Parameters	Total n=108 (%)	Classic type n=97 (%)	Pleomorphic type n=11 (%)	p - value
Age (years)				0.011
<50	60 (55.6)	58 (59.8)	2 (18.2)	
≥50	48 (44.4)	39 (40.2)	9 (81.8)	
Nuclear grade				<0.001
1/2	97 (89.8)	97 (100.0)	0 (0.0)	
3	11 (10.2)	0 (0.0)	11 (100.0)	
Histologic grade				<0.001
I/II	104 (96.3)	97 (100.0)	7 (63.6)	
III	4 (3.7)	0 (0.0)	4 (36.4)	
Pathologic T stage				0.048
T1	64 (59.3)	61 (62.9)	3 (27.3)	
T2/T3	44 (40.7)	36 (37.1)	8 (72.7)	
Lymph node metastasis				0.733
Absent	75 (69.4)	68 (70.1)	7 (63.6)	
Present	33 (30.6)	29 (29.9)	4 (36.4)	
ER				0.484
Negative	6 (5.6)	5 (5.2)	1 (9.1)	
Positive	102 (94.4)	92 (94.8)	10 (90.9)	
PR				0.018
Negative	18 (16.7)	13 (13.4)	5 (45.5)	
Positive	90 (83.3)	84 (86.6)	6 (54.5)	
HER-2				0.002
Negative	101 (93.5)	94 (96.9)	7 (63.6)	
Positive	7 (6.5)	3 (3.1)	4 (36.4)	
Ki-67 LI				0.001
≤14	88 (81.5)	84 (86.6)	4 (36.4)	
>14	20 (18.5)	13 (13.4)	7 (63.6)	
Molecular type				<0.001
Luminal A	82 (75.9)	79 (81.4)	3 (27.3)	
Luminal B	21 (19.4)	14 (14.4)	7 (63.6)	
HER-2	1 (0.9)	0 (0.0)	1 (9.1)	
TNBC	4 (3.7)	4 (4.1)	0 (0.0)	
Tumor recurrence	4 (3.7)	3 (3.1)	1 (9.1)	0.318
Patient deaths	5 (4.6)	2 (2.1)	3 (27.3)	0.007
Duration of clinical follow-up (months, mean ± SD)	72.4±29.3	72.5±29.5	71.3±28.8	0.898

LI, labeling indices; TNBC, triple negative breast cancer; SD, standard deviation.

Statistical analysis

Statistical analysis was carried out using SPSS for Windows version 12.0 (SPSS Inc., Chicago, IL). Student's t test and Fisher's exact test were used for continuous and categorical variables, respectively. 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

Basal characteristics of invasive lobular carcinoma and invasive ductal carcinoma

Clinicopathologic characteristics of 108 ILCs are shown in Table 2. In this study, 108 ILCs were composed of 97 (89.8%) classic types and 11 (10.2%) pleomorphic types. Pleomorphic type was characterized by older age ($p=0.011$), higher nuclear grade ($p<0.001$), higher histologic grade ($p<0.001$), higher T stage ($p=0.048$), PR negativity ($p=0.018$), HER-2 positivity ($p=0.002$), higher Ki-67 LI ($p=0.001$), non-luminal A subtype ($p<0.001$) compared to the classic type. Basal characteristics of 230 IDCs are shown in Table 3.

Expression of DNA methylation-related proteins in ILC according to the histologic type

In ILC, DNMT1 and ISL-1 were exclusively expressed in tumor cells and 5-meC was expressed in both tumor and stromal cells. Expression of DNA methylation-related proteins was not statistically different between classic type and pleomorphic type of ILC (Table 4).

Comparison of the expression of DNA methylation-related proteins between ILC and IDC

Tumoral DNMT3B, 5-meC and ISL-1 differed between ILC and IDC (Table 5, Fig. 1). ILC showed higher positive rate of tumoral DNMT3B and ISL-1 ($p<0.001$) whereas IDC showed higher positive rate of tumoral 5-meC ($p=0.006$). In terms of molecular subtypes, we analyzed the expression of DNA methylation-related proteins in luminal type since most ILC were luminal types (Table 6). In luminal A type, ILC showed higher expression rate of DNMT1 ($p<0.001$), DNMT3B ($p<0.001$) and ISL-1 ($p=0.002$) compared to IDC. In luminal B type, tumoral DNMT3B, and ISL-1 showed high expression rate in ILC ($p<0.001$,

Table 3. Clinicopathologic characteristics of invasive ductal carcinoma.

Parameters	Total (n=230) (%)	Luminal A (n=115) (%)	Luminal B (n=57) (%)	HER-2 (=15) (%)	TNBC (n=43) (%)	p -value
Age (years)						0.087
<50	131 (57.0)	66 (57.4)	39 (68.4)	7 (46.7)	19 (44.2)	
≥50	99 (43.0)	49 (42.6)	18 (31.6)	8 (53.3)	24 (55.8)	
Histologic grade						<0.001
I/II	163 (70.9)	105 (91.3)	36 (63.2)	8 (53.3)	14 (32.6)	
III	67 (29.1)	10 (8.7)	21 (36.8)	7 (46.7)	29 (67.4)	
Pathologic T stage						0.112
T1	146 (63.5)	80 (69.6)	35 (61.4)	10 (66.7)	21 (48.8)	
T2/T3	84 (36.5)	35 (30.4)	22 (38.6)	5 (33.3)	22 (51.2)	
Lymph node metastasis						0.555
Absent	147 (63.9)	69 (60.0)	37 (64.9)	10 (66.7)	31 (72.1)	
Present	83 (36.1)	46 (40.0)	20 (35.1)	5 (33.3)	12 (27.9)	
ER						<0.001
Negative	63 (7.4)	2 (1.7)	3 (5.3)	15 (100.0)	43 (100.0)	
Positive	167 (72.6)	113 (98.3)	54 (94.7)	0 (0.0)	0 (0.0)	
PR						<0.001
Negative	80 (34.8)	10 (8.7)	12 (21.1)	15 (100.0)	43 (100.0)	
Positive	150 (65.2)	105 (91.3)	45 (78.9)	0 (0.0)	0 (0.0)	
HER-2						<0.001
Negative	185 (80.4)	115 (100.0)	27 (47.4)	0 (0.0)	43 (100.0)	
Positive	45 (19.6)	0 (0.0)	30 (52.6)	15 (100.0)	0 (0.0)	
Ki-67 LI						<0.001
≤14	144 (62.6)	115 (100.0)	18 (31.6)	6 (40.0)	5 (11.6)	
>14	86 (37.4)	0 (0.0)	39 (68.4)	9 (60.0)	38 (88.4)	
Tumor recurrence	11 (4.8)	4 (3.5)	2 (3.5)	1 (6.7)	4 (9.3)	0.444
Patient deaths	18 (7.8)	6 (5.2)	3 (5.3)	2 (13.3)	7 (16.3)	0.090
Duration of clinical follow-up (months, mean ± SD)	23.8±10.6	60.2±8.7	58.2±9.3	54.8±15.1	55.0±14.0	0.036

LI, labeling indices; TNBC, triple negative breast cancer; SD, standard deviation.

Table 4. Expression of DNA methylation-related proteins in invasive lobular carcinoma according to the histologic type.

Parameters	Total n=108 (%)	Classic type n=97 (%)	Pleomorphic type n=11 (%)	p - value
DNMT1 (T)				0.319
Low	90 (83.3)	82 (84.5)	8 (72.7)	
High	18 (16.7)	15 (15.5)	3 (27.3)	
DNMT3B (T)				0.740
Low	16 (14.8)	14 (14.4)	2 (18.2)	
High	92 (85.2)	83 (85.6)	9 (81.8)	
5-meC (T)				0.165
Low	22 (20.4)	18 (18.6)	4 (36.4)	
High	86 (79.6)	79 (81.4)	7 (63.6)	
5-meC (S)				n/a
Negative	0 (0.0)	0 (0.0)	0 (0.0)	
Positive	108 (100.0)	97 (100.0)	11 (100.0)	
ISL-1 (T)				0.664
Negative	93 (86.1)	84 (86.6)	9 (81.8)	
Positive	15 (13.9)	13 (13.4)	2 (18.2)	

T, tumor cells; S, stromal cells; n/a, not applicable.

Table 5. Expression of DNA methylation-related proteins in invasive lobular carcinoma and invasive ductal carcinoma.

Parameters	Total n=338 (%)	ILC n=108 (%)	IDC n=230 (%)	p - value
DNMT1 (T)				0.080
Low	297 (87.9)	90 (83.3)	207 (90.0)	
High	41 (12.1)	18 (16.7)	23 (10.0)	
DNMT3B (T)				<0.001
Low	141 (41.7)	16 (14.8)	125 (54.3)	
High	197 (58.3)	92 (85.2)	105 (45.7)	
5-meC (T)				0.006
Low	44 (13.0)	22 (20.4)	22 (9.6)	
High	294 (87.0)	86 (79.6)	208 (90.4)	
5-meC (S)				0.090
Negative	6 (1.8)	0 (0.0)	6 (2.6)	
Positive	332 (98.2)	108 (100.0)	224 (97.4)	
ISL-1 (T)				<0.001
Negative	318 (94.1)	93 (86.1)	225 (97.8)	
Positive	20 (5.9)	15 (13.9)	5 (2.2)	

ILC, invasive lobular carcinoma; IDC, invasive ductal carcinoma; T, tumor cells; S, stromal cells.

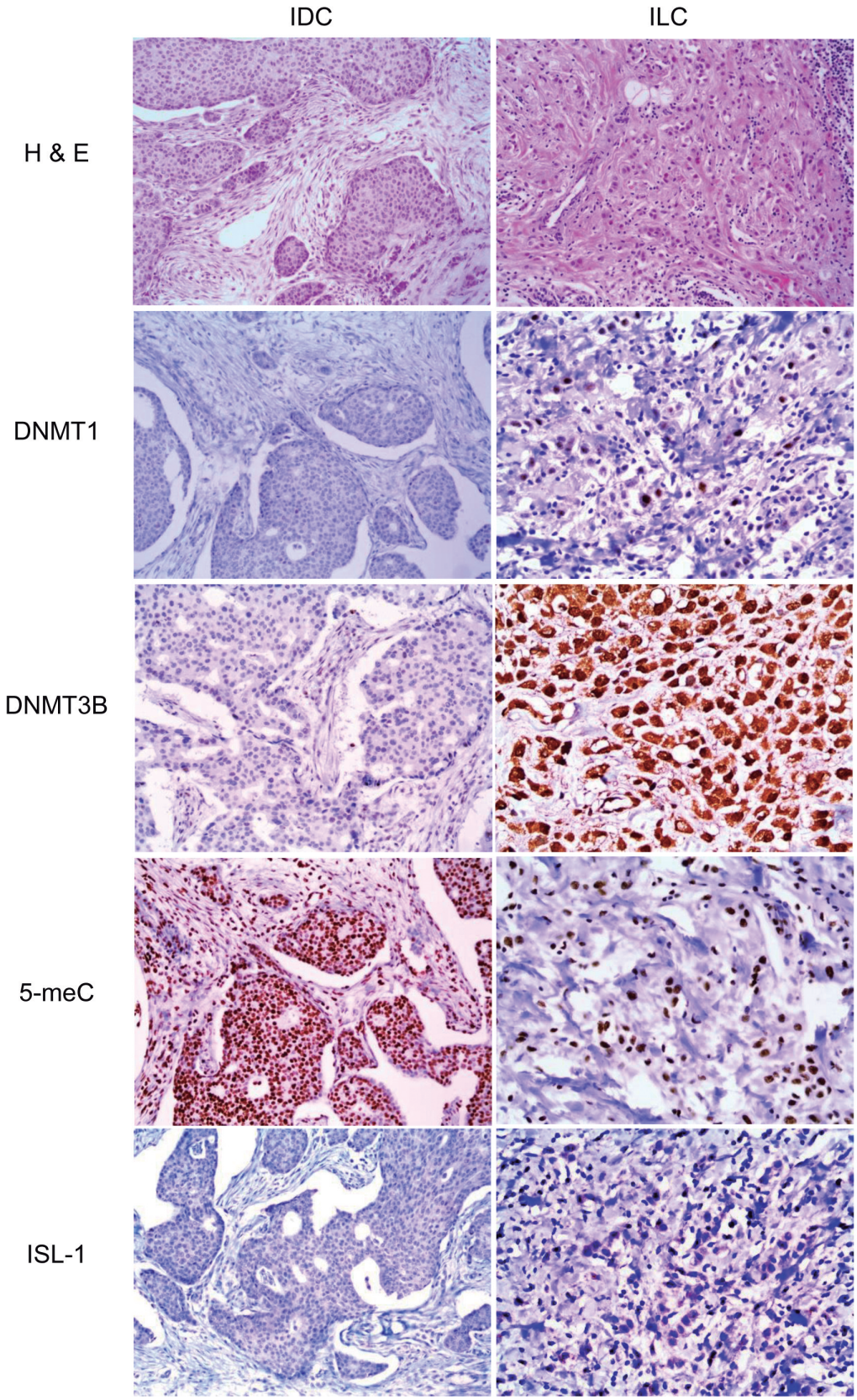

Fig. 1. The expression of DNA methylation-related proteins in invasive lobular carcinoma and invasive ductal carcinoma. DNMT1 and ISL-1 are highly expressed in invasive lobular carcinoma. In invasive ductal carcinoma, 5-meC is highly expressed in tumor cells. x 200

Table 6. Comparison of DNA methylation-related proteins in luminal type of invasive lobular carcinoma and invasive ductal carcinoma.

Parameters	Luminal A type			Luminal B type		
	ILC n=82 (%)	IDC n=115 (%)	p - value	IDC n=57 (%)	ILC n=21 (%)	p - value
DNMT1 (T)			<0.001			0.208
Low	70 (85.4)	114 (99.1)		52 (91.2)	17 (81.0)	
High	12 (14.6)	1 (0.9)		5 (9.8)	4 (19.0)	
DNMT3B (T)			<0.001			<0.001
Low	13 (15.9)	57 (49.6)		2 (9.5)	32 (56.1)	
High	69 (84.1)	58 (50.4)		19 (90.5)	25 (43.9)	
5-meC (T)			0.158			0.039
Low	16 (19.5)	14 (12.2)		4 (7.0)	5 (23.8)	
High	66 (80.5)	101 (87.8)		53 (93.0)	16 (76.2)	
5-meC (S)			0.088			0.541
Negative	0 (0.0)	4 (3.5)		1 (1.8)	0 (0.0)	
Positive	82 (100.0)	111 (96.5)		56 (98.2)	21 (100.0)	
ISL-1 (T)			0.002			0.018
Negative	70 (85.4)	112 (97.4)		57 (100.0)	19 (90.5)	
Positive	12 (14.6)	3 (2.6)		0 (0.0)	2 (9.5)	

ILC, invasive lobular carcinoma; IDC, invasive ductal carcinoma; T, tumor cells; S, stromal cells.

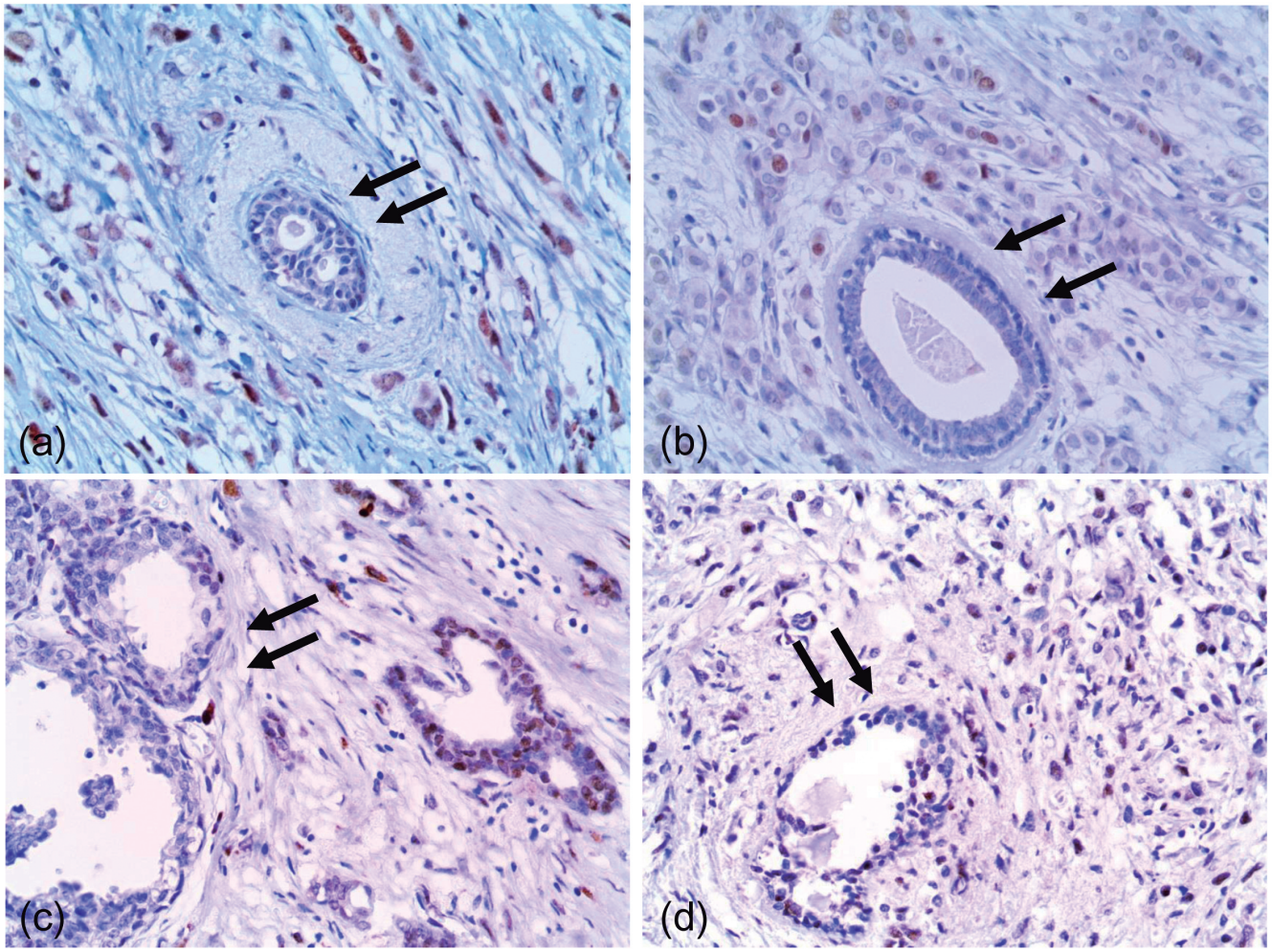

Fig. 2. Expression of DNMT1 and DNMT3B in normal breast tissue. DNMT1 (**a and b**) and DNMT3B (**c and d**) are weakly expressed or almost absent in normal ducts of invasive ductal carcinoma (**a and c**, arrows) and invasive lobular carcinoma (**b and d**, arrows), compared to the tumor cells. x 400

and p=0.018, respectively) and tumoral 5-meC showed high expression rate in IDC (p=0.039). Comparison of ILC and IDC of HER-2 and TNBC type revealed no statistically significant result, probably due to the small sample size of ILC (Table 7). Expression of DNMT1 and DNMT3B was weakly positive or negative in normal ducts and acini (Fig. 2).

Impact of expression status for DNA methylation-related proteins on prognosis in ILC

On univariate analysis, DNMT1 high positivity (p=0.001), and ISL-1 positivity (p=0.018) were associated with shorter DFS in ILC (Table 8, Fig. 3). However, no independent prognostic factor was

Table 7. Comparison of DNA methylation-related proteins in luminal type of invasive lobular carcinoma and invasive ductal carcinoma.

Parameters	HER-2 type			TNBC type		
	ILC n=1 (%)	IDC n=15 (%)	p - value	IDC n=43 (%)	ILC n=4 (%)	p - value
DNMT1 (T)			0.125			1.000
Low	0 (0.0)	14 (93.3)		27 (62.8)	3 (75.0)	
High	1 (100.0)	1 (6.7)		16 (37.2)	1 (25.0)	
DNMT3B (T)			1.000			0.041
Low	1 (100.0)	11 (73.3)		25 (58.1)	0 (0.0)	
High	0 (0.0)	4 (26.7)		18 (41.9)	4 (100.0)	
5-meC (T)			1.000			0.239
Low	0 (0.0)	2 (13.3)		2 (4.7)	1 (25.0)	
High	1 (100.0)	13 (86.7)		41 (95.3)	3 (75.0)	
5-meC (S)			n/a			1.000
Negative	0 (0.0)	0 (0.0)		1 (2.3)	0 (0.0)	
Positive	1 (100.0)	15 (100.0)		42 (97.7)	4 (100.0)	
ISL-1 (T)			n/a			0.239
Negative	1 (100.0)	15 (100.0)		41 (95.3)	3 (75.0)	
Positive	0 (0.0)	0 (0.0)		2 (4.7)	1 (25.0)	

TNBC, triple negative breast cancer; ILC, invasive lobular carcinoma; IDC, invasive ductal carcinoma; T, tumor cells; S, stromal cells; n/a, not applicable.

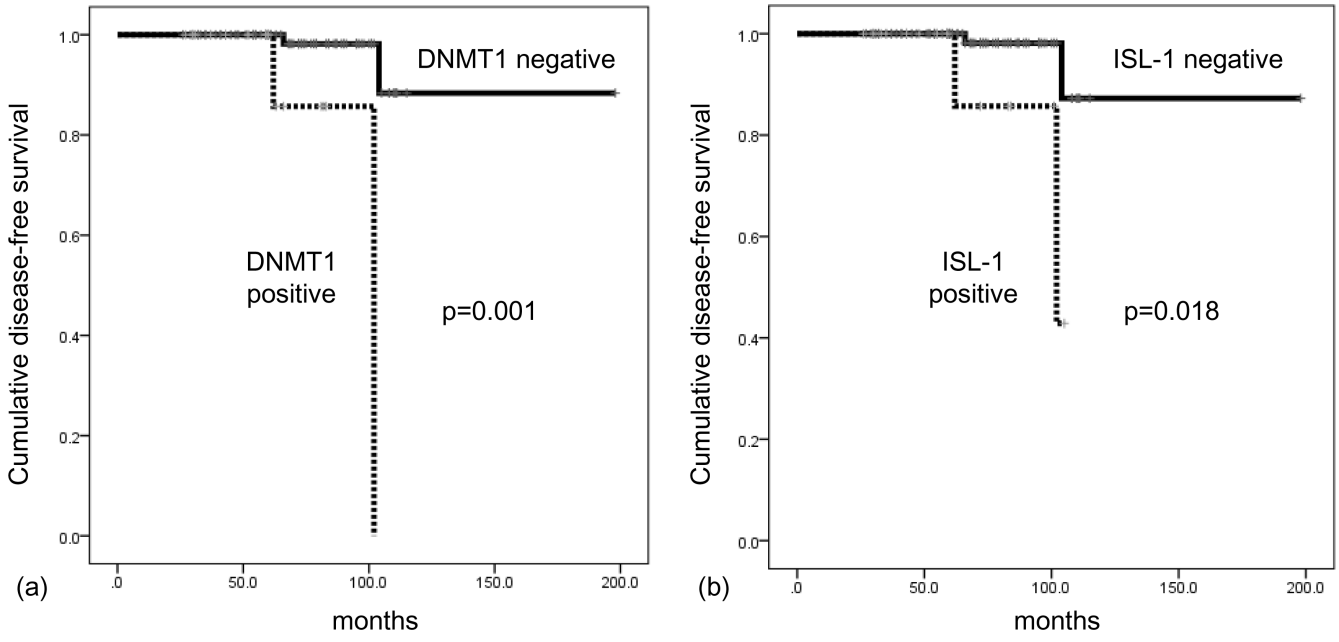

Fig. 3. Disease-free survival according to DNMT1 **(a)** and ISL-1 **(b)** in ILC.

identified on multivariate Cox analysis (Table 9).

Discussion

We evaluated expression of DNA methylation-related proteins of ILC and compared with those of IDC. We found that DNMT1, DNMT3B and ISL-1 were highly expressed in ILC, and 5-meC was highly expressed in IDC. Among the 5 cancer-related genes (*RASSF1A, HIN-1, RAR-beta, Cyclin D2,* and *Twist*), *RASSF1A, HIN-1, RAR-beta, Cyclin D2* had similar promoter methylation status between ILC and IDC, whereas *Twist* was hypermethylated in IDC. Given that result, methylation status of specific genes between ILC and IDC appeared to be different (Fackler et al., 2003). In the present study, DNMT1 was highly expressed in luminal A type ILC, while no difference was found when total ILC and IDC were analyzed. In previous studies, DNMT1 expression was higher in IDC compared to ILC (Agoston et al., 2005, 2007). However, previous studies did neither subcategorization nor specific comparison of luminal A type ILC and those of IDC, which might have led the discordant results between previous studies and present study. Considering higher expression of DNMT1 in TNBC/basal-like type of IDC (Shin et al., 2016; Zhang et al., 2016), luminal A type may have different expression pattern of DNMT1. In breast cancer, DNMT1 has been found to induce miR-152 silencing via promoter methylation, and resulted in loss of E-cadherin/CDH1 expression (Sengupta et al., 2016). Since E-cadherin/CDH1 expression is absent in ILC (De Leeuw et al., 1997), interaction between E-cadherin and DNMT1 in luminal A type ILC may be related to higher DNMT1 expression in ILC than that of IDC. We observed higher positive rate of DNMT3B in both ILC and IDC compared to DNMT1, which was in line with a

Table 9. Multivariate Cox proportional hazard analysis of disease free survival and overall survival in patients with invasive lobular carcinoma.

Variable	Disease free survival		Overall survival	
	HR (95% CI)	p - value	HR (95% CI)	p - value
Pathologic T stage		0.411		n/a
T1	Reference		Reference	
T2/3	7.110 (0.066-761.400)		n/a	
Lymph node metastasis		0.908		0.094
Absent	Reference		Reference	
Present	0.785 (0.013-47.760)		0.088 (0.005-1.510)	
ER		0.655		n/a
Negative	Reference		Reference	
Positive	0.401 (0.007-22.080)		n/a	
PR		0.148		n/a
Negative	Reference		Reference	
Positive	83.410 (0.208-33486.000)		n/a	
Ki-67 LI		0.178		0.341
≤14	Reference		Reference	
>14	12.55 (0.315-499.200)		0.368 (0.047-2.885)	
DNMT1 (T)		0.572		n/a
Low	Reference		Reference	
High	4.015 (0.033-495.700)		n/a	
ISL-1 (T)		0.092		0.098
Negative	Reference		Reference	
Positive	18.497 (0.623-549.400)		0.067 (0.003-1.641)	

HR, hazard ratio; CI, confidence interval; LI, labeling indices; T, tumor cells; S, stromal cells; n/a, not applicable.

Table 8. Univariate analysis by log-rank test of the impact of DNA methylation-related proteins expression in invasive lobular carcinoma on disease free survival and overall survival.

Variable	Invasive lobular carcinoma				Invasive breast cancer including ILC and IDC			
	Disease free survival		Overall survival		Disease free survival		Overall survival	
	Months (95% CI)	p - value	Months (95% CI)	p - value	Months (95% CI)	p - value	Months (95% CI)	p - value
DNMT1 (T)		0.001		n/a		0.182		0.738
Low	186 (168-204)		n/a		181 (163-198)		173 (156-190)	
High	96 (81-110)		n/a		97 (89-105)		116 (107-125)	
DNMT3B (T)		0.628		0.674		0.142		0.134
Low	109 (107-111)		110 (98-121)		104 (100-108)		104 (100-107)	
High	177 (149-205)		173 (152-194)		176 (148-204)		169 (144-194)	
5-meC (T)		0.189		n/a		0.862		0.129
Low	106 (97-115)		n/a		106 (97-115)		101 (91-110)	
High	184 (164-204)		n/a		180 (160-199)		187 (183-192)	
ISL-1 (T)		0.018		0.959		n/a		n/a
Negative	185 (165-204)		176 (159-194)		n/a		n/a	
Positive	97 (86-108)		124 (110-138)		n/a		n/a	
5-meC (S)		n/a		n/a		n/a		n/a
Negative	n/a		n/a		n/a		n/a	
Positive	n/a		n/a		n/a		n/a	

ILC, invasive lobular carcinoma; IDC, invasive ductal carcinoma; CI, confidence interval; T, tumor cells; S, stromal cells; n/a, not applicable.

previous study that showed higher DNMT3B mRNA expression than DNMT1 in breast cancer (Girault et al., 2003). Higher expression rate of DNMT3B in ILC than IDC could be derived from MUC1-C oncoprotein. MUC1-C oncoprotein has been reported to induce the overexpression of DNMT1 and DNMT3B in human breast cancer cells (Rajabi et al., 2016). In addition, approximately 77% of e-cadherin loss in ILC occurred through the methylation of the CDH1 promoter (Droufakou et al., 2001), which could be induced by MUC1-C (Rajabi et al., 2016; Tagde et al., 2016).

In the present study, ISL-1 expression was higher in ILC. ISL-1 is a member of the LIM-homeodomain family, which plays an important role in development of heart, neuron, and pancreas (Bu et al., 2009; Guo et al., 2011; Roy et al., 2012; Wilfinger et al., 2013), and was also found to be involved in carcinogenesis of variable tumors. High expression of ISL-1 has been reported in pancreatic endocrine tumor (Schmitt et al., 2008), lung cancer (Watanabe et al., 2010), breast cancer (Ronneberg et al., 2011), choloangiocarcinoma (Hansel et al., 2003), and malignant lymphoma (Zhang et al., 2014). Aberrant ISL-1 expression is induced by p-STAT3/p-c-JUN/ISL-1 transcription complex, and then upregulates c-myc expression that promotes tumor cell proliferation (Zhang et al., 2014), which needs further evaluation in ILC.

DNA methylation-related proteins in classic type and pleomorphic type ILC did not differ in the present study. Pleomorphic type is an aggressive ILC variant, characterized by adverse biomarker profile such as hormone receptor negative, HER-2 positive, and high Ki-67 LI (Frolik et al., 2001; Jacobs et al., 2012), which reveals different tumor biology from classic type ILC. Pleomorphic type ILC has a similar methylation pattern to IDC by hierarchical cluster analysis rather than classic type ILC (Moelans et al., 2015), which is discordant with the present study. However, molecular analysis studies have demonstrated that both pleomorphic and classic type ILC shared the same genetic pathway and similar genetic profiles (Reis-Filho et al., 2005; Simpson et al., 2008; Vargas et al., 2009). It is an important limitation of the present study that the number of pleomorphic type ILC was too small to perform a proper comparison with classic type. This limitation resulted from lower prevalence of pleomorphic type ILC itself, and cases collected in a single institution. Further studies are required to clarify the methylation status of classic and pleomorphic type ILC.

In the present study, positivity of DNMT1 and ISL-1 were associated with shorter DFS by univariate analysis although multivariate analysis revealed no significance, which is partly compatible with previous results: High DNMT1 expression has been related with poor prognosis in breast cancer (Shin et al., 2016), malignant lymphoma (Zhao et al., 2015), renal cell carcinoma (Li et al., 2014), pancreatic cancer (Zhang et al., 2012), and bladder cancer (Wu et al., 2011). In gastric cancer, ISL-1 positivity was associated with poor prognosis (Guo et al., 2015).

Clinically, DNA methylation-related proteins can be potential targets for targeted cancer therapy. Recently, DNMT1 inhibition has been applied for variable tumors and demonstrated reduction of tumor (Mutze et al., 2011; Amato et al., 2012; Subramaniam et al., 2014; Thottassery et al., 2014), thus DNMT1 may be a therapeutic target in ILC. In conclusion, ILC has a different expression profile of DNA methylation-related proteins from IDC, and has higher expression rate of DNMT1 and ISL-1.

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Conflict of Interest. The authors declare that they have no conflict of interest.

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