Overexpression of Derlin 3 is associated with malignant phenotype of breast cancer cells

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Abstract. Breast cancer (BC) is the most common malignant tumor among women worldwide. Development of novel molecular targets is important to improve prognosis of BC patients. *Derlin 3 (DERL3)* gene is a member of derlin family, and its coding protein is critical to the endoplasmic reticulum-associated degradation mechanism. However, its oncological role in breast cancer remains unclear. This study evaluated *DERL3* expression and function in BC. We analyzed *DERL3* mRNA in 13 BC and two non-cancerous cell lines, and explored effects of *DERL3* knockdown on BC proliferation, invasion and migration. We also evaluated correlation of *DERL3* mRNA expression levels with clinicopathological factors and prognosis in 167 BC patients. *DERL3* mRNA expression was detected in five (38%) BC cell lines. Inhibiting *DERL3* expression significantly decreased proliferation and invasion in BC cells. Specimens from patients with lymph node metastasis had higher *DERL3* mRNA expression than those without (P=0.030). Patients in the highest quartile for *DERL3* mRNA expression (n=42) were more likely to experience shorter overall survival than other patients (P=0.032). These findings indicate that *DERL3* promotes malignant phenotype in BC cells. *DERL3* may serve as a potential prognostic marker and therapeutic target for BC.

Introduction

Breast cancer (BC) is the most common malignant tumor and the highest cause of cancer-related deaths in women

world-wide (1). Because of the development of adjuvant medication therapy such as chemotherapy, endocrine therapy and anti-human epidermal growth factor 2 (HER2) therapy, prognosis of BC patients has improved (2). The 5-, 10- and 15-year relative survival rates are 89, 83 and 78%, respectively (3). However, recurrent BC after resection is still difficult to cure (4). Therefore, development of novel molecular targets is still required. Several commercial multigene expression assays are available (5). Although they can help evaluate prognosis and appropriateness of adjuvant chemotherapy, additional informative biomarkers may improve their accuracy.

Derlin3 (DERL3) gene is located at 22q11.23; its protein locates in the endoplasmic reticulum (EnRt). It belongs to the Derlin family (*DERL1, DERL2* and *DERL3*), which mediates endoplasmic reticulum-associated degradation (ERAD) of misfolded proteins, one of the EnRt stress responses (6-9). Reportedly, EnRt stress responses contribute to cancer progression (10,11). Although *DERL1* overexpression is reported in BC (12), colon cancer (13) and non-small cell lung cancer (NSCLC) (14), no studies have addressed the role of *DERL3* in BC. Referring to EnRt stress responses in BC, previous studies demonstrate that *GRP78*, an EnRt chaperone (15), is overexpressed in high-grade BC (16) and *XBP-1*, a UPR-related transcription factor, is overexpressed in BC (17). The relationship between *DERL3* and these molecules remains elusive.

In searching for a new therapeutic target that is overexpressed in BC, we chose *DERL3* as a candidate. This study therefore investigated *DERL3* expression and functions to determine whether *DERL3* is a potential biomarker and therapeutic target for BC.

Materials and methods

Sample collection. We obtained 13 human BC cell lines (BT-20, BT-474, BT-549, HCC1419, HCC1954, Hs578T, MCF7, MDA-MB-231, MDA-MB-361, MDA-MB-415, MDA-MB-468, SK-BR-3, and ZR-75-1) and two non-cancerous breast epithelial cell lines (MCF-10A, and MCF-12A). BT-549, HCC1419, HCC1954, and Hs578T were purchased from Japanese Collection of Research Bioresources

Cell Bank (Osaka, Japan), BT-474, MCF-7, and MCF-12A were kindly provided by Dr David Sidransky, the Director of the Department of Otolaryngology-Head and Neck Surgery of Johns Hopkins University (Baltimore, MD, USA), and others were from the American Type Culture Collection (Manassas, VA, USA). Cells were stored at -80°C using cell-preservative solution (Cell Banker; Mitsubishi Chemical Medience Corporation, Tokyo, Japan) and cultured in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS), in an atmosphere containing 5% CO_2 at 37°C (18,19).

We acquired 167 primary BC specimens from patients who underwent breast surgery at Nagoya University Hospital between March 2002 and November 2009. All specimens were diagnosed histologically as BC, frozen immediately after resection, and stored at -80°C. Adjacent non-cancerous specimens were resected >3 cm from the tumor edges. BC specimens were classified histologically using the Union for International Cancer Control (UICC) staging system for BC (7th edition). Selected patients received adjuvant chemotherapy, endocrine therapy and anti-HER2 therapy, according to their conditions, pathological factors and physicians' discretion. Patient follow-up data and clinicopathological parameters were collected from medical records.

This study conforms to the ethical guidelines of the Declaration of Helsinki. Enrollees granted written informed consent for use of clinical samples and data, as required by our institutional review board.

Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR). *DERL3* mRNA expression was determined by qRT-PCR. We extracted RNA from cell lines ($8.0x10^6$ cells per each cell line), 167 primary BCs, and adjacent normal tissues using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. cDNA was synthesized from total RNAs (1 μg) by M-MLV Reverse Transcriptase (Invitrogen, Frederick, MD, USA) and Primer 'random' (Sigma-Aldrich). The qRT-PCR of *DERL3*, *GRP78* and *XBP1* was performed using the SYBR Green PCR Core Reagents kit (Applied Biosystems) as follows: 1 cycle of 95°C (10 min); 40 cycles of 95°C (5 sec), and 60°C (60 sec). The primers specific for each gene were as follows: *DERL3*: forward 5'-CTCACTTTCCAGGCACCGT-3'and reverse 5'-TAGTAGATATGGCCCACCGC-3', which generated a 110-bp product; *GRP78*: forward 5'-GACATCAAGTTCTTG CCGTT-3' and reverse 5'-CTCATAACATTTAGGCCAGC-3', which generated a 260-bp product (20); and *XBP1*: forward 5'-CAGACTACGTGCGCCTCTGC-3' and reverse 5'-CTTC TGGGTAGACCTCTGGG-3', which generated a 208-bp product (21). Glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* mRNA levels were quantified to normalize expression levels. Each sample was tested in triplicate; data are shown as (*DERL3* value)/(GAPDH value) (22,23).

Inhibiting DERL3 expression by DERL3-specific small interfering RNAs (siRNAs). Four kinds of siRNAs specific for *DERL3* (si*DERL3*) were used to transfect BT-549 cells. Their sequences were si*DERL3*-1: 5'-GAUUCAGCUUCUUCU UCAATT-3'; si*DERL3*-2: 5'-UUGAAGAAGAAGCUGAAU CCCAGGGTT-3' (from a previous study) (24); si*DERL3*-3:

5'-UUGAAGUAGAGUUGAAAGGGG-3'; and si*DERL3*-4: 5'-UGAAGAAGAAGCUGAAUCCCA-3' (Hokkaido System Science, Sapporo, Japan). AccuTarget Negative Control siRNA Fluorescein-labeled (siControl; Cosmo Bio Co. Ltd., Tokyo, Japan) served as control nontargeting siRNA. The BC cells were seeded into 10-cm dishes with 10 ml of antibiotic-free RPMI-1640 with 10% FBS, and transfected with 400 pmol of siControl or si*DERL3*s in the presence of 40-μl LipoTrust EX Oligo (Hokkaido System Science) 24 h after seeding. After transfection, cells were cultured in antibiotic-free RPMI-1640 with 10% FBS for 72 h.

Western blotting. Cells were incubated in RIPA lysis buffer; lysates were stored at -30°C. Total protein lysates (20 μg/well) were electrophoretically transferred onto polyvinylidene difluoride membranes that were blocked using 5% skim milk in 0.05% PBS-T, and incubated at 4°C overnight with rabbit anti-*DERL3* polyclonal antibody (1:500; Abcam, Cambridge, UK). The membrane was then washed and probed with an anti-rabbit secondary antibody conjugated to horseradish peroxidase (Cell Signaling Technology, Beverly, MA, USA). β-actin served as endogenous control (25).

Proliferation assay. Proliferation was evaluated using Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). After transfection with si*DERL3* (mixture of si*DERL3*-1, -2, -3 and -4), BT-549 cells ($5x10^3$/well) were seeded into 96-well plates with RPMI-1640 containing 2% FBS. Optical density of each well was measured 5 days after seeding, 2 h after adding 10 μl of CCK-8 solution.

Invasiveness assay. Invasiveness in Matrigel was determined using BioCoat Matrigel Invasion Chambers (Corning Inc., Corning, NY, USA) according to manufacturer's protocol. After transfection with mixed si*DERL3*s, BT-549 cells ($2.5x10^4$/well) were suspended in serum-free RPMI-1640 and seeded in upper chambers. After 60 h of incubation, cells on membrane surfaces were fixed, stained, and counted in ten randomly selected microscope fields.

Migration assay. Migration of BT-549 cells was determined using a wound-healing assay. After transfection with mixed si*DERL3*s, BT-549 cells ($3x10^4$/well) were seeded into each well of a 35-mm dish with culture insert (Ibidi, Martinsried, Germany) in RPMI-1640 containing 2% FBS. After 24 h, the insert was removed, and wound widths were measured at 100-μm intervals (20 measurements/well, x40 magnification) at cell-dependent time intervals.

Statistical analysis. Differences in *DERL3* mRNA expression between two groups were evaluated using Mann-Whitney test. Correlations between *DERL3* mRNA levels and those of *GRP78* and *XBP1* were analyzed using the Spearman's rank correlation test. Significance of associations between *DERL3* mRNA expression and clinicopathological parameters was analyzed with χ^2 test. Overall survival (OS) and disease-free survival (DFS) were calculated using Kaplan-Meier method; differences in survival curves were evaluated by log-rank tests. Multivariate regression analysis used Cox proportional hazards model to identify prognostic factors; variables for
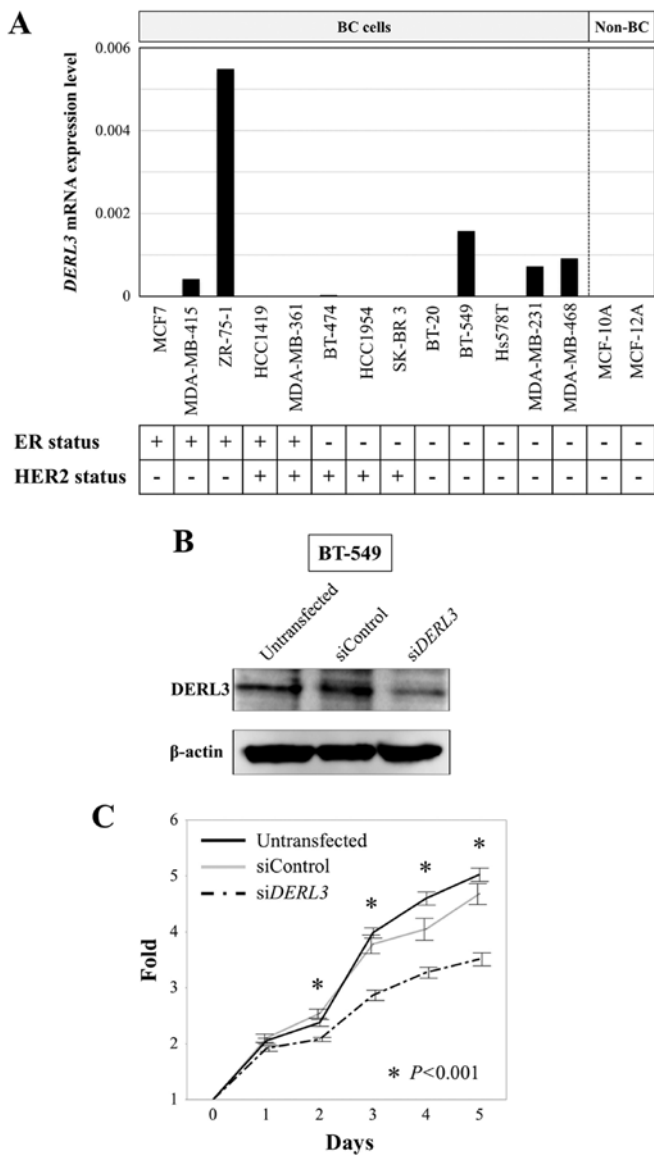
A

B

BT-549

C

Figure 1. (A) *DERL3* mRNA expression in breast cancer and non-cancerous cells. *DERL3* mRNA expression was detectable in MDA-MB-415, ZR-75-1, BT-549, MDA-MB-231, and MDA-MB-468 cells, but not in other tested cell lines. BC cells, breast cancer cell lines. Non-BC, non-cancerous cell lines. (B) Western blotting confirmed inhibition of si*DERL3*s in transfected cells. (C) si*DERL3*-transfected BT-549 cells showed significantly lower proliferation after 2 days than untransfected and control cells. *P<0.001.

which P<0.05 were entered into the final model. All statistical analyses were performed on JMP 12 software (SAS Institute Inc., Cary, NC, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

DERL3 mRNA expression in BC and non-cancerous cell lines. *DERL3* mRNA expression was detectable in five BC cell lines (38%), but not in other BC cell lines or non-cancerous cell lines (Fig. 1A). In BC cell lines, *DERL3* mRNA expression did not differ between estrogen receptor (ER) positive and ER negative, HER2 positive and HER2 negative, or triple-negative and other subtypes. ER and HER2 status of the cell lines were evaluated in previous studies (26,27).

Table I. Associations between *DERL3* mRNA expression and clinicopathological characteristics of 167 patients with breast cancer.

Clinicopathological parameter	High *DERL3* group (n=42)	Others (n=125)	P-value[a]
Age			0.065
≤60 year	32	76	
>60 year	10	49	
Histology			0.265
DCIS	0	7	
IDC	39	109	
ILC	2	4	
Others	1	5	
UICC T factor			0.207
Tis	0	7	
T1	18	52	
T2	19	56	
T3	4	5	
T4	1	5	
Node status			0.054
Negative	16	69	
Positive	26	56	
UICC pathological stage			0.211
0/I/II	30	101	
III/IV	12	24	
ER status			0.424
Positive	30	97	
Negative	12	28	
PgR status			0.062
Positive	24	91	
Negative	18	34	
HER2 status			0.792
Positive	11	28	
Negative	31	88	
Unknown	0	9	
Triple-negative			0.800
True	5	13	
False	37	111	
Unknown	0	1	
Adjuvant therapy			0.460
Endocrine therapy alone	13	44	
Chemotherapy alone	11	19	
Endocrine and chemotherapy	15	49	
None	3	13	

[a]χ^2 test. DCIS, ductal carcinoma *in situ*; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; UICC, Union for International Cancer Control; Tis, carcinoma in situ; ER, estrogen receptor; PgR, progesterone receptor; HER2, human epidermal growth factor 2.
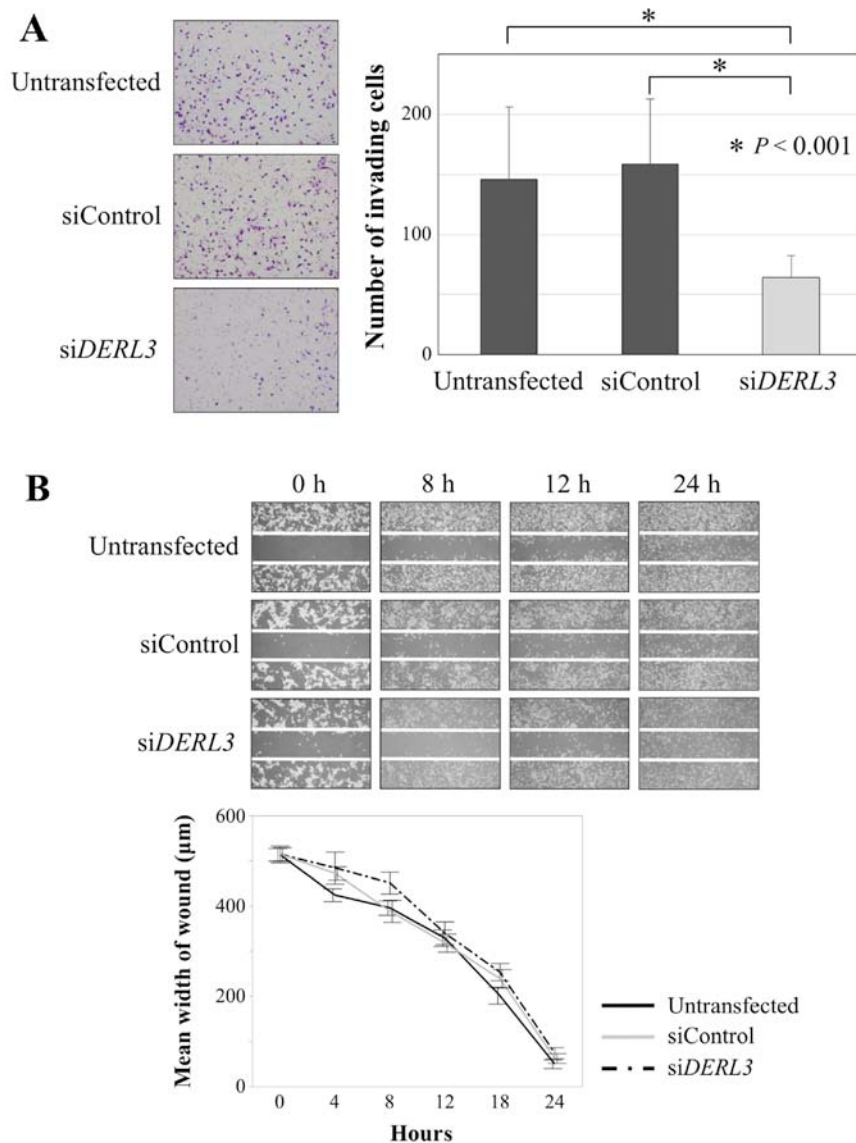
Figure 2. (A) Invasiveness assay: inhibiting *DERL3* expression in BT-549 cells significantly decreased numbers of invading cells. *P<0.001. (B) Migration assay: there were no significant differences.

Expression analysis of DERL3 and genes encoding putative functional partners in BC cell lines. We evaluated the correlation between *DERL3* mRNA expression levels and the genes *GRP78* and *XBP1* that could potentially functionally interact with *DERL3*. There were no correlations between *DERL3* and *GRP78* mRNA expression levels (correlation coefficient 0.253, P=0.405). Although it was not statistically significant, we found a weak correlation between *DERL3* and *XBP1* mRNA expression levels (correlation coefficient 0.335, P=0.263).

Effects of DERL3 inhibition on BC cell phenotypes. BT-549 cell line was transfected with si*DERL3*s to determine *DERL3* functions in BC. Western blot analysis confirmed that *DERL3* was decreased by the transfections (Fig. 1B). We evaluated proliferation, invasion, and migration. *DERL3* inhibition significantly decreased proliferation over days 2-5 compared with the untransfected and siControl cells (P<0.001; Fig. 1C). In the invasion assay, significantly fewer *DERL3*-inhibited cells invaded the Matrigel than did untransfected and siControl

cells (P<0.001; Fig. 2A). In contrast, there was no difference in the migration assay (Fig. 2B).

Patient characteristics. All 167 patients were women, whose mean age was 54.4±11.6 years (range: 26-78 years); and whose disease stages were stage 0: 7; stage I: 47; stage II: 78; stage III: 34; and stage IV: 1. Their median follow-up period was 100.0 months (range: 8-155 months) or until death.

DERL3 mRNA expression levels did not differ significantly between BC tissues and adjacent non-cancerous tissues (P=0.125). *DERL3* mRNA expression levels were higher in BC tissues than in adjacent non-cancerous tissues for 94 (56.3%) of the 167 patients. Of note, *DERL3* mRNA expression levels for several BC specimens were as low as their non-cancerous counterparts, whereas some BC specimens did express high *DERL3* mRNA levels.

Clinical and prognostic significance of DERL3 mRNA expression in BC specimens. Among the 167 patients, we found
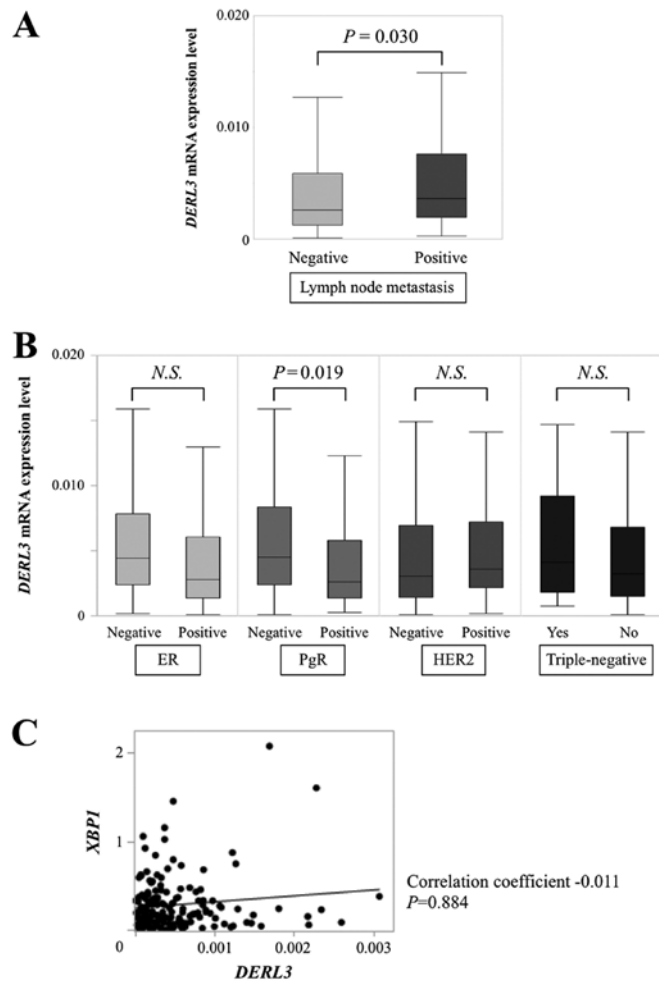
Figure 3. *DERL3* mRNA expression was significantly higher (A) in LN metastasis-positive specimens than in LN metastasis-negative specimens; and (B) in PgR-negative specimens than in PgR-positive specimens. (C) Correlations of mRNA expression levels between *DERL3* and *XBP1* in BC specimens.
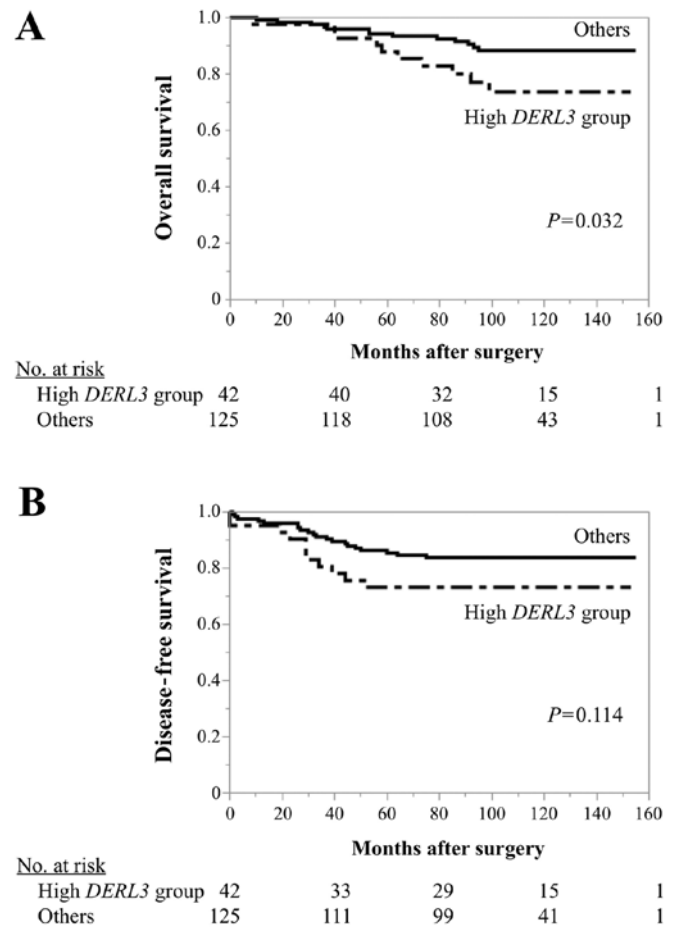

Figure 4. (A) Patients of high *DERL3* group experienced significantly shorter overall survival than others. (B) High *DERL3* group tended to have shorter disease-free survival than others, although the difference was not statistically significant.

DERL3 mRNA expression to be significantly higher in those with lymph node (LN) metastasis (n=82) than in those without LN metastasis (n=85; P=0.030; Fig. 3A). *DERL3* expression did not differ with regard to T categories or UICC stage. Among conventional biomarkers (Fig. 3B), *DERL3* mRNA expression in progesterone receptor (PgR)-negative specimens (n=52) was significantly higher than in PgR-positive specimens (n=115; P=0.019). There were not significant differences between ER-positive (n=127) and -negative (n=40; P=0.070), HER2-positive (n=39) and -negative (n=119; P=0.577; missing data for 9 patients), or triple-negative (n=18) and non-triple-negative specimens (n=148; P=0.467; missing data for 1 patient). We evaluated the correlation between *DERL3* mRNA levels and *XBP1* mRNA levels in the patients' BC specimens. There were no correlations between mRNA expression levels of these genes (correlation coefficient -0.011, P=0.884; Fig. 3C).

The patients with the highest quartile of *DERL3* expression were designated as 'high *DERL3* group' (n=42), and the remaining patients were designated as 'others' (n=125). We found no significant differences with regard to any tested clinicopathological characteristics (Table I). The high *DERL3* group experienced significantly shorter OS than others (5-year

OS, high *DERL3* group: 87.9%; others: 94.3%; P=0.032; Fig. 4A). Moreover, high *DERL3* group tended to have poorer DFS than others, although there were no statistically significant differences (5-year DFS, high *DERL3* group: 73.3%; others: 85.4%; P=0.114; Fig. 4B).

Multivariate analysis of OS identified UICC pathological stage III/IV disease (HR: 6.43; 95% CI: 2.26-20.3, P<0.001) as an independent prognostic factor, but not high *DERL3* expression (HR: 2.27; 95% CI: 0.92-5.47, P=0.074; Table II).

Discussion

In this study, we showed that *DERL3* promotes BC cell proliferation and invasiveness, and that high *DERL3* patients experienced poorer prognosis.

When cells undergo stress through hypoxia and nutrient deprivation, misfolded proteins increase, and activate the unfolded protein response (UPR) (11,28), which upregulates ERAD, and contributes to progression in cancer cells (10,11). Reportedly, *GRP78*, an EnRt chaperone (15), is overexpressed in high-grade BC (16). Furthermore, *XBP-1*, a UPR-related transcription factor, is overexpressed in BC, but is hardly detectable in non-cancerous breast tissues (17). These findings suggest that UPR promotes a malignant BC phenotype.

Table II. Prognostic factors for overall survival in 167 patients with breast cancer.

Variables	n	Univariate			Multivariate		
		Hazard ratio	95% CI	P-value	Hazard ratio	95% CI	P-value
Age (≤60)	108	1.17	0.50-3.03	0.733			
UICC pathological stage (III/IV)	36	7.65	3.35-18.4	<0.001	6.43	2.26-20.3	<0.001[a]
ER status (negative)	40	2.43	1.01-5.54	0.047	2.34	0.52-17.5	0.279
PgR status (negative)	52	2.32	1.01-5.30	0.048	0.95	0.14-3.74	0.951
HER2 status (positive)	39	2.76	1.16-6.40	0.022	1.14	0.38-3.32	0.815
Triple-negative (yes)	18	2.23	0.65-5.95	0.183			
Adjuvant chemotherapy (yes)	94	3.11	1.24-9.43	0.002	0.82	0.23-2.99	0.751
High *DERL3* expression	42	2.40	1.02-5.45	0.044	2.27	0.92-5.47	0.074

[a]P<0.05. Univariate analysis: log-rank test. Multivariate analysis: Cox proportional hazards model. CI, confidence interval; UICC, Union for International Cancer Control; ER, estrogen receptor; PgR, progesterone receptor; HER2, human epidermal growth factor 2.

DERL1 is the most widely studied derlin (6), and regulated by the IRE-XBP1 pathway (8,29). Its overexpression reportedly prevents induction of apoptosis in stressed BC cells and correlates with advanced pathological tumor grade and node-positive status in BC (12). It also contributes to malignant phenotype in colon cancer and in NSCLC by activating the PI3K/AKT and EGRF-EPK pathways, respectively (13,14).

There have been only a few studies on *DERL3*. Although *DERL3* is assumed to be regulated by IRE1-XBP1 pathway such as *DERL1* (8), no significant correlations were found between *DERL3* and *XBP1* mRNA expression levels. There might be another pathway that regulates the expression of *DERL3* in BC cells. According to previous studies, *DERL3* has a protective function for cardiomyocytes through the ERAD mechanism (30), and low *DERL3* expression has been associated with a more malignant phenotype and poorer prognosis in colorectal cancer (31). We have shown that *DERL3* mRNA is scarcely expressed not only in non-cancerous breast cell lines but also in several BC cell lines along with a considerable proportion of BC specimens. These findings suggest that both non-cancerous breast cells and BC cells express little *DERL3* mRNA under slight EnRt stress.

DERL3 inhibition significantly decreased BC cell proliferation and invasion, and patients with high *DERL3* group tended to be LN metastasis-positive (P=0.054; Table I). These results indicate that *DERL3* does promote malignant phenotype when overexpressed in BC. Noteworthy, *DERL1* mRNA overexpression also correlates with positive LN metastasis in BC, colon cancer and NSCLC by the inhibition of apoptosis and the activation of PI3K/AKT, EGRF-EPK pathways, respectively (12-14). *DERL3* might regulate such mechanisms in BC, and it promotes LN metastasis, leading to more malignant phenotype. Further studies are required to determine the underlying mechanisms. We found that the high *DERL3* group experienced shorter OS, independently of ER, PgR and HER2 status, which implies its potential as a prognostic biomarker for all BC subtypes.

These findings have several possible clinical applications. *DERL3* levels in resected samples can help predict prognosis. Patients with high *DERL3* specimens may require more aggressive adjuvant therapy; and if *DERL3* mRNA expression levels can be determined from pre-surgical biopsies, they might help indicate whether neoadjuvant therapy is appropriate. Although further studies are warranted, our study suggests the possibility of developing new therapies for BC that target *DERL3*.

This study has some limitations. First, the roles of *DERL3* in the ERAD pathway of cancer cells, and of ERAD in BC, are unclear. Pathway analyses might elucidate the role of *DERL3* in UPR. Second, interventions such as adjuvant medication might have affected relationships between patient *DERL3* mRNA levels and prognoses. Finally, these results were obtained from *in vitro* data and should be verified by *in vivo* studies.

In conclusion, we found *DERL3* to promote a malignant BC phenotype. High *DERL3* mRNA expression in the BC tissue resulted in poor prognosis. *DERL3* mRNA expression is a potential prognostic marker and *DERL3* protein could be a candidate therapeutic target for BC.

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