Received: 1 May 2018 | Revised: 24 July 2018 | Accepted: 8 August 2018

DOI: 10.1111/cas.13770

ORIGINAL ARTICLE

WILEY Cancer Science

OLFM4, LY6D and S100A7 as potent markers for distant metastasis in estrogen receptor-positive breast carcinoma

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Funding infromation
This work was partly supported by Grant-in-Aid for Scientific Research (25460410 and 26860229) from Japanese Ministry of Education, Culture, Sports, Science and Technology.

Metastatic breast cancer is a highly lethal disease, and it is very important to evaluate the biomarkers associated with distant metastasis. However, molecular features of distant metastasis remain largely unknown in breast cancer. Estrogens play an important role in the progression of breast cancer and the majority of stage IV breast carcinomas express estrogen receptor (ER). Therefore, in this study, we examined molecular markers associated with distant metastasis in ER-positive breast carcinoma by microarray and immunohistochemistry. When we examined the gene expression profile of ER-positive stage IV breast carcinoma tissues (n = 7) comparing ER-positive stage I-III cases (n = 11) by microarray analysis, we newly identified OLFM4, LY6D and S100A7, which were closely associated with the distant metastasis. Subsequently, we performed immunohistochemistry for OLFM4, LY6D and S100A7 in 168 ER-positive breast carcinomas. OLFM4, LY6D and S100A7 immunoreactivities were significantly associated with stage, pathological T factor, distant metastasis and Ki67 status in the ER-positive breast carcinomas. Moreover, these immunoreactivities were significantly associated with a worse prognostic factor for distant metastasis-free and breast cancer-specific survival in ER-positive stage I-III breast cancer patients. However, when we performed immunohistochemistry for OLFM4, LY6D and S100A7 in 40 ER-negative breast carcinomas, these immunoreactivities were not generally associated with the clinicopathological factors examined, including distant metastasis and prognosis of patients, in this study. These results suggest that OLFM4, LY6D and S100A7 immunoreactivity are associated with an aggressive phenotype of ER-positive breast carcinoma, and these are potent markers for distant metastasis of ER-positive breast cancer patients.

KEYWORDS
(10-3) Metastasis-associated gene < (10) Invasion and metastasis, (10-5) Diagnosis of metastasis < (10) Invasion and metastasis, (13-3) Hormones < (13) Growth factors/cytokines/hormones, (14-4) Mammary gland < (14) Characteristics and pathology of human cancer, (15-3) Diagnosis by tumor markers and biomarkers < (15) Diagnosis

1 | INTRODUCTION

Breast cancer is one of the most common malignant tumors among women across the world. Although advancements in detection and treatment of breast cancer have been made, approximately 5% of breast cancer presents metastasis to distant organs, such as bone, lung and liver, at diagnosis (Stage IV).[1] In addition, approximately 30% of breast cancer patients will develop metastasis during the evolution of their disease.[2] Metastatic breast cancer is a highly lethal disease, and the 10-year survival rate remains at only 15%.[1] Estrogens play an important role in the progression of breast cancer, and a majority (e.g. 64% according to Davidson et al[1]) of stage IV breast carcinomas express estrogen receptor (ER). Therefore, it is very important to evaluate the clinical and/or biological markers associated with distant metastasis to improve the prognosis of ER-positive breast cancer patients.

Molecular features of distant metastasis remain largely unknown, and it is still impossible to accurately predict it in breast cancer. Breast cancer is considered a systemic disease at the onset as a consequence of cells entering lymphatics or veins,[3,4] and it is suggested that distant metastasis is not a random event, but is, at least in a part, determined by characteristics of breast carcinoma cells at the primary site. Therefore, in this study, we first examined the gene expression profile of ER-positive stage IV breast carcinoma tissues based on microarray analysis, and newly identified *OLFM4*, *LY6D* and *S100A7* as genes closely linked to distant metastasis.

Olfactomedin 4 (OLFM4) is a member of the olfactomedin family and is a glycoprotein with a single peptide, an N-terminal coil-coil and a C-terminal olfactomedin domain.[5] OLFM4 regulates various signaling pathways and factors, including NF-κB, Wnt and Notch.[5,6] Overexpression of OLFM4 mRNA is detected in various carcinoma tissues, such as colon, breast and lung carcinomas, compared to non-cancerous tissues.[7] In contrast, lymphocyte antigen 6 family member D (LY6D) is a member of the LY6 family, and it is a membrane-bound protein with a glycosylphosphatidylinositol (GPI)-anchor.[8] LY6 family members are reported to play important roles in cancers. LY6K was found to cause metastasis of breast cancer cells,[9] while LY6A/E promoted breast tumorigenesis in mouse models.[10] Finally, S100A7 (S100 calcium-binding protein A7) is a member of the S100 family of calcium-binding proteins and regulates various cellular functions, such as calcium homeostasis, cell proliferation, differentiation, apoptosis and cell invasion.[11] S100A7 enhanced the growth and invasive properties of breast cancer, and it was considered to play an important role in the progression of breast cancer.[12] However, immunolocalization of LY6D has not been examined in breast carcinoma and that of OLFM4 and S100A7 was only very recently reported by Xiong et al[13] and Sakurai et al[14], respectively. Because the significance of these 3 proteins remains largely unknown in breast carcinoma, we subsequently performed immunohistochemistry for OLFM4, LY6D and S100A7 in breast carcinoma tissues according to ER status.

2 | MATERIALS AND METHODS

2.1 | Patients and tissues

Two sets of tissue specimens were evaluated in this study. The first set consisted of 18 specimens of ER-positive invasive ductal carcinoma, not otherwise specified, of the breast (7 stage IV and 11 stage I-III cases) obtained from Japanese female patients (age range; 35-76 years) who underwent surgical treatment from 2010 to 2015 in the Department of Surgery, Tohoku University Hospital (Sendai, Japan). The specimens were stored at −80°C for microarray analysis in addition to being fixed in 10% formalin and embedded in paraffin.

As a second set, 208 specimens of invasive ductal carcinoma, not otherwise specified, of the breast (ER-positive; n = 168, and ER-negative n = 40) were obtained from Japanese female patients (age range; 27-88 years) who underwent surgical treatment. All the specimens were fixed in 10% formalin and embedded in paraffin wax. Among these, stage IV cases (n = 41) were obtained from 1995 to 2015 from Tohoku University Hospital (Sendai, Japan), the National Hospital Organization Sendai Medical Center (Sendai, Japan) and Osaki Citizen Hospital (Osaki, Japan). In contrast, the stage I-III patients (n = 167) were successively treated in Tohoku University Hospital from 2006 to 2008; 91 patients received adjuvant chemotherapy, while 135 patients received adjuvant endocrine therapy after the surgery. The clinical outcome was evaluated by distant metastasis-free survival, which was defined as the time (in months) from primary surgery until the first event of distant metastasis[15] and breast cancer-specific survival, which was defined as the time from surgery to death from breast cancer[16] of the stage I-III patients. The mean follow-up time was 61 months (range, 2-132 months) in this study.

The research protocol was approved by the Ethics Committee at the Tohoku University School of Medicine and review boards of National Hospital Organization Sendai Medical Center and Osaki Citizen Hospital.

2.2 | Microarray analysis

Gene expression profile of ER-positive stage IV breast carcinoma was examined using microarray analysis. Briefly, total RNA was extracted from 18 snap-frozen specimens of ER-positive breast carcinoma tissues (first set) using an RNeasy Mini Kit (QIAGEN, Hilden, Germany). We histologically confirmed that each specimen abundantly included carcinoma cells, although microdissection of carcinoma cells was not performed in this study. A SurePrint G3 Human GE 8×60K v2 Microarray Kit (G4851A, ID 028004 [Agilent Technologies, Waldbronn, Germany]) was used, and sample preparation and processing were performed according to the manufacturer's protocol. Scatterplot analysis of the microarray data was performed using GeneSpring 12.6.1 (Agilent Technologies). In this analysis, when the expression ratio of a probe in the stage IV group compared to that in the stage I-III group was >2.0, we tentatively

determined the probe predominantly expressed in stage IV, according to a cut-off value previously used.[17]

2.3 | Immunohistochemistry

Rabbit polyclonal antibodies for OLFM4 (ab96280) and LY6D (HPA024755) and mouse monoclonal antibody for S100A7 (47C1068) were purchased from Abcam (Cambridge, UK), Sigma-Aldrich (St. Louis, MO, USA) and LifeSpan BioSciences (Seattle, WA, USA), respectively. Immunostaining for these 3 antibodies was automatically performed using Ventana Benchmark XT platform (Roche Diagnostics Japan, Tokyo, Japan). As a positive control, we used human tissue of the prostate, skin and skin for OLFM4, LY6D and S100A7, respectively, based on the data sheet (OLFM4) and database of the Human Protein Atlas (https://www.proteinatlas.org) (LY6D and S100A7).

Immunohistochemistry for ER (CONFIRM anti-ER [SP1]) and progesterone receptor (PR) (CONFIRM anti-PR [1E2]; Roche Diagnostics Japan) was also performed with Ventana Benchmark XT (Roche Diagnostics Japan), and that for HER2 was performed by HercepTest (DAKO). Mouse monoclonal antibody for Ki67 (MIB1) was purchased from DAKO (Carpinteria, CA, USA), and a Histofine kit (Nichirei Bioscience, Tokyo, Japan) was used for the immunohistochemistry.

2.4 | Scoring of immunoreactivity

OLFM4, LY6D and S100A7 immunoreactivity were detected in carcinoma cells, and the cases that had more than 10% positive carcinoma cells were considered positive. Immunoreactivity for ER, PR and Ki67 was detected in the nucleus of the carcinoma cells, and the percentage of immunoreactivity (labeling index [LI]) was determined. Cases with ER LI of more than 1% were considered ER-positive breast carcinoma according to a previous report,[18] while cases with Ki67 LI ≥14% were classified as in the Ki67-high group.[19] HER2 immunoreactivity was evaluated according to the grading system proposed in HercepTest (DAKO). HER2 gene amplification was also investigated by FISH in intermediate scoring (score 2+) cases.

2.5 | Statistical analysis

Association between immunohistochemical status of OLFM4, LY6D and S100A7 and various clinicopathological factors were evaluated using Student's *t* test or a cross-table using the χ^2-test. The statistical analyses were performed using the StatView 5.0J software (SAS Institute, Cary, NC, USA). Distant metastasis-free and breast cancer-specific survival curves were generated according to the Kaplan-Meier method, and statistical significance was calculated using the log-rank test. Univariate and multivariate analyses were evaluated using a proportional hazard model (Cox). *P*-value < .05 and .05 ≤ *P*-value < .10 were considered significant and borderline significant, respectively, in this study.

3 | RESULTS

3.1 | Gene expression profile in estrogen receptor-positive stage IV breast carcinoma

We first examined the gene expression profile of ER-positive stage IV breast carcinoma by microarray analysis in the first set. In Figure 1A, the scatterplot reveals that 88 probes were predominantly expressed in stage IV breast carcinoma. The known functions of the top 10 genes according to the expression ratio (stage IV/stage I-III) are briefly described in Table 1.

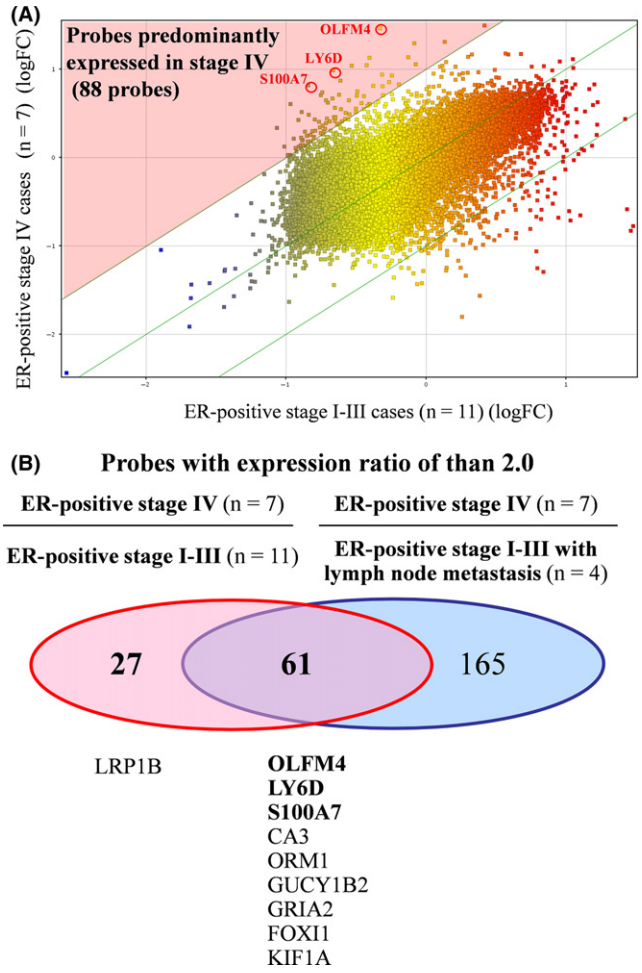

FIGURE 1 Gene expression profile in estrogen receptor (ER)-positive stage IV breast carcinoma. A, Scatterplot analysis in the stage IV breast carcinoma tissues (n = 7) comparing stage I-III cases (n = 11). Probes with an expression ratio of more than 2.0 are located in the left upper section, outside the diagonal lines (area in pink). The top 10 genes predominantly expressed in stage IV breast carcinoma are listed in Table 1. Locations of the top 3 genes, for which immunohistochemistry was performed (ie, *OLFM4*, *LY6D* and *S100A7*), are noted as red circles. B, Venn diagrams representing number of probes predominantly expressed in ER-positive stage IV cases comparing ER-positive stage I-III cases (total 88 probes) and/or that comparing ER-positive stage I-III cases with lymph node metastasis (total 226 probes). All the stage IV cases are lymph node metastasis in this study. The lower panels summarize the location of the top 10 genes. The top 3 genes are listed in both panels and are in bold

All 7 ER-positive stage IV cases examined showed lymph node metastasis, while 4 out of 11 ER-positive stage I-III cases were positive for lymph node metastasis. Among the 88 probes predominately expressed in the stage IV cases, 61 probes were also predominately expressed in the stage IV compared to the stage I-III with lymph node metastasis, suggesting that these were closely associated with distant metastasis in ER-positive breast carcinoma (Figure 1B).

TABLE 1 Brief functions of top 10 genes predominantly expressed in estrogen receptor-positive stage IV breast carcinoma cases

Gene symbol	Fold[a]	Function
OLFM4	3.3	The encoded protein is an antiapoptotic factor that promotes tumor growth and is an extracellular matrix glycoprotein that facilitates cell adhesion.
LY6D	3.1	May act as a specification marker at earliest stage specification of lymphocytes between B-cell and T-cell developments. Marks the earliest stage of B-cell specification.
S100A7	3.1	This gene involved in the regulation of a number of cellular processes such as cell cycle progression and differentiation
CA3	3.0	Carbonic anhydrase III (CAIII) is a member of a multigene family (at least 6 separate genes are known) that encodes carbonic anhydrase isozymes
ORM1	2.9	This gene encodes a key acute-phase plasma protein. Because of its increase due to acute inflammation, this protein is classified as an acute-phase reactant. The specific function of this protein has not yet been determined; however, it may be involved in aspects of immunosuppression.
GUCY1B2	2.9	This gene encodes a member of the low density lipoprotein (LDL) receptor family. These receptors play a wide variety of roles in normal cell function and development due to their interactions with multiple ligands. Disruption of this gene has been reported in several types of cancer.
GRIA2	2.7	The subunit encoded by this gene (GRIA2) is subject to RNA editing (CAG->CGG; Q->R) within the second transmembrane domain, which is thought to render the channel impermeable to Ca(2+).
FOXI1	2.7	This gene belongs to the forkhead family of transcription factors, which is characterized by a distinct forkhead domain. This gene may play an important role in the development of the cochlea and vestibulum, as well as in embryogenesis
KIF1A	2.6	The protein encoded by this gene is a member of the kinesin family and functions as an anterograde motor protein that transports membranous organelles along axonal microtubules.

The gene function was mainly summarized from description of NCBI Gene database (https://www.ncbi.nlm.nih.gov/gene).
[a]Expression ratio (stage IV/stage I-III).

3.2 | Immunolocalization of OLFM4, LY6D and S100A7 in estrogen receptor-positive breast carcinoma

We next performed immunohistochemistry for the top 3 genes (ie, *OLFM4*, *LY6D* and *S100A7*) in ER-positive breast carcinoma tissues using the first set (n = 18). Immunoreactivity of OLFM4 (Figure 2A), LY6D (Figure 2B) and S100A7 (Figure 2C) was detected in the cytoplasm, membrane and both cytoplasm and nucleus of breast carcinoma cells, respectively (left panel in each figure), but it was negative in the stroma or non-neoplastic mammary glands (right panel in each figure). When the cases that had more than 10% of the positive carcinoma cells were considered positive for OLFM4, LY6D and S100A7 in this study, with the 10% cut-off value frequently used as a reproducible evaluation,[20] the frequency of immunohistochemical status in the stage IV and stage I-III cases was 71% and 27% (2.6-fold) for OLFM4, 14% and 0% for LY6D and 29% and 9% (3.2-fold) for S100A7, which is consistent with the results of microarray analysis.

3.3 | Association between immunohistochemical status of OLFM4, LY6D and S100A7 and clinicopathological parameters in estrogen receptor-positive breast carcinoma

To further analyze the clinicopathological significance of these 3 proteins, we immunolocalized OLFM4, LY6D and S100A7 in 168 ER-positive breast carcinoma cases of the second set, which were different from the first set. As shown in Table 2, immunohistochemical OLFM4 status was positive in 45 out of 168 breast carcinomas (27%) and it was positively associated with stage ($P < .0001$), pathological T factor (pT) ($P < .0001$), lymph node metastasis ($P = .0078$), distant metastasis ($P < .0001$) and Ki67 status ($P = .0011$). In contrast, the LY6D status was positive in 19 out of 168 ER-positive breast carcinomas (11%) and it was positively associated with stage ($P = .0022$), pT ($P = .0014$), lymph node metastasis ($P = .010$), distant metastasis ($P = .0012$) and Ki67 status ($P = .0005$) (Table 2). The S100A7 status was positive in 29 out of 168 ER-positive breast carcinomas (17%), and it was positively associated with stage ($P = .0044$), pT ($P = .0038$), distant metastasis ($P = .0064$), histological grade ($P = .0001$) and Ki67 status ($P = .042$), and marginally associated with lymph node metastasis ($P = .090$) (Table 2).

No significant association was detected between OLFM4, LY6D and S100A7 status at the primary site and the metastatic organ in 33 ER-positive stage IV cases in this study (Table S1).

3.4 | Association between OLFM4, LY6D and S100A7 status and clinical outcome of estrogen receptor-positive stage I-III breast cancer patients

As shown in Figure 3A, immunohistochemical OLFM4 status was significantly associated with an increased incidence of distant

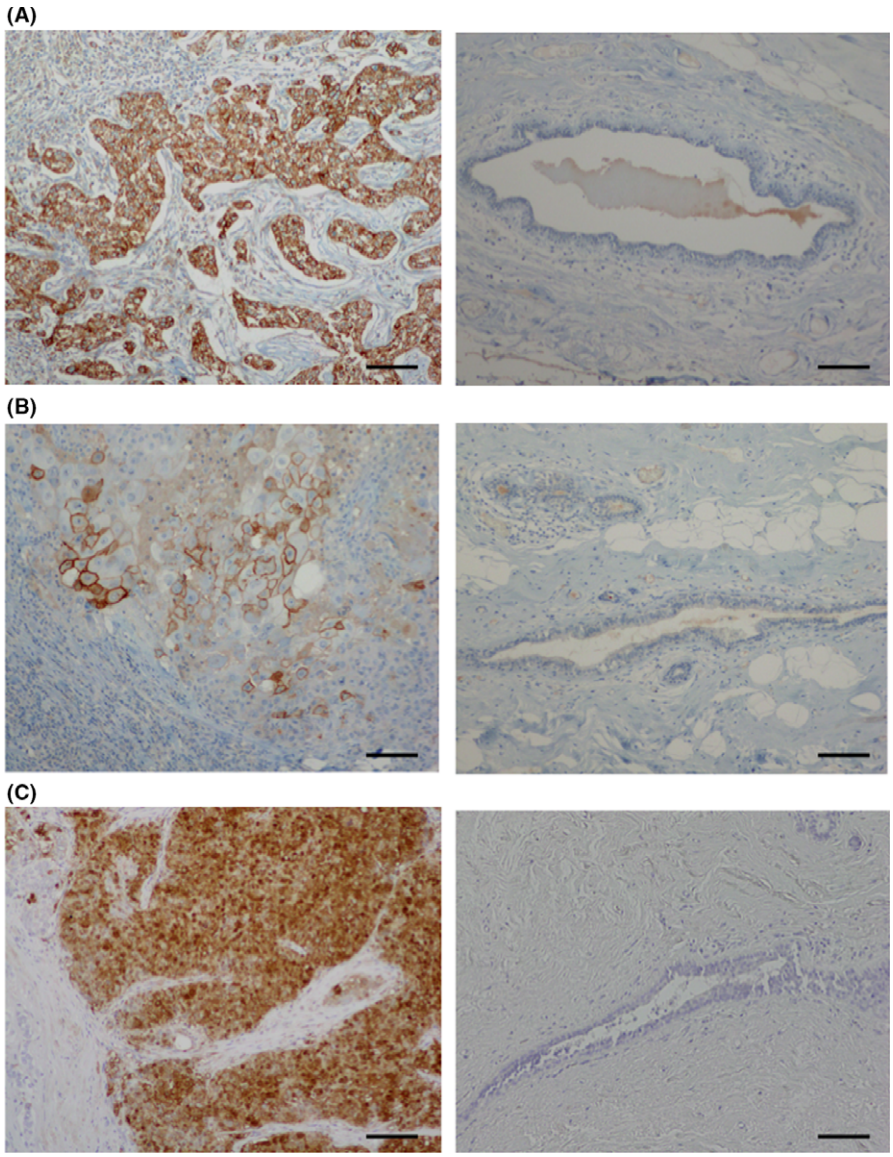

FIGURE 2 Immunolocalization of OLFM4 (A), LY6D (B) and S100A7 (C) in breast carcinoma. In each figure, the left panel displays the immuno-positive case and right panel shows the morphologically normal mammary gland. Bar = 100 μm

metastasis in ER-positive stage I-III breast cancer patients (n = 135; $P < .0001$ by log-rank test). Significant association was also detected between LY6D ($P = .0073$; Figure 3B) or S100A7 ($P = .023$; Figure 5C) status and distant metastasis-free survival of the patients. Results of univariate analysis of distant metastasis-free survival using Cox (Table 3), OLFM4, Ki67, histological grade, pT, lymph node metastasis, LY6D and S100A7 were demonstrated to be significant prognostic factors. The multivariate analysis revealed that Ki67 ($P = .0035$) and OLFM4 ($P = .016$) were the worst prognostic factors for distant metastasis-free survival.

As shown in Figure 4, OLFM4 ($P < .0001$; Figure 4A), LY6D ($P = .0015$; Figure 4B and S100A7 ($P = .0007$; Figure 4C) statuses were significantly associated with adverse clinical outcome of these patients. Univariate analyses for breast cancer-specific survival revealed histological grade, OLFM4, S100A7, LY6D, Ki67 and lymph node metastasis as significant prognostic variables (Table 4). Subsequent multivariate analysis demonstrated that LY6D ($P = .031$) and OLFM4 ($P = .036$) were independent worse prognostic factors, while Ki67 and lymph node metastasis were borderline significant factors, in this study.

3.5 | Immunolocalization of OLFM4, LY6D and S100A7 in estrogen receptor-negative breast carcinoma

We also immunolocalized OLFM4, LY6D and S100A7 in 40 ER-negative breast carcinomas of the second set. The immuno-positivities of OLFM4, LY6D and S100A7 were 58% (23 out of 40 cases), 25% (10 out of 40 cases) and 63% (25 out of 40 cases), respectively, and the OLFM4, LY6D and S100A7 statuses were inversely associated with ER status in the second set in total (n = 208; $P = .0002$, $P = .025$ and $P < .0001$, respectively).

As shown in Table S2, OLFM4, LY6D and S100A7 statuses were not associated with clinicopathological parameters examined in 40 ER-negative cases in this study, except for an inverted association between LY6D and with HER2 ($P = .044$), and a positive association between S100A7 and Ki67 ($P = .042$).

TABLE 2 Association between immunohistochemical OLFM4, LY6D and S100A7 status and clinicopathological factors in 168 estrogen receptor-positive breast carcinomas

	OLFM4 status		
	+(n = 45)	−(n = 123)	*P*-value
Menopausal status			
Premenopausal	18	52	.79
Postmenopausal	27	71	
Stage			
I	5	68	**<.0001**
II	9	30	
III	10	13	
IV	21	12	
Pathological T factor (pT)			
pT1	10	83	**<.0001**
pT2-4	35	40	
Lymph node metastasis			
Positive	26	43	**.0078**
Negative	19	80	
Distant metastasis			
Positive	21	12	**<.0001**
Negative	24	111	
Histological grade			
1-2	35	108	.11
3	10	15	
PR status			
Positive	35	102	.45
Negative	10	21	
HER2 status			
Positive	7	12	.29
Negative	38	111	
Ki67 status			
High	28	42	**.0011**
Low	17	81	
	LY6D status		
	+(n = 19)	−(n = 149)	*P*-value
Menopausal status			
Premenopausal	9	61	.59
Postmenopausal	10	88	
Stage			
I	2	71	**.0022**
II	4	35	
III	4	19	
IV	9	24	
Pathological T factor (pT)			
pT1	4	89	**.0014**
pT2-4	15	60	
Lymph node metastasis			
Positive	13	56	**.010**
Negative	6	93	
Distant metastasis			
Positive	9	24	**.0012**
Negative	10	125	
Histological grade			
1-2	15	128	.42
3	4	21	
PR status			
Positive	16	121	.75
Negative	3	28	
HER2 status			
Positive	3	16	.51
Negative	16	133	
Ki67 status			
High	15	55	**.0005**
Low	4	94	
	S100A7 status		
	+ (n = 29)	−(n = 139)	*P*-value
Menopausal status			
Premenopausal	12	58	.97
Postmenopausal	17	81	
Stage			
I	7	66	**.0044**
II	4	35	
III	7	16	
IV	11	12	
Pathological T factor (pT)			
pT1	9	84	**.0038**
pT2-4	20	55	
Lymph node metastasis			
Positive	16	53	*.090*
Negative	13	86	
Distant metastasis			
Positive	11	22	**.0064**
Negative	18	117	
Histological grade			
1-2	18	125	**.0001**
3	11	14	
PR status			
Positive	22	125	.39
Negative	7	24	
HER2 status			
Positive	4	15	.64
Negative	25	124	

(Continues)

(Continues)

TABLE 2 (Continued)

	S100A7 status		
	+ (n = 29)	−(n = 139)	*P*-value
Ki67 status			
High	17	53	**.042**
Low	12	86	

P-value < .05 and .05 ≤ *P*-value < .10 were significant (in bold) and borderline significant (in italics).

No significant association was detected between OLFM4 (*P* = .94; Figure S1A), LY6D (*P* = .86; Figure S1B) and S100A7 (*P* = .92; Figure S1C) and distant metastasis-free survival in 32 ER-negative stage I-III breast cancer patients in this study. Similarly, no significant association was detected between OLFM4 (*P* = .81), LY6D (*P* = .82) and S100A7 (*P* = .26) and breast cancer-specific survival in these patients.

4 | DISCUSSION

This is the first study to demonstrate the gene expression profile of ER-positive stage IV breast carcinoma tissues, to the best of our knowledge. Gene expression profiling is very important to survey molecular features of a particular group of breast carcinomas,[17,21] and several molecular-based diagnostic systems have been developed, such as MammaPrint[22] and Oncotype DX,[23] and PAM50.[24] In this study, microarray analysis results revealed 88 probes that are predominantly expressed in the stage IV group compared to stage I-III cases. Among the top 10 genes, 9 genes, except for *LRP1B*, were also predominantly expressed in the stage IV compared to stage I-III with lymph node metastasis, which suggests a close link to distant metastasis in ER-positive breast cancer patients. Their function and/or significance are largely unknown in breast carcinoma. However, for instance, carbonic anhydrase III (*CA3*) was immunolocalized in myoepithelial cells of the breast,[25] and it promoted transformation and invasion capability in hepatoma cells.[26] In contrast, ORM1 (orsomucoid 1) was reported as a serum biomarker to predict relapse-free survival of advanced breast cancers[27] and KIF1A (kinesin family member 1A) was overexpressed in ER-negative breast carcinoma cells and it mediated docetaxel resistance.[28] In this study, we immunolocalized OLFM4, LY6D and S100A7, which were listed as the top 3 genes in Table 1, in the second set to clarify their clinicopathological significance in breast carcinoma.

In our study, immunoreactivity of OLFM4, LY6D and S100A7 was detected in 27%, 11% and 17% of ER-positive breast carcinoma tissues, respectively. The immnuoreactivity was significantly associated with distant metastasis, and it is in good agreement with the results from microarray analysis using the first set. Considering that the immunoreactivity of OLFM4, LY6D and S100A7 was negligible in non-neoplastic mammary epithelium, it is suggested that these proteins are abnormally overexpressed in ER-positive stage IV breast carcinoma cases, and these are potent factors associated with distant metastasis in ER-positive breast carcinoma. However, these immunoreactivities were not associated with distant metastasis in ER-negative breast carcinomas in the present study (Table S2), although the number of cases was limited. Therefore, the significance of these proteins may be more evident in the ER-positive group than in ER-negative cases. Our present results suggest that OLFM4, LY6D and S100A7 immunoreactivity are potent markers for the distant metastasis, including the prediction, in ER-positive breast carcinoma tissues, and these proteins may become important therapeutic targets in ER-positive breast cancer patients.

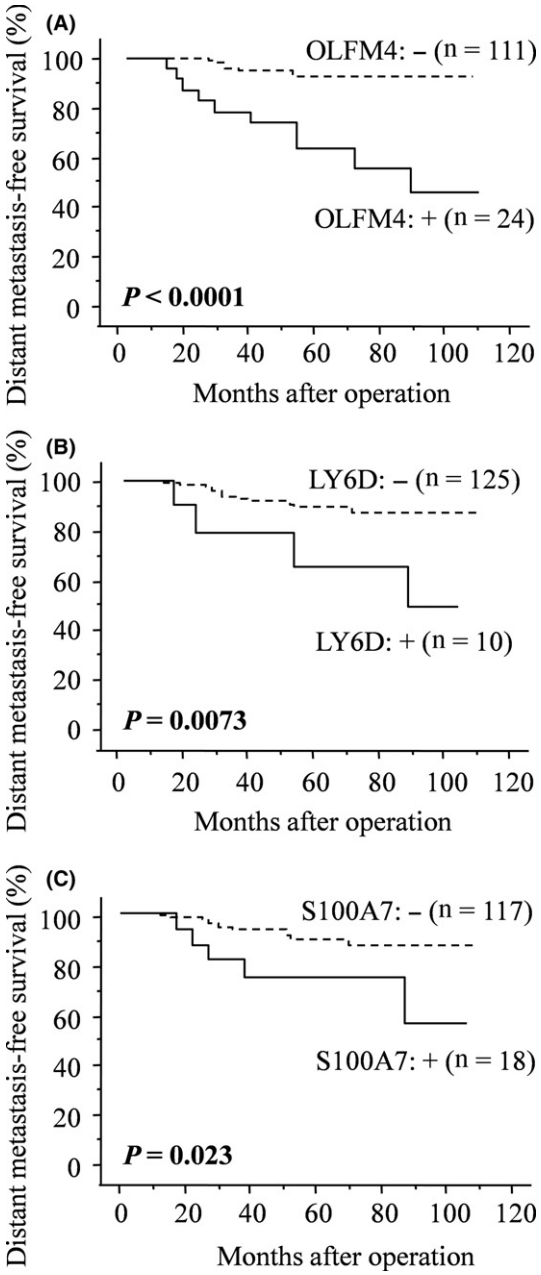

FIGURE 3 Distant metastasis-free survival of ER-positive stage I-III breast cancer patients (n = 135) according to OLFM4 (A), LY6D (B) and S100A7 (C) status. The solid line shows the positive group, and the dashed line shows the negative group. *P*-value < .05 was considered significant (shown in bold)

TABLE 3 Univariate and multivariate analyses of distant metastasis-free survival in 135 estrogen receptor-positive stage I-III breast cancer patients

	Univariate		Multivariate	
Variable	*P*-value	Relative risk (95% CI)	*P*-value	Relative risk (95% CI)
OLFM4 status (negative/positive)	**<.0001**[a]	7.5 (2.9-19.8)	**.016**	4.6 (1.4-15.0)
Ki67 status (low/high)	**.0002**[a]	11.1 (3.2-38.7)	**.0035**	7.8 (2.0-31.0)
Histological grade (1,2 / 3)	**.0007**[a]	5.4 (2.0-14.2)	.24	2.3 (.6-8.9)
pT (pT1 / pT2-4)	**.0019**[a]	5.2 (1.8-14.9)	.18	2.4 (.7-8.5)
Lymph node metastasis (negative / positive)	**.013**[a]	3.4 (1.3-9.0)	.36	1.6 (.6-4.3)
LY6D status (negative/positive)	**.014**[a]	4.1 (1.3-12.8)	.13	3.0 (.7-12.1)
S100A7 status (negative/positive)	**.032**[a]	3.1 (1.1-8.9)	.66	.7 (.2-3.1)
HER2 status (negative / positive)	.73	1.3 (.3-5.6)		

Statistical analysis was evaluated by a proportional hazard model (Cox).
P-value < .05 was considered significant and are listed in bold.
95% CI, 95% confidence interval.
[a]Significant ($P < .05$) values were examined in the multivariate analyses in this study.

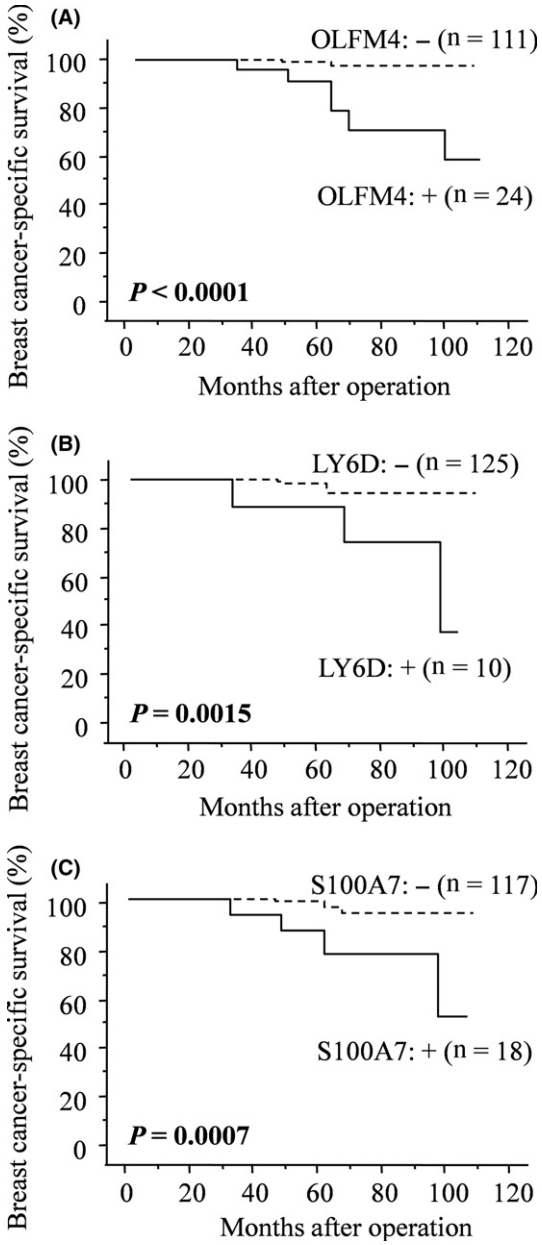

FIGURE 4 Breast cancer-specific survival of estrogen receptor (ER)-positive stage I-III breast cancer patients (n = 135) according to OLFM4 (A), LY6D (B) and S100A7 (C) status. The solid line shows the positive cases and the dashed line shows the negative cases. *P*-value < .05 was considered significant (shown in bold)

Although overexpression of OLFM4 mRNA was previously detected in various carcinoma tissues,[7] the significance of OLFM4 seems controversial. For instance, OLFM4 immunoreactivity was significantly associated with poor prognosis in patients with gastric[29] and pancreatic[30] cancer, and OLFM4 promoted S-phase transition in proliferation[31] and it was involved in suppressing apoptosis[32] of cancer cells. However, Seko et al[33] showed that OLFM4 immunoreactivity was an independent better predictor of survival in patients with colorectal cancer, and, very recently, Xiong et al[13] reported that OLFM4 immunoreactivity was significantly associated with better prognosis of triple negative breast carcinoma, and upregulation of OLFM4 suppressed migration and invasion of the triple negative breast carcinoma cells. In this study, OLFM4 immunoreactivity was significantly associated with stage, pT, lymph node metastasis, distant metastasis and Ki67 status, and, moreover, it was demonstrated to be an independent worse prognostic factor for distant metastasis-free and breast cancer-specific survival for ER-positive stage I-III breast cancer patients. In contrast, no association was detected between OLFM4 and prognosis in ER-negative cases. These results were inconsistent with those of Xiong et al,[13] which may be partly due to differences in the subtype of breast carcinoma, the small number of ER-negative cases in this study, the OLFM4 antibody used, and the evaluation method for the immunoreactivity. Limited information is currently available on OLFM4 in breast cancer, and replication studies with a larger sample set and/or a longer follow-up period are needed to confirm the significance of OLFM4 in breast carcinoma.

This is the first study to demonstrate LY6D immunolocalization in breast carcinoma. In this study, LY6D immunoreactivity was significantly associated with stage, pT, lymph node metastasis, distant metastasis and Ki67 status in ER-positive breast carcinomas, and it was also significantly associated with both distant metastasis-free and breast cancer-specific survival of ER-positive stage I-III breast cancer patients. Luo et al[34] reported that LY6D mRNA expression

TABLE 4 Univariate and multivariate analyses of breast cancer-specific survival in 135 estrogen receptor-positive stage I-III breast cancer patients

Variable	Univariate		Multivariate	
	P-value	Relative risk (95% CI)	*P*-value	Relative risk (95% CI)
Histological grade (1,2/3)	**.0009**[a]	11.3 (2.7-47.6)	.16	5.4 (.5-55.7)
OLFM4 status (negative/positive)	**.0017**[a]	12.9 (2.6-64.3)	**.046**	6.2 (1.0-38.9)
S100A7 status (negative/positive)	**.0040**[a]	7.7 (1.9-30.8)	.67	1.5 (.2-9.2)
LY6D status (negative/positive)	**.0070**[a]	7.2 (1.7-30.3)	**.031**	9.0 (1.2-65.8)
Ki67 status (low/high)	**.010**[a]	15.7 (1.9-128.4)	*.070*	13.0 (.8-206.3)
Lymph node metastasis (negative/positive)	**.019**[a]	6.8 (1.4-33.6)	*.095*	5.2 (.8-36.3)
pT (pT1/pT2-4)	.12	3.2 (.8-13.5)		
HER2 status (negative/positive)	.80	1.3 (.2-10.8)		

Statistical analysis was evaluated by a proportional hazard model (Cox). *P*-value < .05 and .05 ≤ *P*-value < .10 were considered significant and borderline significant, and are listed in bold and italics, respectively.
95% CI, 95% confidence interval.
[a]Significant ($P < .05$) values were examined in the multivariate analyses in this study.

was increased in various carcinoma tissues compared to normal counterpart tissues, and the increased expression was associated with poor outcome in ovarian, colorectal, gastric breast, lung, urinary bladder and brain tumors. Lu et al[35] also reported a gene expression signature which predicts survival of patients with stage I lung cancer, and LY6D was included in one of the overexpressed genes in the high-risk patients. Our present results were consistent with these previous reports, and it is suggested that LY6D immunoreactivity is associated with an aggressive phenotype of ER-positive breast cancer, including distant metastasis.

Previous studies demonstrated that S100A7 was highly expressed in the ductal carcinoma in situ (DCIS) with comedo necrosis[20], and it was involved in transition to invasive breast carcinoma.[12] Very recently, Sakurai et al[14] reported that S100A7 was upregulated by an interaction between breast carcinoma cells and cancer-associated adipocytes, and S100A7 immunoreactivity was significantly associated with histological grade, stage, lymph node metastasis and worse prognosis for relapse-free survival of breast carcinoma, although they did not examine stage IV cases. Our present results are consistent with this report, and it is suggested that S100A7 protein plays an important role in the aggressiveness of ER-positive breast carcinoma, including distant metastasis, and the prognosis of patients.

In summary, we examined the gene expression profile of ER-positive stage IV breast carcinoma by microarray analysis and newly identified that OLFM4, LY6D and S100A7 were closely associated with distant metastasis. A subsequent immunohistochemical analysis revealed that OLFM4, LY6D and S100A7 status were positive in 27%, 11% and 17% of ER-positive breast carcinoma cases, respectively, and these were all significantly associated with stage, pT, distant metastasis and Ki67 status and a worse prognostic factor for distant metastasis-free and breast cancer-specific survival. These results suggest that OLFM4, LY6D and S100A7 immunoreactivity are associated with an aggressive phenotype of ER-positive breast carcinoma, and these are potent markers for distant metastasis in ER-positive breast cancer patients.

CONFLICT OF INTEREST

We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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SUPPORTING INFORMATION

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How to cite this article: Mayama A, Takagi K, Suzuki H, et al. OLFM4, LY6D and S100A7 as potent markers for distant metastasis in estrogen receptor-positive breast carcinoma. *Cancer Sci*. 2018;109:3350–3359. https://doi.org/10.1111/cas.13770