Pathol. Oncol. Res. (2012) 18:161–168
DOI 10.1007/s12253-011-9422-7

RESEARCH

Association of Expression of Kruppel-Like Factor 4 and Kruppel-Like Factor 5 with the Clinical Manifestations of Breast Cancer

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Received: 8 December 2010 / Accepted: 26 May 2011 / Published online: 15 June 2011

Abstract Kruppel-like factors (KLFs) are import modulators of cell proliferation, differentiation, and transformation and have recently been considered possible prognostic factors in breast cancer. In this study, we investigated the correlation between KLF4 and KLF5 expression and the clinical manifestations of breast cancer by immunohistochemical analysis. We observed increased KLF4 and KLF5 expression in tumor cells (invasive and in situ carcinomas), consistent KLF4 and KLF5 expression in in situ and invasive carcinomas, significant associations between KLF4 expression and tumor grade ($p=0.033$), size ($p=0.035$) and stage ($p=0.006$), and an association between KLF5 expression and tumor grade ($p=0.033$). Interestingly, we observed a relationship between increasing age and KLF4 expression ($p=0.007$), with a tendency towards greater expression in tumor cells in patients over 50 years old. Moreover, KLF5 nuclear localization was restricted to non-tumor breast ducts and lobules; however, loss of nuclear expression of KLF5 in in situ and invasive carcinomas was observed. Although the mechanism of the loss of KLF5 nuclear expression is not clear, this phenomenon may imply a possible tumor-suppressor-like role for KLF5 in breast cancer tumorigenesis. The expression of KLF4 and KLF5 in breast cancer patients in Taiwan is similar to that in Western countries, except for the uncertainty surrounding its prognostic significance. Further clarification of the underlying mechanisms of KLF4 and KLF5 expression and their correlations with breast cancer outcomes is necessary.

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Keywords Breast cancer · Immunohistochemistry · KLF4 · KLF5 · Kruppel-like factor

Abbreviations

DCIS ductal carcinoma in situ
KLF Kruppel-like factors
KLF4 Kruppel-like factor 4
KLF5 Kruppel-like factor 5

Introduction

Breast cancer, a leading cause of cancer-related death worldwide, is the fourth-leading cause of cancer deaths in Taiwanese women. A tendency towards increased incidence in younger women (29.3% in those ≤40 years old) has been found in a large study of 830 breast cancer cases in Taiwan, and more aggressive clinical behavior in the younger patients was also noted [1]. New molecular markers of breast cancer have been proposed, and they provide potential targets for new and effective treatment strategies for breast cancer.

Kruppel-like factors (KLFs), a group of zinc-finger-like transcription factors, play important roles in the regulation of cell proliferation, differentiation and embryogenesis [2–5] and have been suggested as possible markers of tumor invasion, metastasis, and prognosis in breast cancers [4–9]. However, most of the studies have been confined to populations from Western countries [6, 7], but none has focused on Asian populations.

KLF4 and KLF5 exert different and often opposing effects on the regulation of gene transcription and cellular proliferation. KLF4 has both tumor suppressor and tumor-promoting functions in different cancers [10–12]; it can promote the proliferation of cancer cells and regulate the production of the extracellular matrix [13]. KLF4 interacts with p53 and p21, causing the cell cycle to remain at the G1/S and G2/M checkpoints [10], and it has an oncogenic effect because of the action of oncogenic RAS^{V12} and the RAS target cyclin-D1 [11]. In breast cancer, increased KLF4 expression in ductal carcinoma in situ and invasive carcinoma has been found in both in vitro and in vivo studies; thus, a possible tumorigenesis role has been suggested [14, 15]. The nuclear localization of KLF4 expression in breast cancer patients may be associated with more aggressive clinical manifestations [6].

KLF5 also possesses both tumor suppressor and oncogenic functions in other human malignancies [2, 10, 16]. KLF5 can facilitate the proliferation and transformation of cancer cells [17]. Some of the results of in vitro and in vivo studies of KLF5 in breast cancer differ, and the expression mechanism remains to be elucidated [16, 18]. Chen and colleagues found that the *KLF5* gene was deleted in about 43% of breast cancer cell lines, and KLF5 has been proposed to play a tumor-suppressor role in breast cancer [16]. According to tissue microarray and immunohistochemistry, however, KLF5 expression is upregulated in clinical breast-tumor samples [18]. Tong et al. found that increased expression of KLF5 is a poor prognostic factor and is positively correlated with the expression of HER-2/neu and Ki-67 in breast cancer [7]. In addition, KLF5 expression is increased in breast cancer patients younger than 50 years of age.

On the basis of the roles of KLF4 and KLF5 in cell proliferation and their possible clinical significance, we hypothesized that KLF4 or KLF5 expression is associated with the clinical manifestations of breast cancer. We analyzed the correlation between KLF4 and KLF5 expression and clinicopathological features in 60 breast cancer patients by immunohistochemistry.

Materials and Methods

Patients and Specimens

Sixty female patients diagnosed with breast invasive carcinoma at the Changhua Christian Hospital between 2003 and 2007 were included in this study. Their clinical data and pathological features were obtained from their chart records and were reviewed. All of the patients underwent primary surgical resection with chemotherapy, radiotherapy, or hormone therapy; the follow-up period ranged from 8 to 59 months (mean, 27 months). Surgical specimens were obtained and fixed in 10% neutral-buffered formalin and embedded in paraffin. Standard hematoxylin and eosin staining was then performed, and one representative paraffin specimen of each case that exhibited the coexistence of ductal carcinoma in situ, invasive carcinoma, and non-tumor breast ducts and lobules was selected for evaluation. This study was approved by the Institutional Review Board and the Ethics Committee, and written informed consent was obtained from all 60 patients.

Immunohistochemical Study of Breast Cancer

The antibodies used in this study were anti-KLF4 (Santa Cruz, sc-20691, dilution: 1:200), anti-KLF5 (Santa Cruz, sc-22797, dilution: 1:50), anti-p53 (Dako, DO-7, dilution: 1:50), and anti-p21 (Santa Cruz, sc-6246, dilution: 1:50).

A manual immunohistochemical procedure was performed using 4-µm-thick tissue sections. After deparaffinization in xylene and rehydration in graded ethanol, heat-induced antigen retrieval was accomplished by immersing the slides in 10 mM citrate buffer, pH 6, at 120°C for 10 min in a pressurized heating chamber (BioCare Medical, Concord, CA). Endogenous peroxidase was blocked with 3% aqueous H_2O_2, and nonspecific binding of the antibody was blocked with 20% protein blocker (Signet Laboratories, Dedham, MA) in Tris-buffered saline. After three washes with phosphate-buffered saline (PBS), the slides were incubated with the appropriate antibodies for 30 min at room temperature. The slides were washed three times in PBS and subsequently incubated with biotinylated anti-goat IgG antibody (Vector Laboratories, Burlingame, CA) at a dilution of 1:200, followed by three washes with PBS. Signal detection was performed, using 3,3′-diaminobenzidine as the chromogen. The slides were counterstained with hematoxylin, cleaned in xylene, and mounted with Permount.

Either nuclear or cytoplasmic expression of KLF4 or KLF5 was considered to be positive labeling. The tumor cells (defined as both in situ and invasive cancer) and non-tumor cells (defined as non-tumor mammary ducts and lobules) of each breast cancer tissue section were evaluated. In the analysis of the p53 and p21 immunostaining, only nuclear staining was regarded as positive expression. The expression intensity (intensity score) of KLF4, KLF5, p21 and p53 was classified as negative (<5% cells), 1+ (≥5% and <25% cells), 2+ (≥25% and <50% cells), or 3+ (≥50% cells); too faint or barely perceptible staining was ignored.

Statistical Analysis

The relationships between the KLF4, KLF5, p53, and p21 expression and clinicopathologic parameters in 60 cases of breast cancer were examined by analysis of variance, or the χ^2 test. The analyses were performed using the Statistical Package for Social Sciences Version 15.0 (SPSS, Version 15.0, Chicago, IL, USA). The data are presented as mean ± SD, mean with a range, or numbers with percentages. Only differences with a p value of less than 0.05 (by two-tailed testing) were considered statistically significant.

Results

Patient Characteristics

A total of 60 female patient samples were analyzed in this retrospective immunohistochemical study. The patients' clinicopathological parameters and KLF protein expression are listed in Table 1.

Table 1 The clinicopathologic features of the 60 patients with breast cancer

Characteristics	Case Numbers (%)
Age (years)	
≤50	37 (66.7)
>50	2 (3.3)
Invasive cancer type	
IDC	54 (90.0)
ILC	3 (5.0)
Mucinous carcinoma	3 (5.0)
Invasive carcinoma grade	
Well	11 (18.3)
Moderate	40 (66.7)
Poor	9 (15.0)
Tumor size	
<2 cm	21 (35.0)
≥2 cm and <5 cm	29 (48.3)
≥5 cm	10 (16.7)
Tumor stage	
I	18 (30.0)
II	26 (43.3)
III	13 (21.7)
IV	3 (5.0)
Lymph node involvement	
No	35 (58.3)
Yes	25 (41.7)
Died of cancer	
No	59 (98.3)
Yes	1 (1.7)
ER expression status	
Negative	13 (21.7)
Positive	47 (78.3)
PR expression status	
Negative	8 (13.3)
Positive	52 (86.7)
Her-2 expression score	
0	23 (38.3)
1+	11 (18.3)
2+	10 (16.7)
3+	16 (26.7)
Follow-up duration (months)	27.5±13.0 (8–59)

Note: The follow-up duration is presented as mean±SD (range)

Abbreviations

IDC invasive ductal carcinoma, *ILC* invasive lobular carcinoma

Immunohistochemical Results of KLF4, KLF5, p21 and p53 in Breast Cancer

KLF4 expression was defined as predominantly cytoplasmic expression (defined as cytoplasmic expression only or mostly cytoplasmic expression with less than 5% of the cells having nuclear expression), combined cytoplasmic and nuclear expression (defined as combined cytoplasmic and nuclear expression in more than 5% but fewer than 25% of the cells with nuclear positivity), and predominantly nuclear expression (defined as nuclear expression in more than 25% of the positive cells). The KLF4 expression pattern (referred to as the “pattern score”) was defined as follows: A, no staining; B, predominantly cytoplasmic expression; C, combined cytoplasmic and nuclear expression; and D, predominantly nuclear expression (Fig. 1).

The KLF5 expression was classified as weak cytoplasmic expression (defined as weak cytoplasmic expression in fewer than 25% of the cells with a paranuclear location), strong cytoplasmic expression (defined as strong cytoplasmic expression in more than 25% of the cells with a paranuclear location), and nuclear expression (defined as nuclear expression in more than 25% of the cells). The KLF5 expression pattern (pattern score) was defined as follows: A’, negative staining; B’, weak cytoplasmic staining; C’, strong cytoplasmic staining; and D’, predominantly nuclear staining (Fig. 1).

In the breast cancers, the expression intensities of KLF4 and KLF5 were stronger in the tumor cells than in the non-tumor cells (see Table 2). We grouped the cases according to the KLF4 staining patterns expression as follows: Group 1 (Patterns A and B, negative expression and predominantly cytoplasmic expression); and Group 2 (Patterns C and D, combined cytoplasmic and nuclear expression and predominantly nuclear expression). Group 2 tended to exhibit greater nuclear expression than Group 1. The expression intensities of p53 and p21 were stronger in tumor cells than in non-tumor cells. The non-tumor mammary ducts and lobules did not express p53 or p21 (see Table 2).

Association of KLF Expression with the Clinicopathological Parameters of Breast Cancer

Significant correlations were observed between the KLF4 expression pattern and tumor stage and size. The Group 2 invasive breast cancers were of a more advanced stage than the Group 1 cancers; the latter tended to be of stage I ($p=0.006$). In addition, an association between group and tumor size was noted: Group 1 tumors tended to be smaller (<2 cm) than Group 2 tumors and tended to belong to the T1 category ($p=0.035$) (see Table 3).

The KLF4 expression intensity in ductal carcinoma in situ (DCIS) and invasive cancers from the same sample was consistent: stronger expression in DCISs was associated with stronger expression in invasive cancers ($p=0.002$), and predominantly nuclear staining in DCISs was associated with the nuclear staining in invasive cancers ($p<0.001$) (data not shown). In cases in which stronger KLF4 expression was observed in the non-tumor cells than the tumor cells, the tumor cells tended to be well differentiated; similarly, when stronger KLF4 expression was seen in the tumor cells, the invasive cancer tended to be of a higher histological grade ($p=0.033$). In the patients over 50 years of age, the expression intensity of KLF4 tended to be stronger in the tumor cells, but the KLF4 expression was stronger in non-tumor cells in the patients under 50 ($p=0.007$). No association of KLF4 expression with that of p53, p21, ER, PR or HER-2/neu was observed in the invasive cancers.

KLF5 expression was correlated with the tumor grade. Invasive breast cancers were better differentiated when the KLF5 expression was negative or weakly cytoplasmic than in cases of stronger cytoplasmic expression ($p=0.035$) (see Table 4). We also observed no nuclear expression in the tumor cells, which was in contrast to the nuclear expression observed in the non-tumor mammary ducts or lobules (16.7%). Consistent KLF5 expression in DCISs and invasive cancers from the same sample was also observed: stronger KLF5 expression in DCISs was correlated with stronger expression in invasive cancers ($p<0.001$) and with concurrent cytoplasmic expression in DCISs and invasive cancers ($p<0.001$). No association between KLF5 expression and ER, PR or HER-2/neu expression, nor any association between the histological type of the invasive carcinomas and KLF4 or KLF5 expression, was observed in this study.

Discussion

In this study, we investigated the expression and significance of two potential biomarkers, KLF4 and KLF5, and their associations with the clinical manifestations of female breast cancer. The results of our study demonstrate that KLF4 expression is significantly correlated with the clinical manifestations of breast cancer and that increased KLF4 nuclear expression is positively associated with tumor size and stage. Tumor size is an important prognostic factor for breast cancer: increased tumor size increases the risk of metastasis to regional lymph nodes [19]. We found that the invasive breast cancers exhibiting KLF4 nuclear expression tended to have higher clinical stages and larger tumor sizes. Pandya et al. first concluded that nuclear expression rather than cytoplasmic expression of KLF4 is associated with more aggressive tumor behavior and is a poor prognostic factor in early-stage breast cancer [6]. The scope of this study did not allow further investigation into why nuclear expression of KLF4 in breast cancer is correlated with poor outcomes; instead, we focused on identifying the specific subcellular locations of KLF4 and their possible distinct biological activity and clinical significance. Nuclear localization of KLF4 plays a role in the malignant transformation of RK3E epithelial cells in vitro and is considered to be important evidence of tumorigenesis in breast cancers [6]. In this study, we observed consistent nuclear expression of KLF4 between DCISs and invasive carcinomas from the same samples, which possibly indicates KLF4-mediated tumor progression in breast cancers. Unfortunately, we were

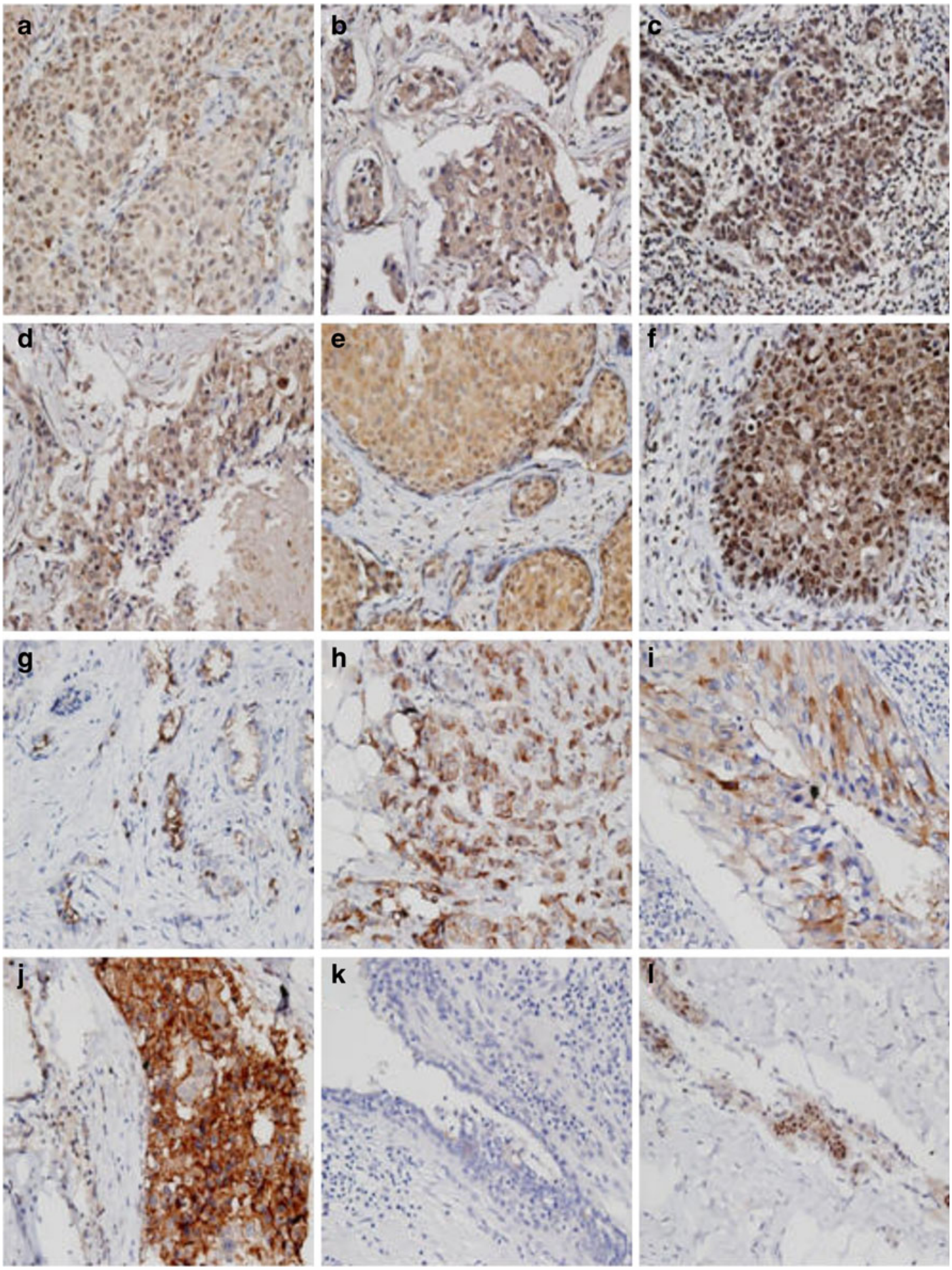

Fig 1 The immunohistochemical analysis of KLF4 and KLF5 in breast tissues. Predominantly cytoplasmic expression of KLF4, pattern B, in invasive carcinoma **a**; combined cytoplasmic and nuclear expression of KLF4, pattern C, in invasive carcinoma **b**; predominantly nuclear expression of KLF4, pattern D, in invasive carcinoma **c**. Predominantly cytoplasmic expression of KLF4, pattern B, in DCIS **d**; combined cytoplasmic and nuclear expression of KLF4, pattern C, in DCIS **e**; predominantly nuclear expression of KLF4, pattern D, in DCIS **f**. Weak cytoplasmic expression of KLF5, pattern B', in invasive carcinoma **g**; strong cytoplasmic expression of KLF5, pattern C', in invasive carcinomas **h**. Weak cytoplasmic expression of KLF5, pattern B', in DCIS **i**; strong cytoplasmic expression of KLF5, pattern C', in invasive carcinoma **j**. Negative cytoplasmic expression of KLF5, pattern A', in non-tumor cells **k**; predominantly nuclear expression of KLF5, pattern D', in non-tumor cells **l**

Table 2 Investigation of KLF4, KLF5, and p53 expression in invasive carcinomas and DCISs of the breast by immunohistochemical analysis

	KLF4				KLF5			
	0	1+	2+	3+	0	1+	2+	3+
Non-tumor	12 (20.0)	12 (20.0)	9 (15.0)	27 (45.0)	48 (80.0)	10 (16.7)	0 (0)	2 (3.0)
Invasive carcinoma	8 (13.3)	10 (16.7)	4 (6.7)	38 (63.3)	23 (38.3)	20 (33.3)	4 (6.7)	13 (21.7)
DCIS	4 (6.7)	10 (16.7)	4 (6.7)	42 (70.0)	28 (46.7)	20 (33)	4 (6.7)	8 (13.3)
	A	B	C	D	A'	B'	C'	D'
Non-tumor	8 (13.3)	42 (70.0)	7 (11.7)	3 (5.0)	48 (80.0)	2 (3.3)	0 (0)	10(16.7)
Invasive carcinoma	7 (11.7)	28 (46.7)	16 (26.7)	9 (15.0)	23 (38.3)	30 (50.0)	7 (11.7)	0 (0)
DCIS	3 (5.0)	38 (63.3)	14 (23.3)	5 (8.3)	28 (46.7)	27 (45.0)	5 (8.3)	0 (0)
	p53				p21			
	0	1+	2+	3+	0	1+	2+	3+
non-tumor	60(100)	0 (0)	0 (0)	0 (0)	60 (100)	0 (0)	0 (0)	0 (0)
invasive carcinoma	52(86.7)	2 (3.3)	3 (5.0)	3 (5.0)	55 (91.7)	5 (8.3)	0 (0)	0 (0)
DCIS	49(81.7)	5 (8.3)	2 (3.3)	4 (6.7)	53 (88.3)	7 (11.7)	0 (0)	0 (0)

Note: Data are presented as number (percentage)

unable to examine the correlation between survival and the nuclear expression of KLF4 because of the short follow-up period for our patients.

Our results show that the cytoplasmic expression of KLF4 is associated with poor cellular differentiation in breast cancers, especially in patients over 50 years of age. To the best of our knowledge, the phenomenon of differing KLF4 expression in different age groups has not previously been reported. The histological grade of tumors is also an important prognostic factor in breast cancer, and breast tumors of a poor histological grade are associated with greater tumor size, a younger age at diagnosis, absent ER expression, and a decreased 5-year survival rate [19]. Our results imply that cytoplasmic expression of KLF4 may be correlated with poor outcome in breast cancer patients.

Most KLF5 expression data from breast cancer cases have been collected from in vitro cell-line studies [16, 17, 20]. To the best of our knowledge, only Chen et al. [18] and our group have used immunohistochemical analysis to evaluate the KLF5 expression in tissue sections of breast cancers. Our findings are similar to those of Chen et al. [18] and show that KLF5 expression seems to be generally weaker in non-tumor cells of the breast than in tumor cells. We also found that KLF5 expression intensity and cyto-

Table 3 The correlation of KLF4 expression with clinicopathologic manifestations

Characteristics	KLF4			KLF4		
	T ≤ N	T > N	*p*-value	A + B	C + D	*p*-value
Age						
≤50 y	26 (70.3)	11 (29.7)	0.007[a]	18 (48.6)	19 (51.4)	0.471
>50 y	8 (34.8)	15 (65.2)		9 (39.1)	14 (60.9)	
Tumor size						
<2 cm	7 (58.3)	5 (41.7)	0.276	9 (75.0)	3 (25.0)	0.035[a]
≥2≤5 cm	25 (61.0)	16 (39.0)		14 (34.1)	27 (65.9)	
≥5 cm	2 (28.6)	5 (71.4)		4 (57.1)	3 (42.9)	
Lymph node metastasis						
No	21 (60.0	14 (40.0)	0.538	17 (48.6)	18 (51.4)	0.511
Yes	13 (52.0)	12 (48.0)		10 (40.0)	15 (60.0)	
Stage						
I	10 (55.6)	8 (44.4)	0.909	13 (72.2)	5 (27.8)	0.006[a]
II+III+IV	24 (57.1)	18 (24.9)		14 (33.3)	28 (66.7)	
Grade						
Well	9 (90.0)	1 (10.0)	0.033[a]	6 (60.0)	4 (40.0)	0.322
Moderate/poor	25 (50.0)	25 (50.0)		21 (42.0)	29 (58.0)	

Note: Data are presented as number (percentage).[a] Correlation is significant at the 0.05 level (two-tailed by the chi-square test). [b] T ≤ N, expression intensity in the tumor cells is weaker than or equal to that in the non-tumor cells; T > N, expression intensity in the tumor cells is stronger than in the non-tumor cells

Abbreviations

T tumor cells, *N* non-tumor cells

Table 4 Correlation of KLF5 expression with clinicopathologic manifestations

Characteristics	KLF5			KLF5		
	T ≤ N	T > N	*p*-value	A' + B'	C' + D'	*p*-value
Age						
≤50 y	18 (48.6)	19 (51.4)	0.164	17 (45.9)	20 (54.1)	0.124
>50 y	7 (30.4)	16 (69.6)		6 (26.1)	17 (73.9)	
Tumor size						
<2 cm	5 (41.7)	7 (58.3)	0.668	5 (41.7)	7 (58.3)	0.919
≥2~<5 cm	16 (39.0)	25 (61.0)		15 (36.6)	26 (63.4)	
≥5 cm	4 (57.1)	3 (42.9)		3 (42.9)	4 (57.1)	
Lymph node metastasis						
No	16 (45.7)	19 (54.3)	0.452	14 (40.0)	21 (60.0)	0.753
Yes	9 (36.0)	16 (64.0)		9 (36.0)	16 (64.0)	
Stage						
I	7 (38.9)	11 (61.1)	0.775	7 (38.9)	11 (61.1)	0.954
II + III + IV	18 (42.9)	24 (57.1)		16 (38.1)	26 (61.9)	
Grade						
Well	6 (60.0)	4 (40.0)	0.294	7 (70.0)	3 (30.0)	**0.035**[a]
Moderate/poor	19 (38.0)	31 (62.0)		16 (32.0)	34 (68.0)	

Note: Data are presented as number (percentage).[a] Correlation is significant at the 0.05 level (two-tailed by the chi-square test)

plasmic expression were consistent in DCIS and invasive carcinoma samples from the same cases, a phenomenon that may imply the involvement of KLF5 in tumorigenesis.

We found that KLF5 expression in invasive carcinomas was significantly associated with histological grade. The cytoplasmic expression of KLF5 was correlated with better differentiation of tumor cells in invasive carcinomas. Interestingly, different expression patterns in the tumor and non-tumor cells of the breast were observed. DCISs and invasive carcinomas showed only cytoplasmic expression, while nuclear expression of KLF5 was mainly restricted to the non-tumor mammary ducts and lobules (10 cases, 16.7%). This phenomenon has not been reported before, and we do not know the exact biological meaning of this phenomenon. Chen et al. have identified homozygous deletion of the *KLF5* gene in breast cancer cell lines [16]. Therefore, in vitro loss of KLF5 nuclear expression in pre-cancerous and cancerous breast lesions may indicate a possible tumor-suppression function of KLF5. However, the rate of nuclear KLF5 expression in non-tumor breast tissue is not high enough to explain this tumor-suppression function. The low rate of nuclear KLF5 expression may be caused by the weaker expression of KLF5 in normal breast tissue [18] or by some underlying genetic alterations of tumor progression already present in the non-tumor tissue adjacent to the malignancy in the same section.

Tong et al. have suggested that the increased level of KLF5 mRNA is a poor prognostic factor for disease-free and overall survival in breast-cancer patients and is also associated with age [7]. However, we were not able to identify any association between survival and KLF5 expression in our patients. The short follow-up period was the main limitation of our study, but no association between KLF5 expression and patient age was observed. This discrepancy between our study and that of Tong et al. may have been caused by different methods of evaluating KLF5 expression: protein level (immunohistochemistry) vs. mRNA level.

No association between KLF4 or KLF5 expression and the three traditional biomarkers (ER, PR and HER-2/neu) of breast cancer was observed in this study. To the best of our knowledge, no studies have reported associations between the expressions of ER, KLF4, and KLF5 in breast cancer [6, 7]. Several genes are upregulated by progestin, including *KLF4*. In T-47D cells, KLF4 is upregulated by progestin [21]. However, no association between KLF4 and PR was noted in our study, and no relationship between PR and KLF5 in breast cancer has been reported. Moreover, no known association has been reported between the expression of HER-2/neu and KLF4 [6]. A positive correlation between HER-2/neu and KLF5 expression in breast cancer has been noted, however [7]. In addition, HER-2/neu over-expression can activate the expression of angiogenic factors, including KLF5 [22]. No association between KLF5 expression and HER-2/neu status was found in our study. The crosstalk among KLF4, KLF5, and traditional prognostic biomarkers, such as ER, remains to be elucidated.

In summary, we found that the expression of KLF4 and KLF5 is associated with the clinical manifestations of breast cancer, including tumor size, stage and differentiation, suggesting possible prognostic roles for these proteins.

Our findings are based on immunohistochemical analysis, but further study is necessary to elucidate the possible prognostic roles of these two markers.

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