

Significant elevation of CLDN16 and HAPLN3 gene expression in human breast cancer

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Abstract. Cancer development involves the destruction of tight junctions, deprivation of cell polarity, and increased cell mobility. Claudin 16 (CLDN16) is a tight junction protein and plays important roles in the maintenance of cell polarity, cellular arrangement, adhesion, paracellular transport, and ionic permeability of various epithelia. A novel link protein, HAPLN3, functions in hyaluronic acid binding and cell adhesion. Both genes are hypothesized to be related to cancer development and metastasis. The purpose of this study was to estimate the roles of the genes CLDN16 and HAPLN3 in breast cancer. A total of 146 samples were collected from breast cancer tissues and their adjacent normal breast tissues. Reverse transcription and real-time polymerase chain reaction were used to estimate gene expression levels. There were significantly increased gene expression of CLDN16 ($p < 0.0001$) and HAPLN3 ($p < 0.0001$) among breast cancer tissues compared with normal tissues, irrespective of clinical pathological parameters. The absolute increased gene expression level of CLDN16 was significantly negatively correlated with estrogen ($r = -0.46$; $p < 0.0001$) and progesterone receptor ($r = -0.384$; $p = 0.001$) staining density. However, a significantly positive correlation ($r = 0.24$; $p = 0.04$) between the absolute increased HAPLN3 gene level and human epidermal receptor 2 staining density was found. There was no significant association between overall survival and the two gene expression levels. The gene up-expression of both CLDN16 and HAPLN3 was suggested to be involved

in the development of breast cancer and to be a biomarker and target treatment for breast cancer.

Introduction

Breast cancer, an epithelial tumor with highly invasive and metastatic potential, is one of the most frequently occurring malignant neoplasms worldwide (1,2), and is the fourth leading cause of cancer death among Taiwanese in Taiwan (3). Mammography screening is used for detection of certain lesions which are considered to be precursors of invasive breast cancer, such as ductal carcinoma *in situ* and atypical ductal hyperplasia (2). Recently, hormone-related factors such as estrogen receptor (ER), progesterone receptor (PR), and human epidermal receptor 2 (HER2) have been considered to be associated with the development and prognosis of breast cancer and suggested to be predictors for response to treatment of breast cancer (4-7). However, poor agreement on these predictors in the detection and prediction of breast cancer development limits their generalizability in clinical application. Therefore, additional biological and clinical studies on their significance in the diagnosis and therapy of breast cancer are strongly suggested.

During cancer development, the destruction of tight junctions (8), as well as the deprivation of cell polarity (9,10) and increased cell mobility (11,12), contribute to cancer cell proliferation, invasion, and metastasis, and a poor prognosis. Claudin-16 (CLDN16), a member of the claudins family, is a tight junction protein and plays important roles in the maintenance of cell polarity, cellular arrangement, adhesion, paracellular transport, and ionic permeability of various epithelia (13-16). Up-expressions of claudin-16 were found in human tissues with ovarian cancer (17) and papillary thyroid carcinomas (18). Moreover, a novel link protein, HAPLN3, belonging to the hyaluronan and proteoglycan link protein family, functions in the aggregation of proteoglycan with hyaluronic acid and cell adhesion (17,19). In the HAPLN family, HAPLN3 is the most abundant and widely expressed in most tissue, including that of the mammary gland, ovary, lymph node, spleen, thymus, heart and lung (19,20), and is believed to play an important role in the construction and stabilization of hyaluronan-dependent extracellular matrix (19). It has been reported that proteoglycan promotes cancer cell mobility and migration through signal transduction by

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binding to cell membrane, which relies on the migratory pathway created by migrating cells themselves and cells of their surrounding tissues, and the connection of proteoglycan and hyaluronan by HAPLN (11). This suggests that over-expression of CLDN16 and HAPLN3 may be related to breast cancer development and metastasis.

The roles of CLDN16 and HAPLN3 in the development of breast cancer have not been fully clarified. We collected 146 samples from breast cancer tissues and their adjacent normal breast tissues to estimate the gene expressions of CLDN 16 and HAPLN3 between non-cancer and cancer tissues using reverse transcription (RT) and real-time polymerase chain reaction (real-time PCR). The association among the expression of both genes, clinicopathological parameters, hormone-related factors, and overall survival of breast cancer patients in relation to increased CLDN16 or HAPLN3 gene expression was also evaluated.

Materials and methods

Subjects and specimen collection. Seventy-three patients with breast infiltrating ductal carcinoma were recruited for our study, and the breast tissue specimens were obtained from patients who underwent surgical treatment at the Department of Surgery at Changhua Christian Hospital between 2002 and 2007. One hundred and forty-six tissue specimens, collected from breast cancer tissues and their adjacent normal breast tissues, were immediately frozen in liquid N₂ and stored at -80°C until further analysis. The histological types of the primary tissues and the clinicopathological stage of the breast cancer were determined by pathology, according to a system based on a modification of the WHO classification and the TNM system, respectively. Associated clinicopathological characteristics, such as clinical stage of breast cancer, cell differentiation status, lymph node metastasis, and distant metastasis, were verified by chart review. The study was performed with the approval of the Changhua Christian Hospital Institutional Review Board and informed written consent was obtained from each individual.

Tissues RNA extraction. Total RNA were extracted from frozen breast cancerous tissues and their adjacent normal breast tissues using RNAzol-B (RNA-Bee™, Tel-Test, Protech Technology Enterprise Co., Ltd., USA), following the instructions in the manual. Tissue samples were homogenized in RNA-Bee. Chloroform was added per 1 ml of RNA-Bee, was vigorously shaken for 15-30 min, and then centrifuged at 4°C. The aqueous phase was transferred to a clean tube, isopropanol added, and the samples stored overnight at -20°C. Then, the samples were centrifuged at 12,000 x g for 5 min at 4°C. RNA precipitate formed a white-yellow pellet at the bottom of the tube. The supernatant was removed and the RNA pellet was washed once with 75% ethanol, and then was centrifuged at 7,500 x g for 5 min at 4°C, and this wash process was repeated twice. At the end of the procedure, the RNA pellet was air-dried briefly for 5-10 min, and then was dissolved in diethyl pyrocarbonate-H₂O and stored at -80°C until reverse transcription.

Generation of cDNA. Total RNA was used to prepare complementary DNA (cDNA) using RT. The reaction mixtures contained RNA, Oligo(dT)₁₅ (Promega, USA) and random primer (Protech Technology Enterprise Co., Ltd.); the volume was adjusted with DEPC-H₂O, heated at 70°C for 5 min, and placed on ice for 3 min. Then, reverse transcriptase mixtures which contained MMLV (Protech Technology Enterprise Co., Ltd.), dNTPs (Protech Technology Enterprise Co., Ltd.), DTT (Protech Technology Enterprise Co., Ltd.), and rRNasin (human ribonuclease inhibitor, Protech Technology Enterprise Co., Ltd.) were added. The reaction was incubated at 37°C for 4 h. The concentration of cDNA was measured by spectrophotometer (BioPhotometer 6131, Eppendorf, Germany) and adjusted to 150 ng/ml for real-time PCR.

Quantitative real-time PCR analysis. CLDN16 and HAPLN3 genes were quantitatively examined for expression levels by real-time PCR using the TaqMan probe real-time PCR assay (Roche Diagnostic, Germany). *Homo sapiens* ribosomal protein S18 (RPS18) was used in the study as an endogenous control gene. Two of the most commonly used endogenous control genes for breast cancer gene expression studies are glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and β -actin (ACTB). The following primer pairs were used for CLDN16 and HAPLN3 gene transcripts: CLDN16 (forward primer, 5'-CTGGGTCTCTGGGTTGCTTT-3'; reverse primer, 5'-TTTCTCTCAGGTCCAACATCTTT-3'; amplicon length 82 bp), HAPLN3 (forward primer, 5'-TTACCGCTGTGAGGTCATTG-3'; reverse primer, 5'-GGGACTGGTAAGGA AAGACCA-3'; amplicon length 89 bp). The amplification of the target genes was as follows: 95°C 10 min, followed by 50 cycles of 95°C for 15 sec and 58°C for 1 min. Each sample was calculated by three repeated analyses.

Immunohistochemistry analysis. Gene expression of ER, PR, and HER2 in cancerous tissue was analyzed based on standard immunohistochemistry. Immunostaining was performed with anti-ER (Dako, ID5), anti-PR (Dako, PGR 636), and anti-HER2 (Dako, Carpinteria, CA), respectively, using an autostaining and semiquantitative scoring system (Ventana Medical System, Inc., AZ, USA). The status of hormone-related receptors was evaluated according to breast pathology guidelines. For estimation of ER and PR gene expression, analysis in which a value was <10% of expression was considered as a negative staining for hormone receptor, and a percentage of expression equal to or >10% of expression was considered as a positive staining. An expression percentage between 10% and <50% was scored as grade 1, between 50 and 75% was scored grade 2, and >75% was scored grade 3. In the assessment of the staining of HER2, no staining or membrane staining <10% of invasive tumor cells was considered a negative and scored grade 0; a barely perceptible membrane staining detected in >10% of invasive tumors was also considered a negative staining and scored grade 1; a weak to moderate complete membrane staining in >10% of invasive tumor cells or <30% with strong complete membrane staining was considered an equivocal staining and scored grade 2; a strong complete membrane staining in >30% of invasive tumor cells was considered a positive



Gene	Normal tissues (n=73)			Tumor tissues (n=73)			P-value
	Mean ± SE	Min, Med, Max	CV of intra-assay (%)	Mean ± SE	Min, Med, Max	CV of intra-assay (%)	
CLDN16	0.59±0.26	0.00, 0.00, 11.25	5.95	8.29±0.82	0.00, 10.49, 18.92	14.75	p<0.0001
HAPLN3	3.76±0.57	0.00, 0.00, 13.64	13.19	8.84±0.58	0.00, 10.41, 17.29	6.28	p<0.0001

The significant differences were analyzed using the Wilcoxon signed-rank test. p-value <0.05 was considered significant. Min, minimum; Med, median; Max, maximum; CV of intra-assay, the variation coefficients of intra-assay.

staining and scored grade 3. In score 2+ cases, fluorescent *in situ* hybridization was performed and the HER2 status was judged on this basis.

Statistical analysis. Experimental results are presented as the mean ± SE, minimum, median, and maximum. The variation coefficients of the intra-assay were estimated for the validation of precision. A non-parametric method was used since the distribution of experimental results was not normal for some variables. A Wilcoxon signed-ranks test was used for evaluation of gene expression in normal and tumor tissues of patients with breast cancer. The comparison of absolute increased gene expression levels between two compared clinical stages was estimated using the Mann-Whitney U test. The correlations were examined with Spearman's rank correlation. The Kaplan-Meier curve model was used for univariate analysis to identify factors most significantly related to overall survival (December 31, 2008). A p-value <0.05 was considered significant. The data were analyzed using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA) statistical software.

Results

Gene expression profiles of CLDN16 and HAPLN3 in tumor tissues versus normal tissues. The mean age (± SE) of our 73 recruited patients with breast cancer was 58.68±1.50 years. The gene expression profiles of CLDN16 and HAPLN3 were determined in tumor tissues with breast cancer, and compared with their adjacent normal breast tissues. Triplicate experiments were carried out for each pair of RNA samples to minimize technical variations and for statistical analysis. The experimental results of mean ± SE, minimum, median, and maximum, and the variation coefficients of the intra-assays are shown in Table I. There were significantly increased gene expression (mean ± SE) of CLDN16 (normal tissues, 0.59±0.26; tumor tissue, 8.29±0.82; p<0.0001) and HAPLN3 (normal tissues, 3.76±0.57; tumor tissue, 8.84±0.58; p<0.0001) among breast cancer samples compared with their adjacent normal breast tissues.

The comparisons of gene expression levels between tumor tissues and normal tissues in different clinical stages. The

significantly different gene expression levels of CLDN16 and HAPLN3 in tumor tissues compared with normal tissues were analyzed based on different clinical stage, respectively, and the results are shown in Table II. There were significantly increased gene expression levels of CLDN16 and HAPLN3 in breast tumor tissues compared with their adjacent normal breast tissues when we analyzed those based on different clinical stages, cell differentiation, lymph node metastasis, and distant metastasis.

The comparisons of absolute increased gene expression levels between different clinical statuses. The absolute increased gene expressions were estimated based on subtracting the gene expression levels of normal tissues from the levels of tumor tissues. The absolute increased gene expression levels (mean ± SE) of CLDN16 were 7.42±0.93 in clinical stage ≤II, 8.47±1.80 in clinical stage ≥III, 6.90±1.04 in good to moderate cell differentiation, 9.23±1.36 in poor cell differentiation, 8.37±1.08 in non-lymph node metastasis, 6.73±1.29 in lymph node metastasis, 7.62±0.90 in non-distant metastasis, and 8.15±2.32 in distant metastasis of each separate group, respectively. In addition, the absolute increased gene expression levels of HAPLN3 were 4.59±0.91, 6.43±1.56, 4.88±1.07, 5.44±1.06, 4.69±1.00, 5.62±1.29, 5.03±0.85, and 5.33±2.14 in each of the above ordinal classifications, respectively. Subsequently, the comparisons of absolute increased gene expression levels of CLDN16 and HAPLN3 between each paired clinical status, such as clinical stage ≤II versus clinical stage (III, good to moderate cell differentiation versus poor cell differentiation, non-lymph node metastasis versus lymph node metastasis, and non-distant metastasis versus distant metastasis were analyzed, respectively. There was no significant difference in absolute increased gene expression levels between each paired comparison for both the CLDN16 gene (Fig. 1A) and the HAPLN3 gene (Fig. 1B).

The correlations between absolute increased gene expression levels and the intensity of hormone-related receptor staining. The correlations between absolute increased gene expression of CLDN16 and absolute increased HAPLN3, estrogen receptor staining density, and progesterone receptor staining density, as well as the correlation between absolute increased HAPLN3 and human epidermal receptor 2 staining density,

Table II. Gene expressions of CLDN16 and HAPLN3 in tumor tissues and normal tissues of 73 patients with breast cancer in different clinical stages.

Variable	Gene	Normal tissues (n=73)		Tumor tissues (n=73)		P-value
		Mean ± SE	Min, Med, Max	Mean ± SE	Min, Med, Max	
Stage ≤II (n=54; 73.9%)	CLDN16	0.80±0.35	0.00, 0.00, 11.25	8.23±0.93	0.00, 10.62, 18.92	p<0.0001
	HAPLN3	4.41±0.70	0.00, 0.00, 13.64	9.01±0.65	0.00, 10.30, 17.29	p<0.0001
Stage ≥III (n=19; 26.1%)	CLDN16	0.00±0.00	0.00, 0.00, 0.00	8.47±1.80	0.00, 10.49, 18.61	p=0.003
	HAPLN3	1.91±0.81	0.00, 0.00, 11.81	8.34±1.30	0.00, 10.82, 15.71	p=0.004
Well to moderate differentiation (n=48; 65.7%)	CLDN16	0.36±0.26	0.00, 0.00, 10.57	7.26±1.07	0.00, 7.12, 18.61	p<0.0001
	HAPLN3	3.26±0.68	0.00, 0.00, 13.64	8.15±0.81	0.00, 9.98, 17.29	p<0.0001
Poor differentiation (n=25; 34.3%)	CLDN16	1.03±0.58	0.00, 0.00, 11.25	10.26±1.17	0.00, 11.33, 18.92	p<0.0001
	HAPLN3	4.72±1.04	0.00, 0.00, 13.41	10.17±0.60	0.00, 10.86, 16.13	p<0.0001
No lymph node metastasis (n=43; 58.9%)	CLDN16	0.59±0.34	0.00, 0.00, 11.25	8.97±1.03	0.00, 11.48, 18.92	p<0.0001
	HAPLN3	4.43±0.80	0.00, 0.00, 13.64	9.13±0.69	0.00, 10.41, 17.29	p<0.0001
Lymph node metastasis (n=30; 41.1%)	CLDN16	0.58±0.41	0.00, 0.00, 10.57	7.32±1.35	0.00, 8.26, 18.61	p<0.0001
	HAPLN3	2.80±0.78	0.00, 0.00, 11.81	8.42±1.03	0.00, 10.36, 16.13	p=0.001
No distant metastasis (n=63; 86.3%)	CLDN16	0.68±0.30	0.00, 0.00, 11.25	8.31±0.89	0.00, 10.13, 18.92	p<0.0001
	HAPLN3	4.09±0.63	0.00, 0.00, 13.64	9.13±0.62	0.00, 10.82, 17.29	p<0.0001
Distant metastasis (n=10; 13.7%)	CLDN16	0.00±0.00	0.00, 0.00, 0.00	8.15±2.32	0.00, 11.02, 18.26	p=0.02
	HAPLN3	1.68±1.14	0.00, 0.00, 9.81	7.01±1.63	0.00, 8.87, 12.92	p=0.04

The significant differences were analyzed with the Wilcoxon signed-rank test. The p-value <0.05 was considered significant. Min, minimum; Med, median; Max, maximum.

are shown in Fig. 2. The significant correlations were 0.5 ($p<0.0001$) between absolute increased CLDN16 and HAPLN3, -0.46 ($p<0.0001$) between absolute increased CLDN16 and estrogen receptor staining density, -0.384 ($p=0.001$) between absolute increased CLDN16 and progesterone receptor staining density, and 0.24 ($p=0.04$) between absolute increased HAPLN3 and human epidermal receptor 2 staining density, respectively.

The association between overall survival and gene expression. During a mean follow-up of 53.8 months (range, 8.4-64.5), 11 (15.1%) of the 73 patients had died. Overall survival was 84.9% in our 73 recruited patients, and in the subgroups, overall survival was 86.0 and 83.3% in the over- and non-overexpression of CLDN16 subgroups, and 83.0 and 88.5% in the over and non-overexpression of HAPLN3 subgroups, respectively. The Kaplan-Meier method found no significant difference in overall survival distributions (CLDN16, $p=0.75$; HAPLN3, $p=0.52$, respectively) between the over-

and non-overexpression subgroups of CLDN16 and HAPLN3 (Fig. 3).

Discussion

CLDN16 is a 36 kDa tight junction protein which has four transmembrane domains and intercellular N- and C-termini, and plays an important role in maintaining the polarity and barrier function of various epithelia (13-16,21). Recently, it has been hypothesized to play a role in carcinogenesis (17,18,22). For breast epithelial cells, the role of the claudin family on breast cancer is still equivocal. Overexpression of CLDN4 was found in breast tumor cells compared to normal epithelial cells, irrespective of tumor size, presence or absence of lymph node metastasis, and histology type (23,24). However, significant down-expression of CLDN2 was found in advanced breast carcinoma (25). In the present study, we found a significantly increased gene expression of CLDN16 in breast cancer tissues compared to their adjacent

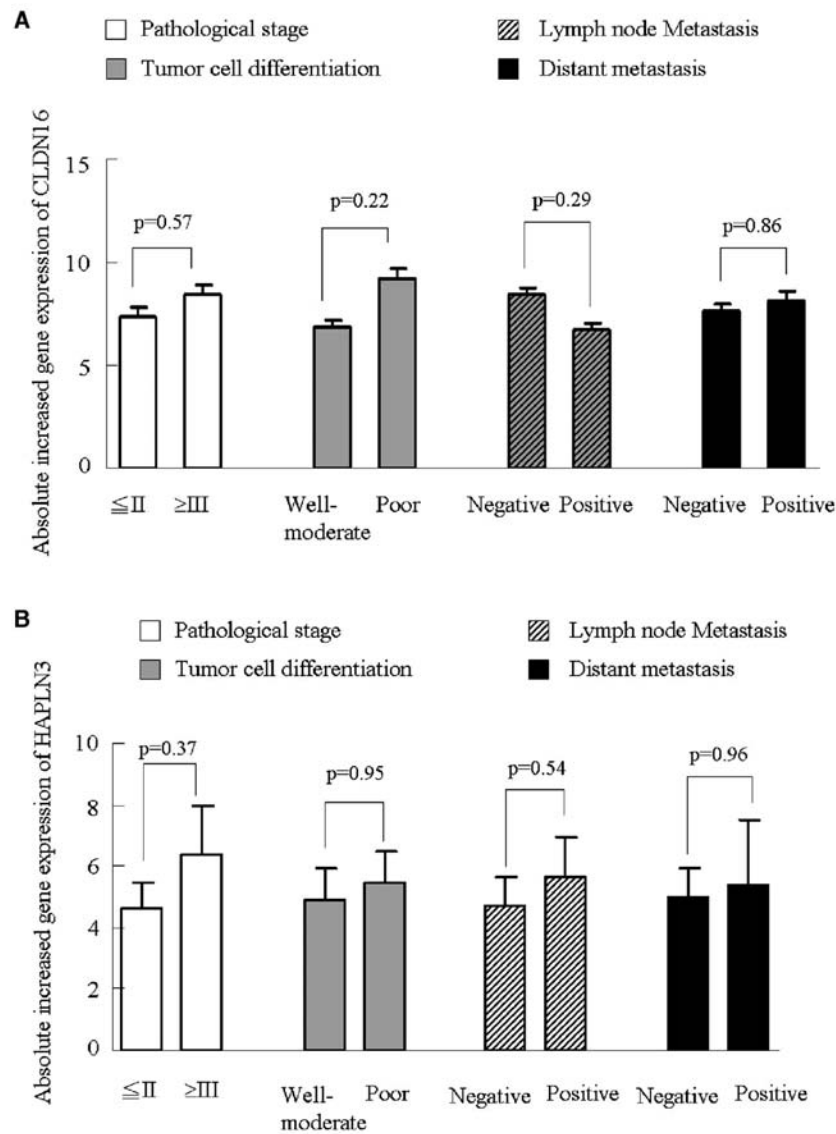


Figure 1. The comparisons of absolute increased gene expression levels of CLDN16 and HAPLN3 between two compared clinical stages were estimated using the Mann-Whitney U test. There was no significant difference of absolute increased gene expression levels between each paired comparison for either the CLDN16 gene (A) or the HAPLN3 gene (B).

normal breast tissues, irrespective of clinical pathological parameters, such as clinical stage, cell differentiation, lymph node metastasis, and distant metastasis among the 73 recruited subjects. Our results are similar to those of Rangel *et al* and Fluge *et al*. In their studies, they found up-expressions of CLDN16 in human tissues with ovarian cancer (17) and papillary thyroid carcinomas (18), respectively. Moreover, up-expression of CLDN16 was considered to be correlated with the alteration of cell polarity in dedifferentiated papillary thyroid carcinomas (18). To the best of our knowledge, only one study (26) has demonstrated that CLDN16 induced changes in cell morphology, increased trans-epithelial persistence, reduced paracellular permeability, and reduced the invasive phenotypes of human breast cancer cells after transfection of CLDN16 gene into those cells (27). The authors also carried out an immunohistological assay to estimate the degree of staining of CLDN16 using 10 pairs of matched human breast tissues (tumor and normal tissue), and found that the staining for

CLDN16 within the tumor sections was significantly decreased compared to normal tissues. However, they found no significantly different gene expression between tumor and normal tissue at the mRNA level using quantitative PCR. We consider that the small sample sizes of their study (28) was the reason for the conflicting result in their data and between their data and ours. Although the CLDN16 gene was transfected into the cell lines to investigate the possible mechanism of CLDN16-induced morphological change, phenomenon of gene transfection in an *in vitro* study is limited in fully revealing the fact phenomena of human breast tissue. Numerous other explanations for the different findings are possible, including genetic variance in different racial populations, different age distributions between the two studies, environmental factors, and interaction between CLDN16 and the linked gene.

Recently, link proteins have been considered to be associated with the mobility and migration of cancer cells (11). HAPLN3, a novel link protein, is widely found in

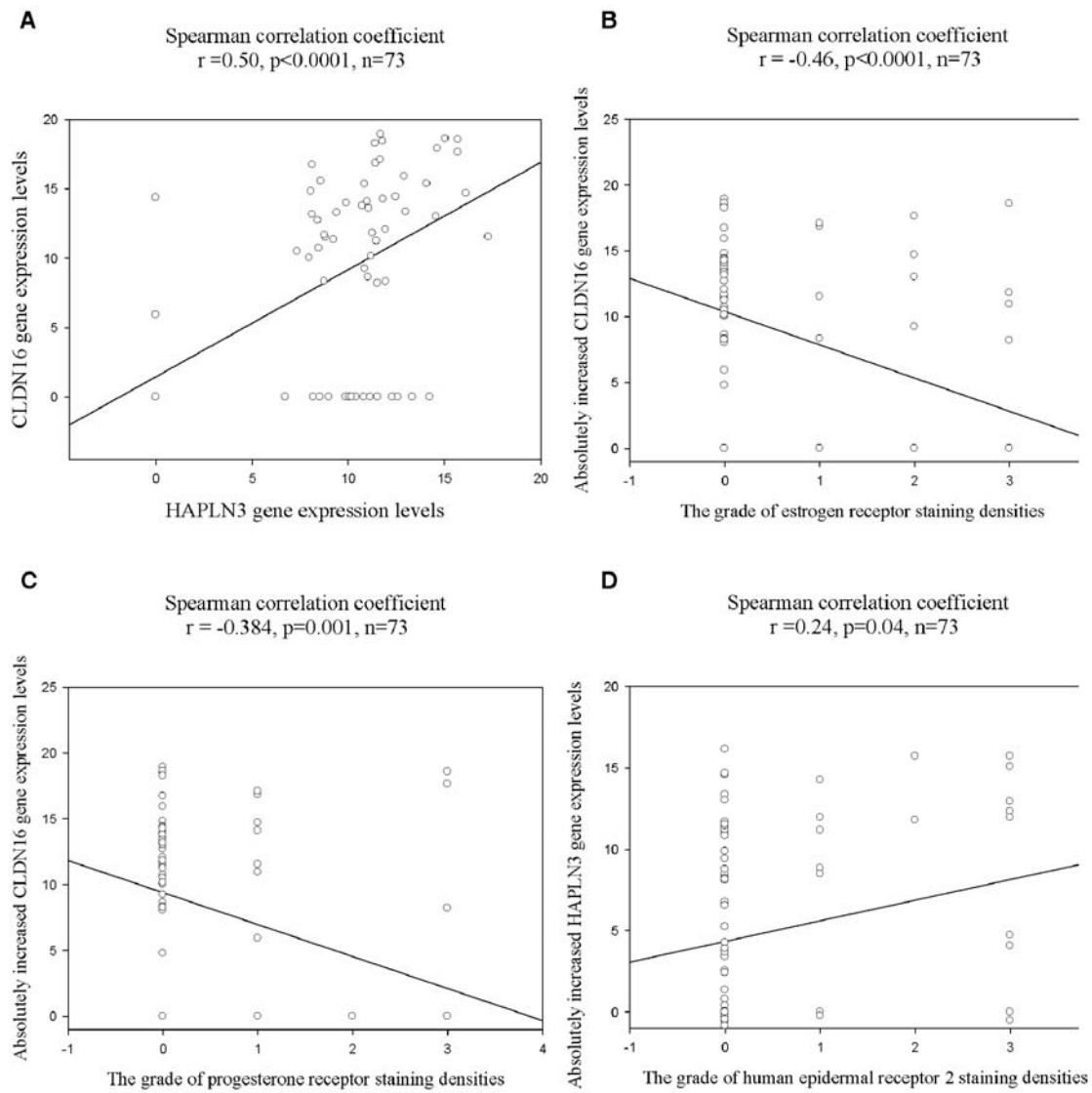


Figure 2. The significant correlations between each parameter were estimated using Spearman's rank correlation. (A) The significant correlation between CLDN16 and HAPLN3 ($r=0.5$; $p<0.0001$) gene expressions. (B) The significantly negative correlation between the absolute increased gene expression level of CLDN16 and estrogen receptor staining density ($r=-0.46$; $p<0.0001$). (C) The significantly negative correlation between the absolute increased gene expression level of CLDN16 and progesterone receptor staining density ($r=-0.384$; $p=0.001$). (D) The significantly positive correlation between the absolute increased gene expression level of HAPLN3 and human epidermal receptor 2 staining density ($r=0.24$; $p=0.04$).

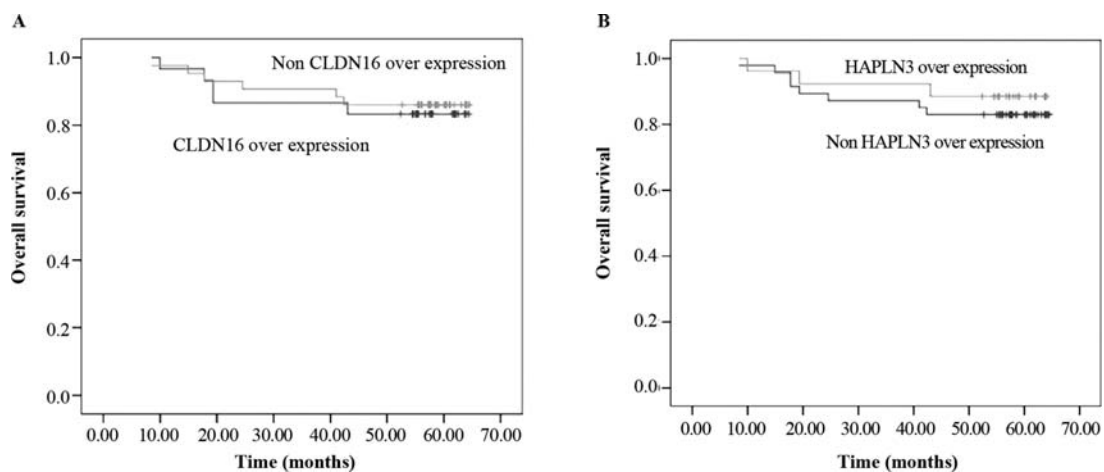


Figure 3. Kaplan-Meier curves for overall survival in patients with breast cancer based on over versus non-overexpression of CLDN16 (a, $p=0.75$) and HAPLN3 (b, $p=0.52$), respectively. The log-rank test was used for statistical significance.

SPANDIDOS/ gland, ovary, lymph node, spleen, thymus, heart
PUBLICATIONS tissues (19,20). To our knowledge, this is the first

study to investigate the role of HAPLN3 in breast cancer. We found that the HAPLN3 gene had significantly increased expression in breast cancer tissues compared to normal breast tissues, but was not associated with clinicopathological parameters, such as clinical stage, cell differentiation, lymph node metastasis, and distant metastasis. Moreover, the absolute increased gene expression level of HAPLN3 was significantly correlated with that of CLDN16. The role of HAPLN3 in carcinogenesis still remains to be determined. Only Ogawa *et al* have reported a coordinated up-regulation of HAPLN3 and versican by platelet-derived growth factor in primary cultured rat arterial smooth muscle cells (20). Versican, expressed in the extracellular matrix of epithelial and non-epithelial tumors, is a large chondroitin sulfate proteoglycan and is considered to play a role in the development of breast cancer by enhancement of cell proliferation, differentiation, adhesion, and migration, as well as having potential effects on angiogenesis (26-29). Moreover, it has been suggested that hyaluronan, conjugated with HAPLN3 for aggregating with proteoglycan, had significantly increased expression in malignant breast tumors, either through secretion of diffusible factors or cell-cell contact. Subsequently, the up-expression induced the accumulation of hyaluronan and contributed to cancer cell mobility by forming an expanded space, and enhanced tumor growth by inducing vascularization (30). We suggested that up-expression of HAPLN3 could contribute to the promotion of cell proliferation, migration, and angiogenesis by cooperation with versican or hyaluronan. Moreover, over-expression of CLDN16 dominated the alteration of cell polarity in breast cancer (9,18,31), and mutual up-expression of both CLDN16 and HAPLN3 could contribute to the development of breast cancer, although those mechanisms require further clarification.

The role of hormone-related receptors in breast cancer is still controversial. Nevertheless, it has been demonstrated that an invasive grade of breast cancer was negatively correlated to ER and PR expression (23,31,32), but positively correlated with HER2 expression (33,34). Similar to Munoz-Guerra *et al* (22) and Lanigan *et al* (24), we found that the absolute increased gene expression level of CLDN16 was significantly negatively correlated with estrogen and progesterone receptor staining density. However, a significantly positive correlation between absolute increased HAPLN3 and human epidermal receptor 2 staining density, and an insignificant association between overall survival and both gene expression levels were found.

In view of this study, up-regulation of both CLDN16 and HAPLN3 is suggested to be involved in the development of breast cancer. Despite the fact that the exertion of both genes on breast cancer is unclear, we have provided a novel finding for further application in the biomarker and targeted treatment of breast cancer.

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