

Reduced Expression of Claudin-7 Correlates with Invasiveness and Nuclear Grade of Breast Carcinomas

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Background : Claudins are important components of the tight junctions in the intercellular barriers and cell polarity. Among them, claudin-7 is down-regulated in breast cancers compared with the normal breast epithelium. The aim of this study was to determine the expression pattern and prognostic value of claudin-7 in breast carcinomas. **Methods :** Claudin-7 expression was evaluated immunohistochemically in 42 cases of ductal carcinoma in situ (DCIS) and in 142 cases of invasive breast carcinoma (IBC) using a tissue microarray (TMA). **Results :** Claudin-7 was strongly expressed in the normal luminal epithelial cells in the breast lobule. The level of claudin-7 expression was significantly lower or absent in 45.2% (19/42) of DCIS and 72.5% (103/142) of IBC. A loss or reduced expression of claudin-7 correlated with the invasiveness ($p=0.001$) of breast carcinomas and a high nuclear grade ($p=0.013$) in IBC. **Conclusion :** Claudin-7 is an important tight junction protein in the breast and a loss of expression may assist in the dissociation and invasion of tumor cells.

Key Words : Claudin-7; Breast cancer; Immunohistochemistry; Microarray analysis

Adherens junctions and tight junctions are the two main types of junctions that maintain the cell-to-cell adhesion in epithelial cell sheets. It is widely accepted that a loss of cell-to-cell adhesion in neoplastic cells is essential for an invasion of the surrounding stromal elements and subsequent metastatic events. E-cadherin is a major functional component of the adherens junctions and has also been examined extensively in various types of cancer. A correlation between the loss of E-cadherin expression and the metastatic potential and poor prognosis of invasive breast carcinomas has been reported.¹⁻³ Tight junctions consist of two main molecular components, occludin and claudins, which have important roles in controlling paracellular transport and in maintaining the cell polarity. While occludin does not have any subtypes, the claudin family consists of approximately 23 proteins and appears to be more important for the selective and site specific functions of the tight junctions.^{4,5}

Normal cells typically express multiple claudin proteins. However, some family members exhibit a tissue specific distribution. For example, in mice, claudin-2 is not found in the lung tissue but is found in the liver and kidney, while claudin-4 is found in the lung and kidney but not in the liver.⁶ Claudin-5 is the main

form of the claudin protein in endothelial cells.⁷ While there have been relatively few histopathological studies on the expression of the tight junction protein in the tumor tissues, changes in the claudin proteins have been observed in several types of cancer. Claudin-3 and -4 are overexpressed in breast, ovary and prostate cancers.⁸⁻¹⁰ Claudin-4 is overexpressed in pancreatic cancer.¹¹ On the other hand, claudin-7 is underexpressed in breast and head and neck cancer but overexpressed in stomach cancer.¹²⁻¹⁴ Since claudin-7 is down-regulated in breast cancer compared with the normal breast epithelium¹² and its decreased expression in cancer cells is consistent with the generally accepted hypothesis that tumorigenesis is accompanied by a disruption of the tight junctions, it was hypothesized that a loss of expression might play a role in the process of invasion and metastasis of breast cancer cells by allowing the liberation of individual cancer cells from the primary tumor. Therefore, we examined the expression of the tight junction protein, claudin-7, in ductal carcinoma in situ (DCIS) and invasive breast carcinoma (IBC) immunohistochemically, and correlated the results with the clinicopathological parameters i.e. the invasiveness, tumor size, histological grade, lymph node status, stage, and patients' survival, to determine

the expression pattern and prognostic value of claudin-7 in breast carcinomas.

MATERIALS AND METHODS

Case selection and clinicopathological parameters

A total of 179 IBC and 42 DCIS samples were obtained from patients who had undergone surgery between 1995 and 1997 at Yeungnam University Hospital. Among the archives, consecutive cases with well-preserved representative tumor blocks and clinical follow-up data were selected. The samples were fixed in 10% buffered formalin and embedded in paraffin. Information on the clinicopathological parameters of IBC: age at initial diagnosis, tumor size, histological subtype, histological grade, nuclear grade, number of positive lymph nodes, tumor stage, tumor recurrence and distant metastasis, patients' overall and the disease-free survival, were obtained from the pathology reports and patients' medical records. The histological grade of IBC was assessed according to a modified Bloom-Richardson-Scarff grading system,¹⁵ and the nuclear grade of DCIS was evaluated according to the criteria presented at a consensus conference on the classification of ductal carcinoma in situ.¹⁶

Construction of tissue microarrays (TMAs)

All the hematoxylin-eosin-stained slides for each case were reviewed and representative tumor regions were selected for the TMA construction. The TMAs were constructed in our laboratory using a 2.0 mm diameter dermal punch biopsy needle. To account for the cancer tissue heterogeneity, 2 cylindrical core biopsies were carefully taken from different sites of each tumor block and arrayed in two different recipient TMA blocks. For DCIS, a whole tissue section was used for the immunohistochemical staining of claudin-7 in order to overcome the tumor heterogeneity. Samples of the normal colonic mucosa, gastric mucosa, liver, kidney and prostate were contained in the TMAs as internal controls. Sixty-four tumor tissues and 8 control tissues were arrayed in a single recipient TMA block.

Immunohistochemistry

Immunohistochemical staining for claudin-7, estrogen receptor (ER), progesterone receptor (PR), HER2, and p53 was performed on the TMA sections in IBC. Claudin-7 staining was

performed on the whole tissue sections in DCIS. Four-micrometer tissue sections were cut from the recipient TMA blocks and mounted on poly-L-lysine-coated slides. The sections were deparaffinized in xylene and hydrated in a graded series of alcohol. Heat-induced epitope retrieval was performed at 120°C for 10 min in an ethylene diamine tetraacetic acid buffer, pH 8.0. The endogenous peroxidase activity was inactivated by incubation in 4% H₂O₂ for 5 min. After rinsing the sections in phosphate-buffered saline, the nonspecific binding sites were blocked by incubating them in normal goat serum for 6 min. Tissue sections were then incubated with the primary antibodies for claudin-7 (1:70; Zymed, San Francisco, CA, USA), ER (1:50; Zymed, San Francisco, CA, USA), PR (1:60; Zymed, San Francisco, CA, USA), and p53 (1:200; Novocastra, Nottingham, UK) for 60 min at room temperature, and then treated with a DAKO EnVision Plus-HRP detection kit (DAKO, Glostrup, Denmark) according to the manufacturer's instructions. The level of HER2 expression was evaluated using a HercepTest kit (DAKO, Carpinteria, CA, USA).

Membranous staining for claudin-7 was considered in the evaluation, and the result was scored on a semiquantitative scale from 0 to 3+ as follows: 0, no staining; 1+, <50% cells positive and incomplete membranous staining; 2+, 50-75% cells positive and complete or incomplete membranous staining; 3+, >75% cells positive and complete membranous staining. Zero and 1+ were considered reduced expression (negative).

Tumors were considered to be positive for ER, PR, and p53 when nuclear reactivity was observed in more than 10% of the tumor cells at any intensity. For HER2, only those cases with a membranous staining score of 3+ were considered positive.¹⁷

Statistical analysis

Statistical analysis was performed using SPSS for Windows version 11.0. The correlation between claudin-7 expression and the clinicopathology parameters was evaluated using a χ^2 test. The survival curves were drawn using the Kaplan-Meier method and the differences were assessed using a log-rank test. A *p* value <0.05 was considered significant.

RESULTS

Characteristics of tissue samples

For IBC, only 142 cases had interpretable cores for the immu-

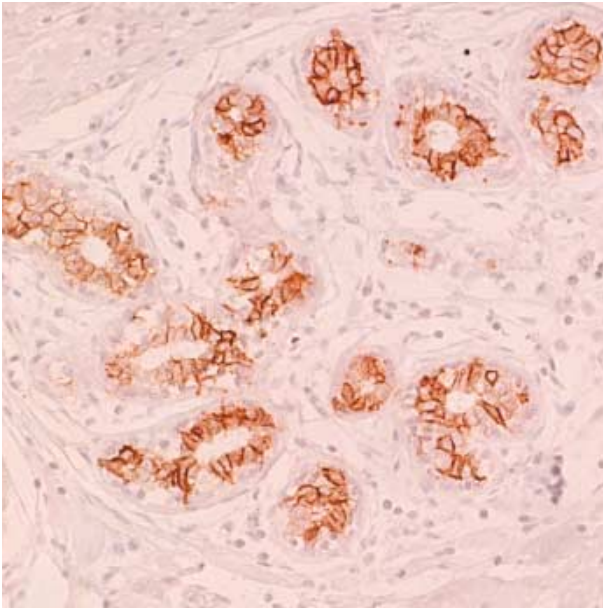


Fig. 1. Luminal epithelial cells in the normal breast lobule show strong expression for claudin-7 immunostain.

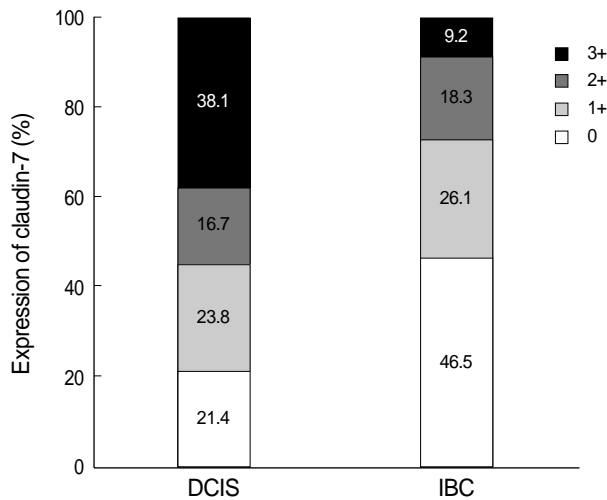


Fig. 3. Frequencies of claudin-7 expression according to staining pattern are statistically different between ductal carcinoma in situ (DCIS) and invasive breast carcinoma (IBC) ($p=0.001$ by χ^2 test).

nohistochemical stains. The analysis was performed on 142 informative cases. The patients' age ranged from 24 to 77 years (mean, 46.4 years). The tumor sizes varied from 0.2 to 10 cm, with a mean size of 2.6 cm. Fifty-five (38.7%) tumors were pT₁, 78 (54.9%) were pT₂, and 9 (6.3%) were pT₃. The histological types of the 142 IBC were 133 (93.7%) invasive ductal carcinomas, NOS, 3 (2.1%) mucinous carcinomas, 3 (2.1%) invasive lobular carcinomas, 2 (1.4%) medullary carcinomas, and 1 (0.7%) invasive micropapillary carcinoma. Information on the histological grade was obtained for 124 tumors: 23 (18.5%) were

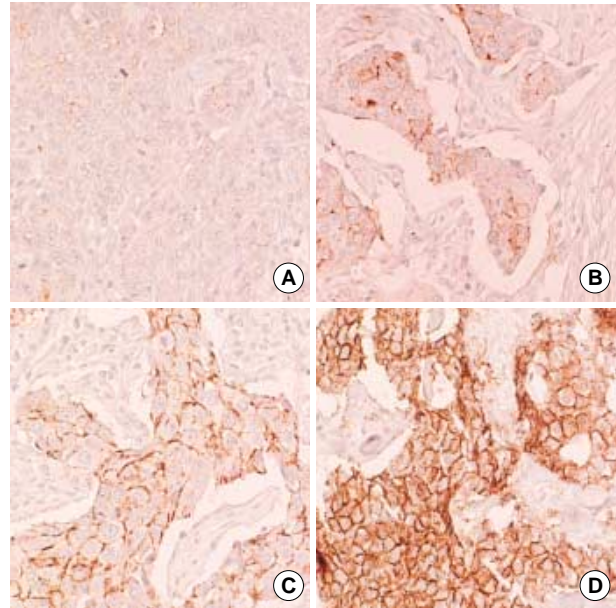


Fig. 2. Invasive breast carcinomas show various expression for claudin-7. We scored immunostains semiquantitatively as 0 (A), 1+ (B), 2+ (C), and 3+ (D).

grade 1, 42 (33.9%) were grade 2, and 59 (47.6%) were grade 3. The nuclear grade was obtained for 140 tumors: seven (5%) were grade 1, 37 (26.4%) were grade 2 and 96 (68.6%) were grade 3. All the IBC were treated surgically, which consisted of a lumpectomy with an axillary node dissection or modified radical mastectomy. At the time of surgery, 84 (59.1%) patients showed positive axillary lymph node(s). Regarding adjuvant therapy, 108 (76.1%) patients were treated with tamoxifen and 122 (85.9%) patients received chemotherapy. Radiotherapy was administered to 46 (32.4%) patients. The mean follow-up period was 79.4 months (range, 9-165 months). During the follow-up period, 10 patients suffered from local recurrences, 30 experienced distant metastasis and 24 died from breast cancer.

The patients' age of DCIS ranged from 33 to 74 years (mean, 47.3 years). There were 11 (26.2%) nuclear grade 1, 10 (23.8%) nuclear grade 2, and 21 (50%) nuclear grade 3 cases. Twenty-eight (66.7%) cases showed punctate or comedo necrosis. The tumors were classified by their architectural pattern as follows; 15 (35.7%) comedo, 7 (16.7%) cribriform, 10 (23.8%) micropapillary, 7 (16.7%) papillary, and 3 (7.1%) solid pattern.

Results of immunohistochemical stains in breast carcinomas

The normal breast epithelium showed diffuse staining (2+ or 3+) for claudin-7 (Fig. 1). Breast carcinomas, both DCIS and

Table 1. Relationship between reduced expression of claudin-7 and clinicopathological parameters in invasive breast carcinoma

	Cases with reduced expression of claudin-7 (%)	p
Tumor size (cm)		0.076
≤2	42/55 (76.4)	
2 < and ≤5	52/78 (66.7)	
> 5	9/9 (100)	
Lymph node metastasis		0.159
0	45/58 (77.6)	
1-3	39/52 (75.0)	
≥4	19/32 (59.4)	
Histological grade		0.093
1	13/23 (56.5)	
2	32/42 (76.2)	
3	47/59 (79.7)	
Nuclear grade		0.046
1	4/7 (57.1)	
2	22/37 (59.5)	
3	76/96 (79.2)	
Stage		0.431
I	21/27 (77.8)	
II	68/98 (69.4)	
III	14/17 (82.4)	
Vascular invasion		0.783
Present	28/40 (70.0)	
Absent	68/94 (72.3)	
ER		0.938
Positive	60/83 (72.3)	
Negative	43/59 (72.9)	
PR		0.019
Positive	46/72 (63.9)	
Negative	57/70 (81.4)	
HER2		0.744
Positive	21/28 (75.0)	
Negative	82/114 (71.9)	
P53		0.366
Positive	51/67 (76.1)	
Negative	52/75 (69.3)	
Recurrence/Metastasis		0.407
Yes	31/40 (77.5)	
No	72/102 (70.6)	
Death		0.425
Yes	19/24 (79.2)	
No	84/118 (71.2)	

IBC, showed aberrant claudin-7 expression (Fig. 2). Decreased claudin-7 expression (0 or 1+) was observed in 45.2% (19/42) and 72.5% (103/142) of DCIS and IBC, respectively. When the staining results were correlated with the invasiveness (DCIS vs IBC), the frequency of reduced claudin-7 expression was significantly higher in the IBC than DCIS ($p=0.001$) (Fig. 3). Considering only those cases with a high nuclear grade, there were significant differences in the frequency between IBC and DCIS (79.2% vs 52.4%, $p=0.01$).

Table 2. Relationship between reduced expression of claudin-7 and pathological parameters in ductal carcinoma in situ

	Cases with reduced expression of claudin-7 (%)	p
Nuclear grade		0.639
1	4/7 (36.4)	
2	4/10 (40)	
3	11/21 (52.4)	
Necrosis		0.293
Present	14/28 (50)	
Absent	5/14 (35.7)	
Architectural pattern		0.136
Comedo	10/15 (66.7)	
Cribriform	3/7 (42.9)	
Papillary	1/7 (14.3)	
Micropapillary	3/10 (30)	
Solid	2/3 (66.7)	

Correlation of claudin-7 expression with clinicopathological parameters of breast carcinoma

Table 1 gives a summary of the clinicopathological and immunohistochemical features of IBC according to the level of claudin-7 expression. A decrease in claudin-7 was correlated with the nuclear grade ($p=0.046$), and PR negativity ($p=0.019$). When IBC was divided into two nuclear groups, low to intermediate (NG 1 and 2) and high (NG 3) nuclear groups, claudin-7 expression was significantly lower in the high-grade tumors ($p=0.013$). There was no correlation between claudin-7 expression and the tumor size, lymph node metastasis, histological grade, stage, immunohistochemical results for ER, HER2 and p53, recurrence/metastasis or death. In DCIS, reduced expression of claudin-7 was observed more frequently in tumors with high-grade nuclei and necrosis. However, the differences were not significant. The architectural pattern of growth was not correlated with claudin-7 expression in DCIS even though the comedo type, which was frequently combined with tumor necrosis, showed a higher frequency of reduced claudin-7 expression (Table 2).

Claudin-7 expression and prognosis in IBC

Claudin-7 expression was not associated with the overall survival and disease-free survival (Fig. 4). Considering only those patients who had received adjuvant chemotherapy, claudin-7 expression was not associated with the patients' survival.

DISCUSSION

Cell-to-cell adhesion molecules are known to play an impor-

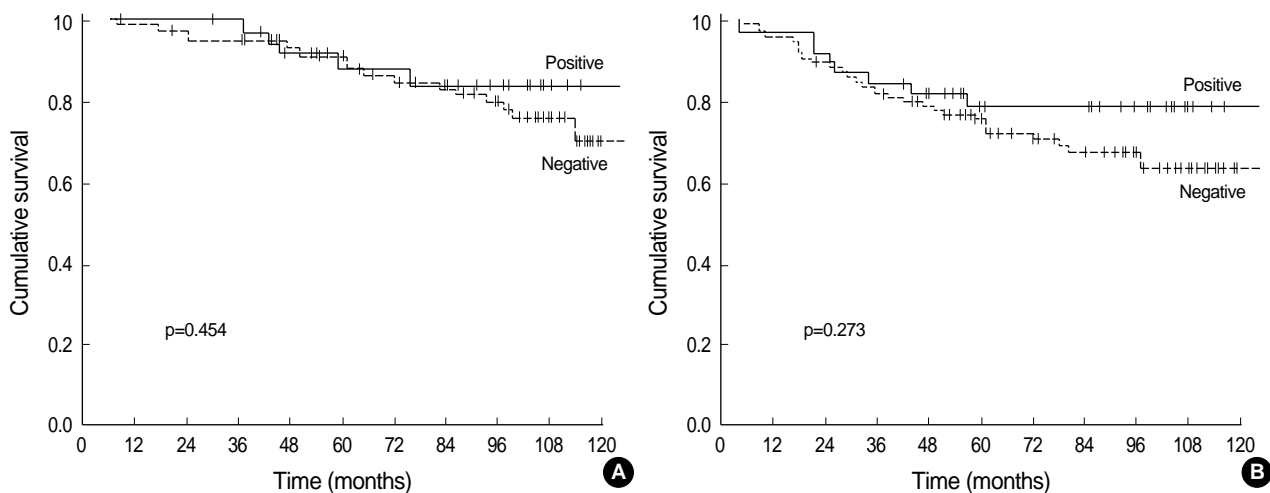


Fig. 4. Kaplan-Meier curves show overall (A) and disease-free survival (B) according to the expression of claudin-7 in invasive breast carcinomas.

tant role in tumor invasion and metastasis because cancer cells need to be detached from the primary tumor in order to establish a metastasis.^{1-3,18} Although the precise role of claudin-7 has not yet been established in breast cancer, its function in cell-to-cell adhesion as a tight junction molecule, suggests that a loss of expression might play a role in tumor progression and metastasis. In this study, the decrease in claudin-7 expression was observed in a high percentage of primary breast carcinomas, when compared with the normal mammary epithelial cells, which showed strong claudin-7 expression. It was concluded that a decrease in claudin-7 is a common event in breast carcinogenesis and is related to tumor progression. This assumption is supported by the fact that the frequency of reduced claudin-7 expression was significantly higher in IBC than in DCIS.

Previous studies reported that the claudin proteins are expressed in a site-specific pattern.^{7,8,19} In this study, claudin-7 expression was observed in the normal mammary and colon epithelia, but not in the gastric epithelium (data not shown). In tumor tissues, a decrease in claudin-7 expression was observed in a considerable number of breast carcinomas, while most colorectal carcinomas showed strong claudin-7 expression (data not shown). This suggests that claudin-7 functions differentially in breast and colon carcinogenesis, and would not be involved in colon cancer progression.

It was observed that the reduced expression of claudin-7 correlated with the nuclear grade, occurring more frequently in grade 3 tumors than in grades 1 or 2 tumors. This is consistent with a previous study reported by Kominsky *et al.*¹² but contrasts with the findings reported by Park *et al.*²⁰ Park *et al.*²⁰ suggested that their failure to demonstrate a correlation between claudin-

7 expression and the histological grade was due to the inclusion of not enough grade 1 tumors and all lymph node-positive cases. This study also included many more grade 3 tumors than grades 1 and 2 tumors. As Park *et al.*²⁰ postulated, case selection bias might contribute to the correlation between claudin-7 expression and the PR status. It was found that the frequency of reduced claudin-7 expression increased as the breast lesions progress from a low-grade to a high-grade, and from DCIS to IBC (38.1% in low-grade DCIS, 52.4% in high-grade DCIS, 59.1% in low-grade IBC, and 79.7% in high-grade IBC). Considering the process of breast cancer progression, this finding suggests that a loss of claudin-7 expression may potentiate tumor cell dissociation from each other and allow the successful invasion of the surrounding stroma in primary breast carcinomas. Claudin-7 expression was not evaluated in the metastatic lesions of the same patients. Park *et al.*²⁰ reported that invasive ductal carcinomas showed significantly higher expression levels of adhesion molecules (E-cadherin, α -catenin, β -catenin, γ -catenin, and claudin-7) in the metastases than in the primary tumors. Although the mechanism for the re-expression of the adhesion proteins in metastatic sites is unknown, they suggested that re-expression of the proteins enables the circulating tumor cells to resettle at the metastatic sites by facilitating the intercellular adhesion process for metastatic tumor progression. Kominsky *et al.*¹² reported a correlation between a loss of claudin-7 expression and the hypermethylation of the promoter in breast cancer cell lines, but not in primary breast carcinomas. These two studies suggest that claudin-7 expression is reversibly regulated so that breast carcinomas with reduced claudin-7 expression in the primary tumors can re-express claudin-7 in metastatic tumors. If irreversible genet-

ic or epigenetic events caused the reduced expression of claudin-7 in the primary tumor, its re-expression in the metastatic tumor of the same patient cannot be easily explained. Therefore, further studies on the mechanism of the reduced expression will be needed to reveal the precise role of claudin-7 in breast cancer progression.

Sauer *et al.*²¹ reported reduced claudin-7 expression in 87% (13/15) of invasive breast carcinomas with an axillary lymph node metastasis, and also reported that this loss is associated with distant metastatic disease and locoregional recurrence. Kominsky *et al.*¹² demonstrated a loss of claudin-7 expression in 77% (10/13) of grade 3 invasive ductal carcinomas and in 70% (7/10) of invasive ductal carcinomas with a lymph node metastasis. Kominsky *et al.*¹² attempted to demonstrate that claudin-7 expression in primary invasive ductal carcinomas is associated with the ability of the tumors to metastasize. They also performed immunostaining for claudin-7 on TMAs for a large number of breast carcinomas and found no correlation between claudin-7 expression and the ER, PR status, tumor size, or lymph node status. Their finding corresponds to our results, except for PR. They suggested that the lack of a correlation with the lymph node status was due to an error in TMA, an absence of an internal control and small sample from each tissue punch. However it is believed that TMA analysis is a high-throughput study that has been confirmed by previous studies,²²⁻²⁶ and the numbers of cases in their study and the present study (612 and 142, respectively) were large enough to validate the results. In addition, Park *et al.*²⁰ recently reported no correlation between reduced claudin-7 expression and the lymph node status in 196 invasive breast carcinomas. We present possible explanations for the absence of a correlation between claudin-7 expression and the lymph node status. First, the loss of one tight junction protein, claudin-7 only, might not have a significant impact in increasing the metastasis. Numerous claudins exist in the epithelial tissues, and some of them, claudin-1, -3, and -4, are found to be unchanged in breast carcinomas.¹² Therefore, another claudin family might compensate for the loss of claudin-7. Second, claudins may have more complicated functions in cancer cells. Claudin-7 has been found to be overexpressed in stomach cancer but down-regulated in breast and head and neck cancer.¹²⁻¹⁴ The overexpression of claudin-7 in stomach cancer can not be explained by its role in cell-to-cell adhesion and may pertain to roles that are unrelated to the tight junction formation.

The prognostic significance of claudin-7 expression in terms of the survival of breast cancer patients is unclear. We and Park *et al.*²⁰ found no association between claudin-7 expression in

primary breast tumors and the clinical outcome, even though there was a trend towards a shorter overall and disease-free survival of patients with reduced expression compared to those with strong expression. Further studies with a larger number of cases will be needed to validate the clinical significance of the reduced expression of claudin-7 in breast carcinomas.

In conclusion, the expression of the tight junction protein, claudin-7, was markedly lower in a majority of DCIS and IBC, occurring more frequently in IBC than DCIS and in high-grade than in low-grade tumors. Claudin-7 is an important tight junction protein in the breast and its loss of expression might assist tumor cells in the invasion of the stroma well. However, its relationship with the metastatic potential and prognosis requires further study.

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