Reduced expression of Claudin-7 in fine needle aspirates from breast carcinomas correlate with grading and metastatic disease

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Objective: To study the immunocytochemical expression of the tight junction protein Claudin-7 in smears from breast carcinomas and correlate with grading, nodal status, locoregional and distant metastases and the cellular cohesion.

Methods: The material consisted of 52 air-dried smears from fine needle aspirates of breast carcinomas, both primary and metastatic and smears from seven benign lesions. A primary antibody to Claudin-7 was used for immunocytochemical staining. The degree of staining was recorded as negative, reduced or full, with full expression meaning equivalent to the staining pattern found in the fibroadenomas used as benign control. Staining intensity and the percentage of stained cells were evaluated. The control smears revealed a strong membrane and cytoplasmic positivity in all luminal epithelial cells. Cellular cohesion was graded as: (1) mainly cohesive groups, (2) groups and single cells and (3) mainly single cells.

Results: All primary and recurrent/metastatic breast lesions expressed Claudin-7. Full expression was demonstrated in 46% of the cases. Reduced expression was found in 54%. In cases with reduced expression, the percentage of stained cells were usually high, and no smear showed <50% stained tumour cells. The staining pattern was heterogeneous and always mixed membrane/cytoplasmic. Claudin-7 expression showed a significant correlation ($P < 0.05$) with grading, locoregional and distant metastases, nodal involvement and cellular cohesion in invasive carcinomas, but not with tumour size or subtype.

Conclusion: Reduced expression of Claudin-7 correlated with higher tumour grade, metastatic disease, including loco-regional recurrences and with cellular discohesion.

Keywords: Claudin-7, tight junction, breast carcinomas, fine needle aspiration cytology, cell cohesion

Introduction

Metastasis is the primary cause of death from breast cancer. Numerous events are thought to contribute to the metastatic process, including loss of cell-to-cell adhesion in neoplastic cells. In breast epithelial cells adhesion in cell sheets is maintained mainly through two types of junctions, namely adherens junctions and tight junctions. Dysfunction of the adherens molecules has been implicated in the invasiveness and carcinogenesis of tumour cells,[1] and reduced expression of E-cadherin has been found to correlate with dedifferentiation, tumorigenicity and invasiveness. The cadherin-mediated cell adhesion system has been shown to act as an invasion suppressor system.[2] E-cadherin has been studied extensively in breast carcinomas and has been found to be reduced in a large number of cases.[3–9]

Claudins are a family of transmembrane proteins that seal tight junctions. They have two main functions. First they prevent the paracellular transport of solutes and ions for the purpose of maintaining paracellular transport. Second they prevent the diffusion of membrane proteins and lipids from the apical

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to the basolateral layer of the epithelial sheets, thus helping to maintain cell polarity.[10] Claudins have been found to consist of four transmembrane domains and two external loops through which they bind to Claudin molecules on adjacent cells.[11] They have been found to be the main sealing proteins of the tight junctions.[12] Their role in carcinogenesis has been scarcely studied. Kramer *et al.*[13] found decreased levels of claudin-1 cDNA in a number of breast tumours and breast cancer cell lines. In a recent study, Kominsky *et al.*[14] demonstrated loss of Claudin-7 to correlate with histological grade in both ductal carcinoma *in situ* (DCIS) and invasive ductal carcinomas.

In clinical cytology, cellular dissociation, with intact, single lying neoplastic cells is one of a number of features associated with malignancy. Previous studies have demonstrated that regional and distant metastases as well as local recurrence correlate with decreased cellular cohesion.[15–18] A dysfunctional, intercellular adhesion system may be responsible for the tumour cell dispersion pattern seen on fine needle aspiration cytology (FNAC). Yu *et al.* found the degree of cellular discohesion from smears of fresh operation specimens to correlate with reduced expression of one or several members of the cadherin/catenin complex.[8] Likewise, we have found that reduced or negative expression of E-cadherin correlate with the degree of dissociation of breast carcinoma cells on FNAC.[19]

The aim of this study was to investigate immunocytochemical expression of the tight junction protein Claudin-7 in FNAC from breast carcinomas and correlate with grading, nodal status, locoregional and distant metastases and cellular cohesion.

Materials and methods

The material consisted of 59 air-dried FNAC smears from 48 primary malignant breast tumours, four locoregional recurrences from breast carcinomas, five fibroadenomas (FA), one intraductal papilloma and one benign NOS (not otherwise specified). The smears had been kept at −20 °C until immunostaining.

TNM, HER-2 and grading were retrieved from the histo- and cytopathological files. Cohesion was graded as: (1) mainly cohesive groups, (2) groups and single cells and (3) mainly single cells.

The smears were fixed in 4% buffered formalin. The primary antibody (mouse-anti-Claudin-7, clone 5D10F3; Zymed Laboratories Inc., San Francisco, CA, USA) was diluted 1 : 200. Antigen retrieval was carried out by microwave treatment in a specific solution, during 3 minutes at 750 W, followed by 7 minutes at 160 W. Immunocytochemical staining was done on the Autostainer from DAKOCytomation (DakoCytomation Glostrup, Denmark) using a dextran-polymer technique and with DAB as visualization. The degree of staining was recorded as negative, reduced or full, with full expression meaning equivalent to the staining pattern found in the fibroadenomas used as benign control and equivalent to the staining pattern expected in normal breast tissue. Both staining intensity and the percentage of stained cells were evaluated. The control smears revealed a strong membrane and cytoplasmic positivity in all luminal epithelial cells, whereas the myoepithelial cells were negative.

Statistical analyses for correlations between the different parameters were done using SPSS 12.0 (*P*-values, chi-square test).

Results

All primary and recurrent/metastatic breast lesions expressed Claudin-7. Full expression was demonstrated in 46% of the cases (Figure 1). Reduced expression was found in 54% (Figure 2). All benign cases showed a strong membrane and cytoplasmic staining defined as full expression (Figure 3).

In cases with reduced expression, the percentage of stained cells was usually high, and no smear showed <50% stained tumour cells. The staining pattern was always mixed membrane/cytoplasmic. The staining intensity varied from weak (+) to strong (+++), and

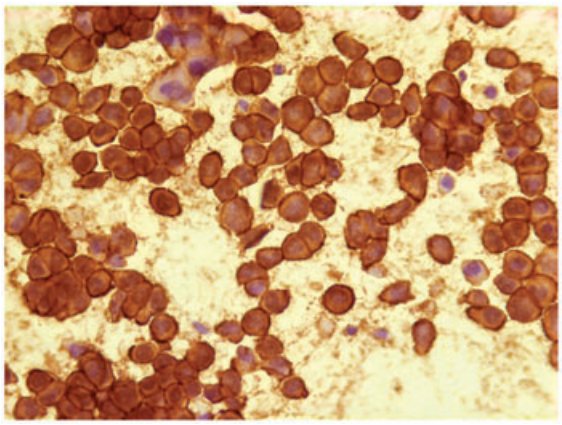

Figure 1. Original magnification × 40. Breast carcinoma cells showing a strong, full expression of Claudin-7.

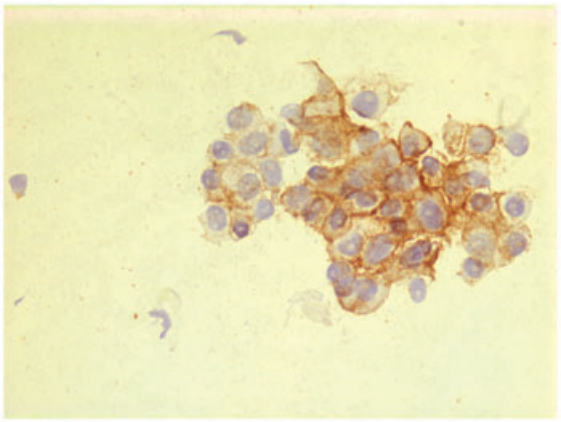

Figure 2. Original magnification × 40. Breast carcinoma cells showing reduced expression of Claudin-7.

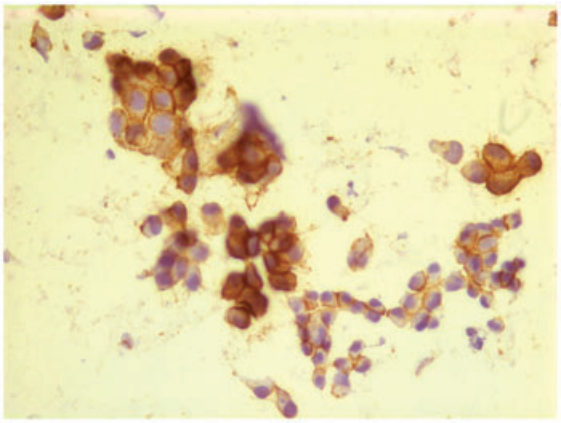

Figure 4. Original magnification × 40. Breast carcinoma cells with heterogeneous expression of Claudin-7.

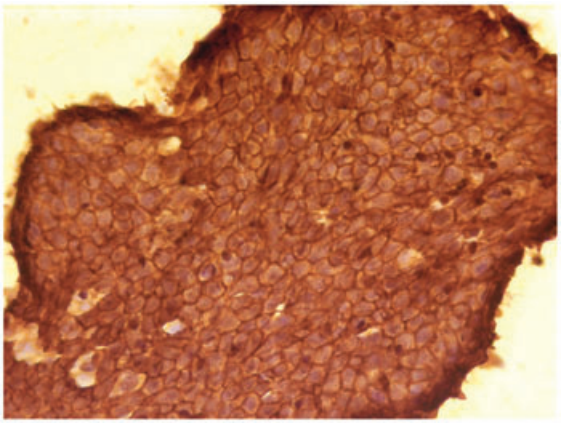

Figure 3. Original magnification × 40. Sheet of epithelial cells from a fibroadenoma showing a strong, full expression of Claudin-7.

was heterogeneous in cases with reduced expression (Figure 4).

Claudin-7 expression according to tumour (sub)-types, grade, nodal (N) status, HER-2 status and cohesion is shown in Tables 1 and 2. Reduced Claudin-7 expression showed a significant correlation with grading ($P = 0.031$), nodal involvement ($P = 0.037$) and cohesion ($P = 0.001$) in invasive carcinomas, but not with tumour size or subtype. HER-2 amplification correlated also ($P = 0$–017), but the numbers investigated were small. Three of four cases from locoregional recurrences revealed reduced expression. In addition, three of four primary breast carcinomas with simultaneous or later detected metastases other than axillary lymph node involvement, had reduced expression (Table 3).

Table 1. Claudin-7 expression according to tumour (sub)-type

Tumour type	Full Claudin-7 expression	Reduced Claudin-7 expression
IDC (38), primary	17	21
DCIS (3), grade 3	3	
Loco-reginal recurrence (4)	1	3
Medullary, G3		1
Micropapillary, invasive, G3		1
Lobular, G2		1
Mucinous, G2	1	
Papillary, intracystic, invasive, G1	1	
Papillary, intracystic, *in situ*, G1		1
Ductal with endocrine differentiation, G3	1	
FA, papilloma and benign NOS (7)	7	
subtotal	31 (24 malignant)	28

There were four *in situ* carcinomas. One intracystic, papillary carcinoma *in situ* showed staining of all tumour cells, but had areas of reduced staining intensity and was recorded as reduced expression (G1, cohesion grade 2) (Figure 5). Three DCIS grade 3 revealed full expression (cohesion grade 1, 2 and 3, respectively) (Figure 6).

Table 2. Claudin-7 expression in carcinomas according to grade, N and HER-2 status, and cohesion

	Full Claudin-7 expression	Reduced Claudin-7 expression
Grade 1	2	4
Grade 2	9 (39.1)	14 (60.9)
Grade 3	5 (29.4)	12 (70.6)
NX (node status unknown)	11	10
N0 (node negative)	6	10
N+ (node positive)	2 (13.3)	13 (86.7)
HER-2 amplified		5
HER-2 negative	7	3
Cohesion grade 1	5	2
Cohesion grade 2	11 (29.7)	26 (70.3)
Cohesion grade 3	3 (37.5)	5 (62.5)

Histological grading unknown in six cases. Values in parentheses are in percentage.

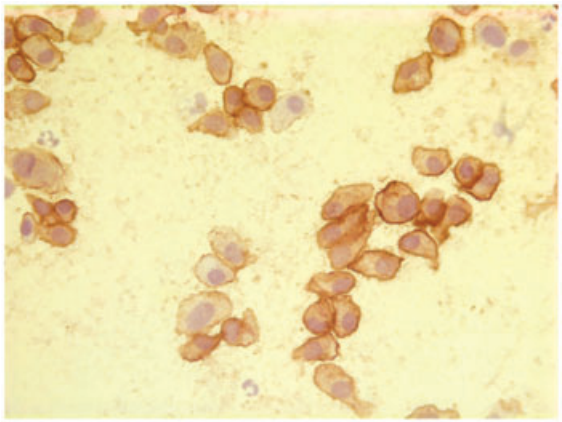

Figure 5. Original magnification × 40. Cells from an intracystic papillary carcinoma with focally reduced expression of Claudin-7.

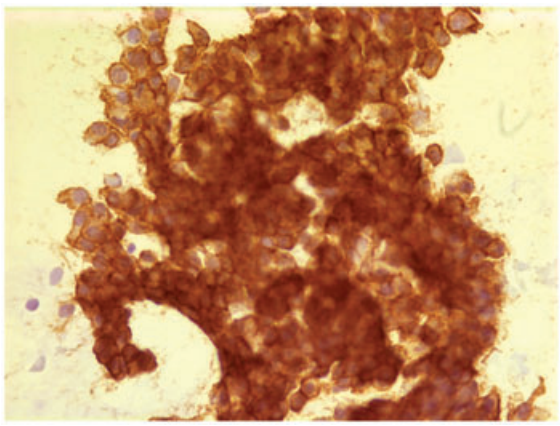

Figure 6. Original magnification × 40. Cells from a ductal carcinoma *in situ* (DCIS) grade 3 with a full expression of Claudin-7.

Discussion

Both adherens and tight junctions contribute to interepithelial cohesion. Loss of cohesion is a complex process and may involve one or both systems. Reduced expression of Claudin-7 was found mainly in carcinomas revealing a distinct population of single lying cells. Nevertheless, up to 39% of the carcinomas in this group showed full Claudin-7 expression. In these cases, other markers, as for example E-cadherin and/or catenins may be responsible for the loss of cohesion. In a previous study we found reduced expression of E-cadherin in 85% of invasive ductal carcinomas.[19] It seems therefore that Claudin-7 plays a minor role in the dissociation pattern seen in FNAC from breast carcinomas compared with E-cadherin.

In accordance with Kominsky *et al.*[14] we found an inverse correlation of Claudin-7 expression with grading. We observed reduced expression in both G2 and G3 invasive carcinomas, 61% and 70%, respectively, whereas Kominsky *et al.*[14] found loss of expression mainly in G3 carcinomas (77% of their cases).

Reduced expression of Claudin-7 also correlated with metastatic disease, namely with the presence of axillary lymph node metastases, loco-regional recurrences, distant metastases and HER-2 positivity. Kominsky *et al.*[14] found contradicting results on the

Table 3. Claudin-7 expression in smears from primary breast carcinomas with metastases to other locations than axillary LN

Case no.	TN + grading	Metastatic site	Claudin-7 expression
1: ductal	pT4 pN1 G2	Lung and columna	Reduced
2: ductal	pT2 N2a G3	Lever	Reduced
3: micropapillary, invasive	pT3 N2a G3	Supraclavicular LN	Reduced
4: ductal	pT2 N1a G2	Supraclavicular LN	Full

correlation between Claudin-7 expression and axillary lymph node status. Their case-by-case analysis showed reduced expression in 7/10 (70%) of invasive ductal carcinomas with positive axillary lymph node status. We observed an even higher percentage in which 87% (2/15) of cases with metastatic axillary lymph nodes revealed reduced expression of Claudin-7. Half of NX and 60% of NO cases also had reduced expression. Follow-up is too short (2 years at the most) to make any assumptions on the prognostic value of reduced Claudin-7 in these cases.

Reduced expression of Claudin-7 was demonstrated in 3/4 (75%) of loco-regional recurrences and in 3/4 (75%) of distant metastases. Even if the numbers are small, these findings are consistent with the assumption that Claudin-7 might play a major role in the metastatic process in the malignant cells.

This study included four *in situ* carcinomas, one papillary grade 1 and three ductal (DCIS) grade 3. Papillary carcinomas, both *in situ* and invasive, often have an abundance of single lying tumour cells on FNAC smears, consistent with a distinct loss of cohesion. Therefore, it is not surprising that the papillary *in situ* lesion revealed reduced expression. However, the invasive papillary carcinoma showed full expression of Claudin-7. Both had cohesion grade 2 and there is no obvious explanation for the difference between the two other than inter-tumour heterogeneity.

All three DCIS grade 3 revealed full expression of Claudin-7. This is in contrast to Kominsky *et al.*[14] who found inverse correlation between DCIS grade 3 and Claudin-7 expression. The number in our study is too small to make firm conclusions, but show that there is a population of DCIS grade 3 cases which have no reduction in Claudin-7. Whether this in any way could reflect the metastatic capacity, has, to the best of our knowledge, not been investigated. Almost 50% of DCIS grade 3 show a distinct single cell population on FNAC smears,[20] as did two of the DCIS cases in this study. Women harbouring a DCIS grade 3 in the breast have a 10–15 times risk of developing an invasive carcinoma.[21] A possible role of Claudin-7 in these cases is unresolved.

HER-2 positivity with gene amplification is found in a subgroup of invasive ductal carcinomas and is a marker of poor prognosis.[22] Therefore, it is not surprising that all HER-2 positive cases revealed reduced expression of Claudin-7.

In conclusion, reduced Claudin-7 expression in invasive breast carcinomas is correlated with grading, metastatic disease, including loco-regional recurrences and with the dissociation pattern.

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