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ORIGINAL PAPER

Expression of Tight Junction Protein Claudin-4 in Basal-Like Breast Carcinomas

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Abstract Claudins (CLDN) are tight junction proteins which contribute to the paracellular transport and ionic permeability of various epithelia. In recent years they came into focus for their suggested role in carcinogenesis and possible role in cancer therapy. According to our previous studies, in breast tissue CLDN4 is also related to the level of cellular differentiation. Thirty-eight estrogen (ER) and progesterone receptor (PgR) negative, HER2/neu negative, but cytokeratin 5/6 positive basal-like—mainly grade 3—breast carcinomas were compared with twenty-one grade 1, twenty-five grade 2 and twenty grade 3 non-basal-like invasive breast carcinomas, in respect to their CLDN4 expression. The immunohistochemical reactions were evaluated both semiquantitatively and by morphometrical analysis. Statistically significant difference (p=0.001) was observable regarding CLDN4 expression in the basal-like group as compared to grade 1 and 2 cancers. Further, CLDN4 expression was significantly higher (p=0.017) in the basal-like compared with the non-basal-like grade 3 carcinomas. Our results suggest that basal-like carcinomas are a subset of breast cancer with high level of CLDN4 protein expression. The finding is in accordance with our former observation that CLDN4 is indeed related to cellular differentiation. This observation may be seen as a further proof that basal-like carcinomas represent a separable group amongst grade 3 breast carcinomas.

Keywords Breast carcinoma · Basal-like · Tissue microarray · Immunohistochemistry · Claudin-4

Abbreviations

CLDN4	claudin-4
EGFR	epidermal growth factor receptor
ER	estrogen receptor
FFPE	formalin-fixed, paraffin-embedded
FISH	fluorescent in-situ hybridisation
PgR	progesterone receptor
TMA	tissue microarray

Introduction

Basal-like breast carcinomas have been identified on the basis of their genetic profile [1]. Since the identification of this subgroup mostly consisting of grade 3 invasive breast carcinomas, many attempts have been made to establish adequate identification method both histologically and

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immunohistochemically [2, 3]. The reason for these efforts is the sharp difference shown upon comparison of the survival data of the basal-like genetic subgroup and the other genetically identified breast carcinoma groups [2–5]. In genetic profile studies, the basal-like carcinomas show the poorest prognosis. Also, at present there is no tailored therapy available for these tumors. Basal-like carcinomas have a distinct gene expression and protein profile, with their typical histological features as well as immune-profile described recently [1, 2, 4]. This subgroup shows a "triple-negative" receptor status (ER, PgR and HER2/neu negative). On the other hand, many of these carcinomas over-express HER1 (epidermal growth factor receptor—EGFR) [6]. Basal-like cancers are usually high grade tumors with high mitotic activity. Bryan and colleagues showed immunohistochemical evidence of high grade ductal carcinoma in situ with an analogous immunophenotype to that of invasive basal-like carcinomas [7]. There is considerable amount of data supporting the idea that there are different pathways for the development of high and low grade breast cancers [8]. The CLDNs, as a tight junction protein family, consist of 24 known members in humans [9]. According to our previous studies, CLDN4 is related to cellular differentiation [10, 11]. The aim of the present study was to explore the possible differences in CLDN4 expression between grade 3 basal-like and grade 1, 2 and grade 3 but not basal-like invasive breast carcinomas.

Material and Methods

A cohort of 104 patients with surgically resected primary invasive breast carcinoma was investigated. Archival material was used from formalin-fixed and paraffin-embedded (FFPE) breast carcinoma tissues. The permission was granted for this study by the Ethical Committee of the Semmelweis University. Immunohistochemical analysis of the different tumor groups was performed using the antibodies and conditions shown in Table 1.

The mean percentage of positive tumor cells in the different tumor groups was calculated for ER, PgR, Ki67 and p53. The HER2/neu status was evaluated according to breast pathology guidelines: score 0 and 1+ cases were considered negative, and score 3+ cases—strong, continuous membrane reaction in >10% of the tumor cells—were considered positive. In score 2+ cases FISH was performed and the HER2/neu status was judged on that basis.

Tissue Microarrays

Tissue microarrays (TMAs) were assembled using a simple device (Histopathology Ltd., Pécs, Hungary), with the recipient blocks able to contain 24 cores. From the formalin-fixed, paraffin-embedded tumor blocks three to four cores from each case were put into one recipient block, thus permitting maximum six cases per block. The basal-like breast carcinomas were selected from a larger series of 60 "triple-negative" invasive carcinomas diagnosed in 2005 and 2006 in our department. The identification of this tumor type was performed using the strictest definition: only tumors with ER-/PgR-/HER2-/CK5/6+ immunophenotype were included. Accordingly, 38 cases were found which fulfilled the above criteria. A control group consisting of 21 cases of grade 1 and 25 cases of grade 2 plus 20 grade 3 non-basal-like tumors were collected in consecutive TMAs for routine immunohistochemistry purposes. These TMAs were prepared in the same manner as those containing the basal-like cancers: maximum six cases were put in one TMA block.

Immunohistochemistry for Claudin-4 Detection

The CLDN4 immunohistochemical reactions were performed on 5 μm thick FFPE sections obtained from the TMA blocks. After the deparaffination steps, the slides were treated for 30 min in a retrieval solution (Target from Dako, Glostrup, Denmark) in a microwave oven. The reactions were carried out in a Ventana ES automatic immunostainer (Ventana Medical Systems Inc., Tucson, AZ, USA) using the reagents provided by the manufacturer. The monoclonal mouse anti-CLDN4 antibody (#18-7341; Zymed, South San Francisco, CA, USA) was used in 1:100

Table 1 The type of the antibodies, dilutions and antigen retrieval methods used in this study

Antibodies	Dilution	Antigen retrieval	Type of antibody/company
Estrogen receptor (ER)	1:120	TRIS/EDTA 30 min MW	ER-6F11 Novocastra
Progesteron receptor (PgR)	1:200	Vector 12 min MW	PGR-312 Novocastra
CB11	1:100	Vector 12 min MW	NCL-LCB11 Novocastra
Ki-67	1:120	TRIS/EDTA 30 min MW	Ki-67 MIB-1 DAKO
CK5/6	1:600	TRIS/EDTA 30 min MW	D5/16B4 DAKO
P53	1:200	TRIS/EDTA 30 min MW	DO/7 DAKO
Claudin 4	1:100	TARGET (DAKO) 30 min MW	18-7341 Zymed

Table 2 General assessment of carcinomas in this study

Tumor groups	Mean age	Mean ER%	Mean PgR%	Mean Ki67%	Mean p53%
Grade 1	62.4	86.5	49.8	5.9	2.2
Grade 2	62.4	78	41	14	11.3
Grade 3	62.4	8	0	33.3	63.3
Basal-like	61.6	0	0	52.5	33.3

Mean percentage is given for the analyzed immunohistochemical reactions.

dilution (as shown in Table 1). The sections were counterstained with hematoxylin. A negative control was included with the omission of the primary antibody. Colon carcinoma and endometrial tissue samples previously shown to express different CLDNs served as positive controls.

The results of the immunohistochemical reactions were evaluated semiquantitatively by two independent pathologists (JK and LM) and the results were recorded on a Microsoft Excel worksheet. Since CLDN4 reaction shows a membranous staining, the semiquantitative evaluation was performed similarly to the HER2/neu immunohistochemistry: 0, 1+, 2+, 3+ scores were given depending upon the intensity, continuity and extent of the positive reaction. Following the semiquantitative evaluation, five representative digital photos were taken from each case at a magnification of x600. The images were then analyzed using Leica Qwin V3 morphometrical software (Leica Microsystems Imaging Solutions Ltd., Cambridge, UK), enabling the objective quantification of the immunohistochemical reactions.

Statistical Analysis

Analysis was performed using SPSS 15.0 version. The CLDN4 expression (morphometrical data) in breast carcinomas of different grades was analyzed by student's t-test. 'p' values less than 0.05 were considered as statistically significant.

Results

General Assessment of Patient Data

The mean age of patients with non-basal type breast carcinomas was 62.4 year, and that of women with basal-like cancer was 61.6 year at the time of breast surgery.

The grade 1 tumors showed a high percent of ER, PgR positivity (86.5% and 49.8%, respectively) low proliferative activity (Ki67=5.9%) and very low ratio of mutated p53 (2.2%). Grade 2 carcinomas showed similar ER and PgR expression (78% and 41%, respectively) with slightly higher proliferation rate (Ki67=14%) and positive p53 immunohistochemical reaction (11.3%). Grade 3 tumors presented ER positivity in 8%, and showed no PgR positivity, while having a much more accelerated proliferative potential (Ki67=33.3%) and a mean of 63.3% of p53 positive cells. Fifteen percent of the grade 3 carcinomas showed HER2/neu positivity either by immunohistochemistry or FISH. No CK5/6 reactivity was observed among the non-basal carcinomas.

Thirty-two of the 38 basal-like tumors were grade 3 (84.2%), ER, PgR and HER2/neu negative (out of the remaining six basal-like carcinomas 2 (5.3%) were grade 1 and 4 (10.5%) grade 2, and all 38 cancers were at least partially positive in the CK5/6 immunohistochemical reaction). The proliferative potential was the highest in this carcinoma group: mean ratio of Ki67 positive cells was

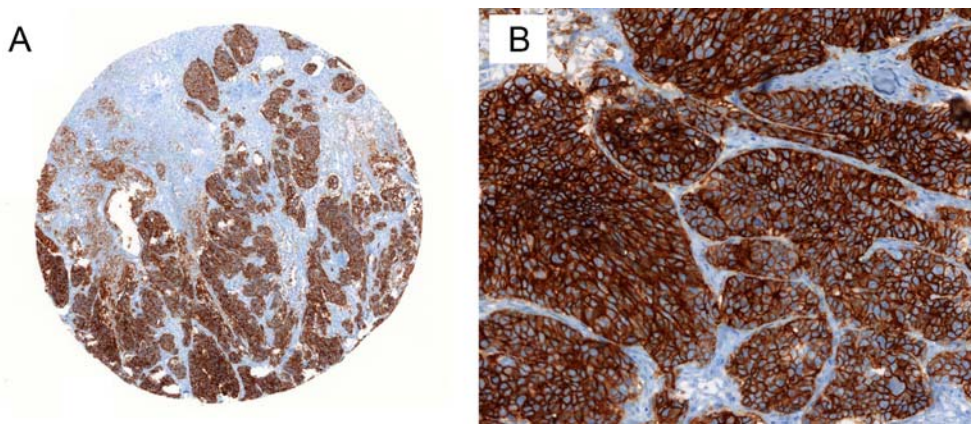

Fig. 1 Claudin-4 expression in basal-like breast carcinoma. The immunohistochemical reaction as seen on a representative sample of a basal-like carcinoma core at magnification ×50 (**a**). Strong CLDN4 expression of cancer cells is noted: the reaction shows a membrane bound positivity on the ×200 magnification (**b**); chromogen: DAB

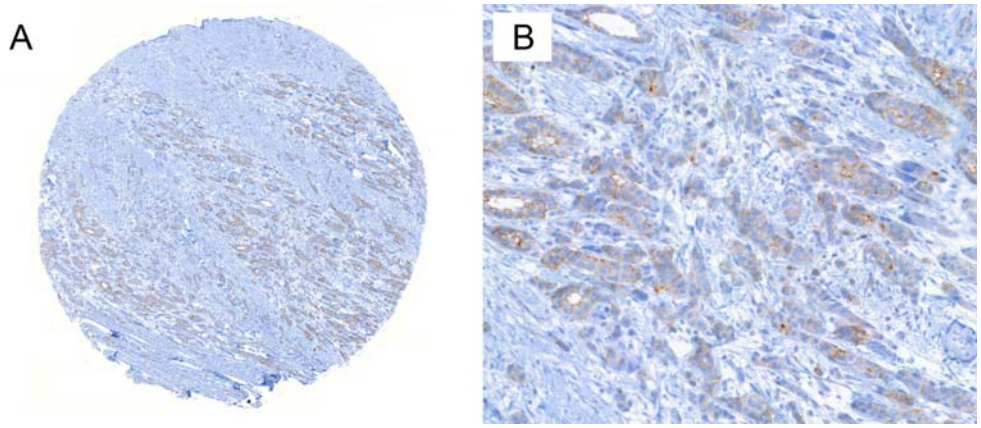

Fig. 2 Claudin-4 expression in the non-basal, grade 1 carcinoma of the breast. A representative core of the control invasive carcinoma group shows a decreased immunohistochemical reaction as compared to the basal-like carcinomas (**a**), magnification ×50. Weak membrane bound CLDN4 expression: positive reaction at the level of tight junctions on the cell membranes at ×200 magnification (**b**); chromogen: DAB

52.5%. P53 positivity (p53 mutation) was observed in one third of the carcinomas of this group (Table 2.).

From the 38 basal-like carcinoma, 19 cases were pN0, nine patients had one to three metastatic lymph nodes, five patients had more than four metastatic lymph nodes; in the remaining five cases the axillary nodal stage was not known. In the grade 3 non-basal carcinoma group, all but two patients had proven axillary lymph node metastasis. As far as tumor stage is concerned, pT1 tumors were represented in equal ratio in both the basal-like and the non-basal grade 3 carcinoma group. However, while 25% of the grade 3 non-basal breast carcinomas were in advanced, T4 stage, amongst the basal-like carcinomas only two stage T4 cases were present (5.3%).

Expression of Claudin-4

By immunohistochemistry, CLDN4 expression was marked in most of the basal-like carcinoma cases examined. Only four CLDN4 negative basal-like carcinomas were found in the series, which showed the following features: two of them were grade 1, well-differentiated tumors (one of these cases was a rare special type tumor, a low grade adenosquamous carcinoma), the third tumor was examined following primary systemic chemotherapy, and responded with considerable regression. The fourth CLDN4 negative basal-like breast carcinoma showed histiocytoid morphology associated with a lobular type infiltration pattern. In 18 cases strong, 3+ positive reaction was detected (Fig. 1).

CLDN4 expression was decreased or absent in many tumor cells of the grade 1 or 2 carcinomas (Fig. 2), whereas moderately increased expression was observed in the majority of grade 3 non basal-like breast carcinomas. (Table 3)

Subjecting the immunohistochemical results to morphometrical analysis, the four groups analyzed (grade 1, 2, 3 non-basal and basal-like) could be separated in regard to their CLDN4 immunopositivity (Table 4; Fig. 3).

Statistical analysis revealed significant differences regarding CLDN4 association between the tumors of different grade. CLDN4 expression was significantly higher in the basal-like breast carcinomas when compared to grade 1, 2 and 3 non-basal-like carcinomas.

Discussion

The definition of the basal-like subgroup of breast carcinomas is not fully unequivocal. In the present study, the following immunohistochemical criteria were included: ER/PgR/HER2 negative, CK5/6 positive tumors. The majority of these carcinomas were grade 3. In the control group histological grade 1, 2 and 3 breast carcinomas were included, irrespective of any other characteristics. It is to be mentioned that within the grade 3 control group no CK5/6 positive tumors were present.

Knowledge on the claudin family has increased during the last few years, since their first identification by Furuse et al. [12]. There have been attempts to find links between claudin expression, or changes of claudin expression and the development of various carcinomas (uterine cervix, ovarium, pancreas, prostate). Some studies have proved that claudin expression and/or claudin expression profile is related to a certain stage of carcinogenesis (uterine cervix) or to a certain subtype of carcinoma (endometrium, ovarium) [13, 14]. Rather surprisingly, the attempt to find a link between claudin expression profile and presence or absence of lymph node metastasis in breast cancer cases has been so far unsuccessful [10, 11].

Table 3 CLDN4 expression in basal-like and grade 1, 2 and 3 non-basal-like breast carcinomas

CLDN4 expression	Grade 1	Grade 2	Grade 3	Basal-like
neg	12	11	1	4
1+	8	8	4	6
2+	–	3	8	10
3+	1	3	7	18
All	21	25	20	38

Table 4 Results of morphometrical evaluation of the CLDN4 immunohistochemical analysis

Tumor groups	Number of cases	Mean surface area % of positive CLDN4 membrane reaction	Standard error
Grade 1	21	0.6856	0.49434
Grade 2	25	1.3251	0.70191
Grade 3	20	1.6678	0.31298
Basal-like	38	3.7041	0.76910

The present study revealed different CLDN4 protein expression levels between the basal-like and non-basal grade 3 tumors. At protein level, differences were observed between basal-like breast carcinomas and tumors of grade 1 and 2. The difference was also noted between the basal-like and the non-basal grade 3 carcinomas. The CLDN4 negative "basal-like" breast carcinomas in this series showed the following features: two of them were grade 1, well differentiated tumors. Of those, one was a special type, so-called low grade adenosquamous carcinoma, the other a very poorly cellular invasive carcinoma with lobular type infiltration pattern (i.e. discohesive tumor cells in abundant stroma). A third tumor was examined following primary systemic chemotherapy. Considerable regression occurred, therefore, a small area of the tumor could be investigated. It may be that due to the heterogeneity in CLDN4 staining the remaining cells happened to be negative. But, another theoretical explanation may be that the CLDN4 positive poorly differentiated cell population disappeared—it is known from the literature, but also from our own experience, that basal-like breast carcinomas are most likely to regress to preoperative systemic chemotherapy [3]. The fourth CLDN4 negative basal like breast carcinoma showed a particular histological appearance: histiocytoid cells were infiltrating in a lobular type pattern. Taking into consideration that CLDNs are parts of the tight junction, one may imagine that tumors with such diffuse infiltration pattern had lost the majority of the tight junction components.

A considerable amount of data supports the idea that the pathways for the development of high and low grade breast carcinomas differ. It has been postulated that basal-like breast

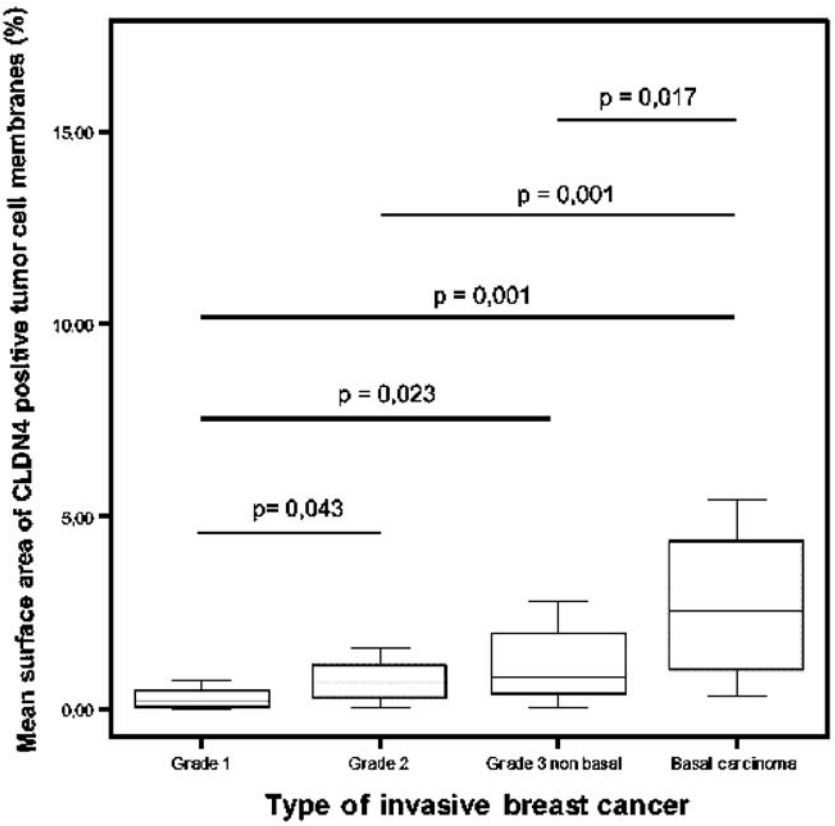

Fig. 3 Graph showing the results of the morphometrical evaluation of claudin-4 expression. Morphometrical analysis of CLDN4 expression in grade 1, 2, and non-basal grade 3 and basal-like breast carcinomas showed increasing tendency of CLDN4 expression with grade and highest CLDN4 expression in basal-like breast carcinomas. The differences between the tumor groups are statistically significant as shown on the box-plot diagram

carcinomas arise from the myoepithelial cell compartment or originate from progenitor/stem cells. Up to now, there has been no totally acceptable answer to this question.

Additionally, when comparing the pT and pN stage of the grade 3 non-basal and the basal-like carcinomas we found considerable differences: more T4 cases were present amongst the non-basal carcinomas (25% vs. 5.3% in the basal-like group). The majority of the grade 3 non-basal like cancers possessed axillary lymph node metastases at the time of the operation, while more than 80% of the basal-like carcinomas were node negative. These data are in accordance with recent literature data [2].

It is generally accepted that basal-like breast carcinomas have a distinct genomic and protein profile. The results of the present study support this observation.

CLDN4 and CLDN3 have mostly been examined together; the main reason for this is that these two molecules are in fact the receptor molecules for the Clostridium perfringens enterotoxin. In cell cultures, CLDN4 expressing pancreatic carcinoma cells disintegrated following infection of the cell culture with Clostridium perfringens [15, 16]. This and a few similar experiments call attention to a new direction of tailored therapies. If we can find the controlled mode, toxin producing bacteria could be used to produce antitumor effect in those cases where the receptor is expressed by the tumor cells. Knowing that both CLDN3 and CLDN4 are such receptors, theoretically, those cancers which express one or both of these tight junction proteins, might be subjects of this new and yet experimental method of anti-cancer treatment. According to the literature, basal-like breast carcinomas have poor prognosis [17], or, according to the experience of Fulford et al. [2], even though they have a relatively good long-term survival, survival after metastases is poor and currently they cannot be treated individually. A large proportion of these carcinomas are EGFR positive [7, 18]. This finding seems promising as far as tailored treatment is concerned. A further possible chance in the future for the targeted therapy of basal-like breast carcinomas might be via the high likelihood of their CLDN4 positivity.

Conclusions

In the present study significant differences were found between grade 3, basal-like breast carcinomas and grade 1, 2 and 3 non basal-like invasive breast carcinomas regarding their CLDN4 expression: basal-like breast carcinomas were mainly strongly CLDN4 positive. This study provides further evidence that basal-like breast carcinomas represent a biologically distinct disease entity, probably requiring different therapeutic strategies. In theory, these tumors could be subjects of the yet experimentally investigated Clostridium perfringens enterotoxin based anti-cancer therapy.

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Competing interests The authors declare no competing interests. The study was reviewed and approved by the Institutional Ethical Board under the licence number 172/2003.

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