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RESEARCH

Expression of Tight Junction Molecules in Breast Carcinomas Analysed by Array PCR and Immunohistochemistry

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Abstract In the past few decades an enormous amount of data became known to clarify the molecular composition and architecture of tight junctions (TJs). Despite the efforts, the expression and function of several TJ genes and proteins in breast carcinoma are still not known and some of the data are contradictory. The expression of forty-four TJ associated genes was examined at mRNA level in eighteen invasive ductal breast carcinoma samples and corresponding normal breast tissues by using low density array PCR. Expressions of claudins (CLDNs) 5, 10, 16, 17, and 18, and ZO-1, ZO-2 were evaluated by immunohistochemistry as well. Using immunohistochemical phenotype as a surrogate for the genetic subtype, 11 luminal A, 3 luminal B, 3 triple negative and one HER2+ cases were included. Ten genes were significantly downregulated in tumors compared with normal breast tissues (CLDNs 5, 10, 16, 18, 19, CTNNAL1, JAM-

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B, ZO-1, ZO-2 and PARD3), whereas one gene (CLDN17) was significantly up-regulated in tumors when compared with normal breast. At protein level CLDNs 5, 10, 16, 18, ZO-1 and ZO-2 were downregulated in tumors as compared with normal breast tissue. CLDN17 showed variable expression in tumor tissues in comparison to normal breast. In the single HER2+ tumor when compared with the other subtypes CLDNs 5, 16, 17, 18, CTNNAL1, JAM-B, ZO-1, ZO-2 and PARD3 genes were found to be upregulated. We found altered TJ genes and proteins whose expression has not yet been associated with breast carcinoma. Our findings show a tendency of TJ genes and proteins to be downregulated in breast cancer. Further studies are necessary to examine whether the downregulation of the above mentioned TJ associated genes and proteins may contribute to the malignant progression of invasive ductal breast carcinomas.

Keywords Breast carcinoma · Tight junction · Claudin · Array · Immunohistochemistry

Abbrevations

AJ-	Adherens junctions
BSA-	Bovine serum albumine
CDC42-	Cell division cycle 42 (GTP binding protein, 25 kDa)
CGN-	Cingulin
CLDN-	Claudin
CLDN1-	Claudin 1
CLDN10-	Claudin 10
CLDN11-	Claudin 11 (oligodendrocyte transmembrane protein)
CLDN12-	Claudin 12
CLDN14-	Claudin 14
CLDN15-	Claudin 15
CLDN16-	Claudin 16
CLDN17-	Claudin 17
CLDN18-	Claudin 18
CLDN19-	Claudin 19
CLDN2-	Claudin 2
CLDN20-	Claudin 20
CLDN3-	Claudin 3
CLDN4-	Claudin 4
CLDN5-	Claudin 5
CLDN6-	Claudin 6
CLDN7-	Claudin 7
CLDN8-	Claudin 8
CLDN9-	Claudin 9
CLDND2-	Claudin domain containing 2
CRB3-	Crumbs homolog 3 (Drosophila)
CTNNAL1-	Catenin (cadherin-associated protein), alpha-like 1
CTNNBIP1-	Catenin, beta interacting protein 1
DAPI-	4′-6-Diamidino-2-phenylindole’
F11R-	F11 receptor
GAPDH-	Glyceraldehyde-3-phosphate dehydrogenase
HPRT1-	Hypoxanthine phosphoribosyltransferase 1
IDC-	Invasive ductal carcinoma
JAM-B-	Junctional adhesion molecule B
JAM-C-	Junctional adhesion molecule C
MAGI1-	Membrane associated guanylate kinase, WW and PDZ domain containing 1
MAGIX-	MAGI family member, X-linked
MARK2-	MAP/microtubule affinity-regulating kinase
MLLT4-	Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 4
MPDZ-	Multiple PDZ domain protein
MPP5-	Membrane protein, palmitoylated 5 (MAGUK p55 subfamily member 5)
OCLN-	Occludin
PARD3-	par-3 partitioning defective 3 homolog
PARD6A-	par-6 partitioning defective 6 homolog alpha
PBS-	Phosphate buffered saline
RHOA-	Ras homolog gene family, member A
RPL13A-	Ribosomal protein L13a
SDHA-	Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)
SYMPK-	Symplekin
TGFB1-	Transforming growth factor, beta 1
TJ-	Tight junction
ZO-1-	Tight junction protein 1 (zona occludens 1)
ZO-2-	Tight junction protein 2 (zona occludens 2)
ZO-3-	Tight junction protein 3 (zona occludens 3)
TLDA-	TaqMan Low Density Array

Introduction

In epithelial tissues cell-cell interaction is mediated by various junctional complexes. Each of these complexes—tight junctions (TJs), adherens junctions (AJs), desmosomes and gap junctions—have typical morphology, composition and function. TJs are the most apical intercellular junctions in epithelial cells with diverse functions. It is generally accepted that TJ proteins can be categorised into three groups: integral membrane proteins (occludin, claudins, junctional adhesion molecule—JAM, tricellulin and crumbs), peripherally associated cytoplasmic proteins (zonula occludens—ZO, partitioning-defective molecules—PAR, MAGUK inverted—MAGI, cingulin, symplectin and others) and signaling proteins (protein kinase A, protein kinase C, heterotrimeric G-proteins) [1-4]. There have been decades of evidence regarding altered TJs in cancerous epithelia like attenuations as well as lack of [5] increased permeability to paracellular tracers [6, 7]. The expression and function of

several TJ proteins in breast carcinoma, however, are not known and some of the published data are contradictory. The relevant publications in this field, without claim of completeness, are presented in Supplementary Table 1.

Gene expression profiling analyses have allowed the identification of genes that are differentially expressed for example in ovarian cancer [8]. Later on studies have also identified changes in the expression of several TJ proteins in numerous carcinomas [9-14].

To date only a few CLDN proteins have been investigated in breast carcinomas and in a relatively limited number [1, 10, 11, 15-20]. While some CLDNs are silent in certain cancers, the same protein can be over-expressed in others, suggesting that both, expression and function of CLDNs are tissue specific. In breast carcinomas, the majority of available data on TJs have shown the expression of multiple TJ and AJ proteins to be downregulated or absent [15, 16, 21]. In our previous studies we found significant loss of CLDN1 protein in breast cancer cells compared with normal breast tissue, with downregulation of CLDN4 noted in ductal carcinoma grade 1, in special types of breast carcinoma (mucinous, papillary, tubular) and in areas of apocrine metaplasia [22]. Contrary to the above presented we have found that in the basal-like group as compared with the non-basal-like grade 3 breast carcinomas the CLDN4 expression was significantly higher (p=0.017) [19]. Recent studies showed that CLDN16 expression was also reduced in human breast cancer, particularly in patients developing aggressive tumors with high mortality rate [22, 23]. Furthermore, JAM-A is robustly expressed in normal human mammary epithelium, and its expression is downregulated in metastatic breast cancer [24, 25]. Morohashi and coworkers (2007) analysed the expression patterns of CLDN1/CLDN4 in recurrent and non-recurrent breast carcinomas, with the finding that the recurrent group showed decreased expression of CLDN1 (p<0.001) as compared with the non-recurrent group [26]. Recently, a new subgroup called “claudin-low” was described with aggressive behaviour and poor prognosis based on gene expression profiling, which was initially declared to be a subgroup of triple negative breast carcinomas [27-30].

The present study focuses on the mRNA and protein expressions of several TJ components in human breast carcinomas and corresponding normal breast tissues from the same patients.

Material and Methods

Altogether, breast carcinomas and corresponding normal breast tissues of eighteen patients were included in the study. Tissue samples were collected in conformity with the national law (23/2002.V.9—Hungarian Ministry of Health) regarding human studies. The work was approved by the Regional Ethical Committee (reg. no. 77/2007 and 185/2007).

RNA and protein expressions were analysed by TaqMan Low Density Array (TLDA) and fluorescent immunohistochemistry. The histopathological diagnosis of the selected

Table 1 Histopathologic data of the analysed breast carcinomas

PATIENT nr.	TNM	grade	ER%	PR%	KI-67%	p53%	HER2 score
1	T3N0	2	80	60	10	10	0
2	T1cN0	3	0	0	90	0	0
3	T2N1	3	0	0	100	100	0
4	T2N0	2	100	100	30	5	0
5	T1cNx	1	100	100	5	30	0
6	T1cN0	2	100	100	5	0	0
7	T1cN1a	2	70	50	10	0	0
8	T2N1a	3	90	0	30	10	0
9	T2N1	2	90	80	10	30	0
10	T2N2a	3	100	100	20	5	0
11	T2N1a	3	0	0	30	100	0
12	T1cN0	2	50	90	10	5	0
13	T3cN0	2	100	20	30	0	0
14	T1cN1	3	0	0	90	90	+
15	T1cN0	1	80	90	10	0	0
16	T2N2a	3	70	40	20	100	+
17	T2N2a	1	80	90	20	30	+
18	T2N2a	2	80	0	10	60	+

breast lesions was pure invasive ductal carcinoma (IDC), all other cases were omitted.

The histological grade was defined according to the modified Elston-Bloom-Richardson (Nottingham) grading system. The estrogen and progesteron receptor (ER and PgR) status as well as the HER2 status (determined by immunohistochemistry, and FISH when staining was scored 2+) were available for all patients (Table 1.). Patients were divided into 4 biological subtypes: 1) triple-negative (ER, PgR and HER2 negative), 2) HER2 (HER2 positive, ER and PgR negative), 3) luminal B (ER positive and/or PgR positive and/or HER2 positive), and 4) luminal A (ER and PgR positive, HER2 negative) [31].

PCR-based Array Evaluation

RNA Extraction and Reverse Transcription

RNA was isolated from the eighteen frozen tumor samples and their corresponding normal tissue. The percentage of tumor cells was evaluated for each case on H&E stained slides, with the threshold set to a minimum of 70%.

Samples were taken from the tumorous component by a pathologist, while normal breast tissue specimens were obtained from non-tumorous breast tissue, sufficiently far from the tumor containing fatty tissue and normal breast epithelial cells, after grossing the fresh specimen. The selected breast tissue samples, obtained in accordance with current local and ethical recommendations, were snap frozen in liquid nitrogen and then stored at −80°C.

The tissues (50–100 mg), after homogenization with a Polytron homogenizer (Kinematica AG, Littau/Lucerne, Switzerland), were subsequently treated with Trizol (Invitrogen, Carlsbad, CA, USA) to extract RNA according to the manufacturer's instructions. Briefly, the RNA was precipitated with 0.5 ml isopropyl-alcohol in the aqueous phase. The pellet was then washed in 70% ethanol once, dried and resuspended in 50 μl of RNAse-free water and kept at −80°C until further use. Total RNA integrity was verified by electrophoresis using ethidium-bromide staining and NanoDrop ND-1000 was used to measure RNA concentration (NanoDrop Products, Wilmington, DE, USA). One microgram of total RNA was reverse transcribed in a total volume of 20 μl using High capacity cDNA Reverse Transcription Kit (Applied Biosystems—ABI, Foster City, CA, USA) according to the manufacturer's protocol.

TaqMan Low Density Array (TLDA)

Gene expression levels were measured for genes associated with TJs and selected control genes; targets were chosen based on literature reviews of TJs (presented in the Introduction section). The list of assays used in the study is given in Table 2. Each set of 48 genes (Table 2.) also contained four housekeeping genes: GAPDH, HPRT1, RPL13A and SDHA.

Custom TaqMan® Low Density Arrays (48 TaqMan® Gene Expression assay Part n° 699973 microfluidic cards, ABI) were applied on Applied Biosystems 7900 HT instrument. Thermal cycler conditions were as follows: 50°C for 15 min, 95°C for 2 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s.

All samples were run in duplicates and expressed as mean +/− SD. The threshold cycle Ct was automatically given by SDS2.2 software package (ABI). Relative quantification was determined using the equation: $2^{-\Delta\Delta Ct}$. The non-parametric, Mann–Whitney test was applied to compare groups. All statistical tests were two-sided and differences were considered to be statistically significant at $p<0.05$.

Materials Used for Immunohistochemistry and Immunofluorescent Staining

The expression of the following proteins was analysed by immunohistochemistry: CLDNs 5, 10, 16, 17, 18, ZO-1 and ZO-2. The *Human Protein Atlas (HPA)* was considered as one of the references (http://www.proteinatlas.org).

Indirect immunofluorescent staining was performed on 4 to 8 μm thick frozen sections of fixed breast carcinomas and corresponding normal breast tissues (in −20°C methanol for 20 min., air-dried and incubated with 5% BSA/PBS for 30 min. at room temperature) followed by incubation with the primary antibodies at 4°C overnight under conditions presented in Supplementary Table 2. Excess primary antibodies were removed by washing in PBS and slides were then incubated with the secondary antibodies conjugated to Alexa Fluor probe (Molecular Probes, Carlsbad, CA, USA) in dark for 30 min. at room temperature. The following Alexa Fluor dyes were used: goat anti-mouse Alexa Fluor 568, goat anti-rabbit Alexa Fluor 568, goat anti-mouse Alexa Fluor 488 and goat anti-rabbit Alexa Fluor 488 (Supplementary Table 2). Cell nuclei were counter-stained with DAPI (Vectashield mounting medium for fluorescece with DAPI, Vector Laboratories, Inc., Burlingame, CA, USA). For each TJ protein a negative control with omission of the primary antibody was included. Stained specimens were analysed by laser scanning confocal microscopy (BioRad Radiance 2100 Laser Scanning Confocal Microscope System, BioRad Lab. Ltd., Hercules, CA, USA).

Results

Forty-eight (44 TJ genes and 4 endogenous controls) genes were successfully evaluated in eighteen invasive ductal breast carcinomas and corresponding normal breast tissue

Table 2 List of assays used in the study

Nr.	Gene symbol	Gene name
1.	CDC42-Hs00741586_mH	cell division cycle 42 (GTP binding protein, 25 kDa)
2.	CGN-Hs00430426_m1	cingulin
3.	CLDN10-Hs00199599_m1	claudin 10
4.	CLDN11-Hs00194440_m1	claudin 11 (oligodendrocyte transmembrane protein)
5.	CLDN12-Hs00273258_s1	claudin 12
6.	CLDN14-Hs00273267_s1	claudin 14
7.	CLDN15-Hs00204982_m1	claudin 15
8.	CLDN15-Hs00370756_m1	claudin 15
9.	CLDN16-Hs00198134_m1	claudin 16
10.	CLDN17-Hs00273276_s1	claudin 17
11.	CLDN18-Hs00212584_m1	claudin 18
12.	CLDN19-Hs00326959_s1	claudin 19
13.	CLDN1-Hs00221623_m1	claudin 1
14.	CLDN20-Hs00378662_m1	claudin 20
15.	CLDN2-Hs00252666_s1	claudin 2
16.	CLDN3-Hs00265816_s1	claudin 3
17.	CLDN4-Hs00533616_s1	claudin 4
18.	CLDN5-Hs00533949_s1	claudin 5 (transmembrane protein deleted in velocardiofacial syndrome)
19.	CLDN6-Hs00607528_s1	claudin 6
20.	CLDN7-Hs00600772_m1	claudin 7
21.	CLDN8-HS00273282-s1	claudin 8
22.	CLDN9-Hs00253134_s1	claudin 9
23.	CLDND2-Hs00380745_m1	claudin domain containing 2
24.	CRB3-Hs00373616_m1	crumbs homolog 3 (Drosophila)
25.	CTNNAL1-Hs00169384_m1	catenin (cadherin-associated protein), alpha-like 1
26.	CTNNBIP1-Hs00172016_m1	catenin, beta interacting protein 1
27.	F11R-Hs00170991_m1	F11 receptor
28.	GAPDH-Hs99999905_m1	glyceraldehyde-3-phosphate dehydrogenase
29.	HPRT1-Hs99999909_m1	hypoxanthine phosphoribosyltransferase 1 (Lesch-Nyhan syndrome)
30.	JAM-B-Hs00221894_m1	junctional adhesion molecule B
31.	JAM-C-Hs00262270_s1	junctional adhesion molecule C
32.	MAGI1-Hs00191026_m1	Membrane associated guanylate kinase, WW and PDZ domain containing 1
33.	MAGIX-Hs00227501_m1	MAGI family member, X-linked
34.	MARK2-Hs00250126_m1	MAP/microtubule affinity-regulating kinase
35.	MLLT4-Hs00291852_s1	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 4
36.	MPDZ-Hs00187106_m1	multiple PDZ domain protein
37.	MPP5-Hs00223885_m1	membrane protein, palmitoylated 5 (MAGUK p55 subfamily member 5)
38.	OCLN-Hs00170162_m1	occludin
39.	PARD3-Hs00219744_m1	par-3 partitioning defective 3 homolog (C. elegans)
40.	PARD6A-Hs00180947_m1	par-6 partitioning defective 6 homolog alpha (C. elegans)
41.	RHOA-Hs00357608_m1	ras homolog gene family, member A
42.	RPL13A-Hs01926559_g1	ribosomal protein L13a
43.	SDHA-Hs00417200_m1	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)
44.	SYMPK-Hs00191361_m1	symplekin
45.	TGFB1-Hs99999918_m1	transforming growth factor, beta 1
46.	ZO-1-Hs00268480_m1	tight junction protein 1 (zona occludens 1)
47.	ZO-2-Hs00178081_m1	tight junction protein 2 (zona occludens 2)
48.	TJP3-Hs00274276_m1	tight junction protein 3 (zona occludens 3)

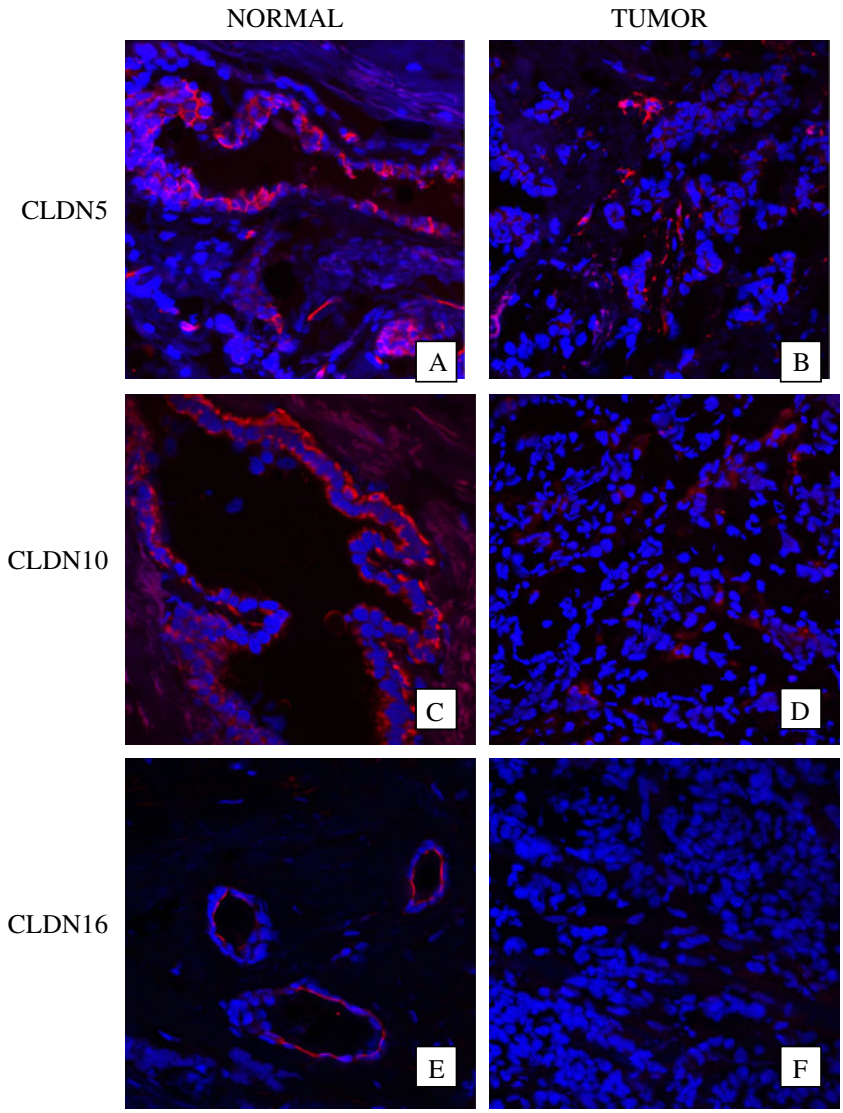


Fig. 1 The expression of claudin 5, 10 and 16 proteins in normal breast epithelium and invasive ductal breast carcinomas. Images were obtained using laser scanning confocal microscopy. **a** Claudin-5 (CLDN5) positivity in normal breast epithelium. Shown is the immunohistochemical reaction of normal breast duct using monoclonal CLDN5 antibody. The secondary antibody is IgG conjugated to Alexafluor 488. Cell nuclei were counter-stained with DAPI (Original magnification: 400×). **b** The significant loss of CLDN5 positivity in invasive breast carcinoma (Original magnification: 400×). **c** Claudin-10 (CLDN10) expression in benign breast epithelium. Intense CLDN10 positivity in benign breast epithelium. Shown is the immunohistochemical reaction of benign breast tissue using polyclonal CLDN10 antibody (Original magnification: 400×). **d** CLDN10 expression in invasive ductal breast carcinoma. Note the membrane staining in few scattered tumor cells only (Original magnification: 400×). **e** Intense Claudin-16 (CLDN16) positivity in benign breast epithelium. Shown is the immunohistochemical reaction of benign breast tissue using polyclonal CLDN16 antibody (Original magnification: 400×). **f** CLDN16 expression in invasive ductal carcinoma of the breast. Complete loss of CLDN16 expression was observed in this case of invasive ductal breast carcinoma (Original magnification: 400×)

by TLDA. Successful results were found in 17 tumor-normal pairs and in an additional tumor. The histopathological data of the analysed cases are presented in Table 1.

When the mean value of the four analysed reference genes was used for relative quantification, seven transcripts were notable showing significantly different expression in tumors, as compared with normal breast tissues. Six genes were significantly downregulated (CLDNs 5, 16, 18, 19, JAM-B, ZO-2) and only one, CLDN17, was significantly upregulated in tumors when compared with normal breast tissues. By using only GAPDH as reference gene, eleven significantly differentially expressed transcripts in tumors compared with normal breast tissues were found. Ten genes were significantly downregulated in tumors compared with normal breast tissues (CLDNs 5, 10, 16, 18, 19, CTNNAL1, JAM-B, ZO-1, ZO-2 and PARD3), whereas one gene (CLDN17) was significantly upregulated in tumors when compared with normal breast tissues (Figs. 4 and 5).

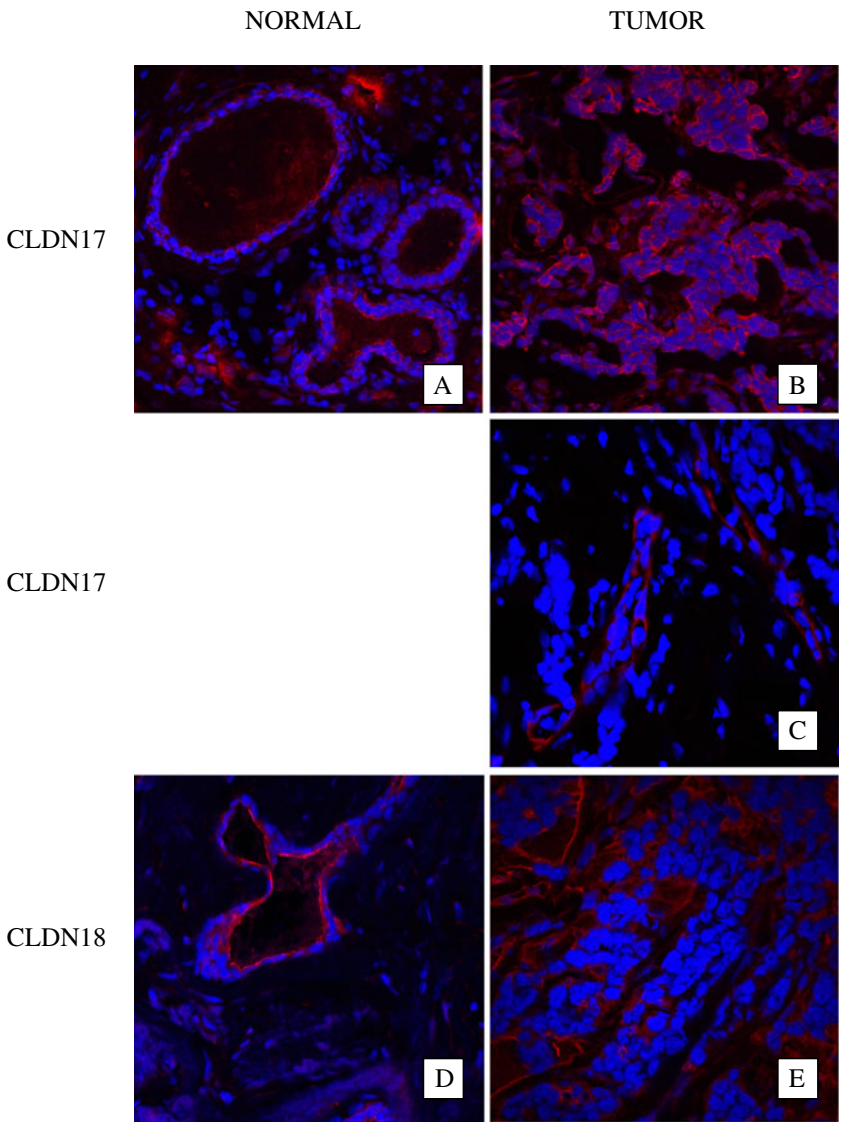


Fig. 2 The expression of claudin 17 and 18 proteins in normal breast epithelium and invasive ductal breast carcinomas. Images were obtained using laser scanning confocal microscopy. **a** Claudin-17 (CLDN17) positivity in normal breast epithelium. Shown is the immunohistochemical reaction of normal breast tissue using polyclonal CLDN17 antibody. The secondary antibody is IgG conjugated to Alexafluor 488. Cell nuclei were counter-stained with DAPI (Original magnification: 400×). **b** The expression of CLDN17 protein in concordance with PCR results was not uniform. In some cases overexpression was observed (Original magnification: 400×), whereas **c** in other cases the loss of this protein was detected (Original magnification: 600×). **d** Claudin-18 (CLDN18) expression was intense in benign breast epithelium. The immunohistochemical reaction is shown using polyclonal CLDN18 antibody (Original magnification: 600×). **e** CLDN18 expression in invasive ductal breast carcinoma. Note the significant loss of this protein as compared with normal epithelial cells (Original magnification: 400×)

Although several of the genes analysed in our study have previously been implicated in breast cancer (eg. CLDNs 1, 4, 7 and 16), quite a few still have unclear roles in breast cancer. Analysing the gene expression results case by case, the expression was not uniform throughout the studied cases. It was intriguing to find the eventual differences in TJ components between the different breast carcinoma subgroups. In this study 11 luminal A, 3 luminal B, 3 triple negative and one HER2+ tumors were included. In the single HER2+ tumor when compared with the other subtypes CLDNs 5, 16, 17, 18, CTNNAL1, JAM-B, ZO-1, ZO-2 and PARD3 were found to be upregulated, whereas the rest of the cases showed a clear tendency for the above mentioned TJ components to be downregulated in tumors as compared with the normal breast epithelium (case 14, Table 1.).

In the normal tissues the analysed proteins appeared as strong membrane reaction of the epithelial cells. In tumors, the positivity when present was variable in both intensity

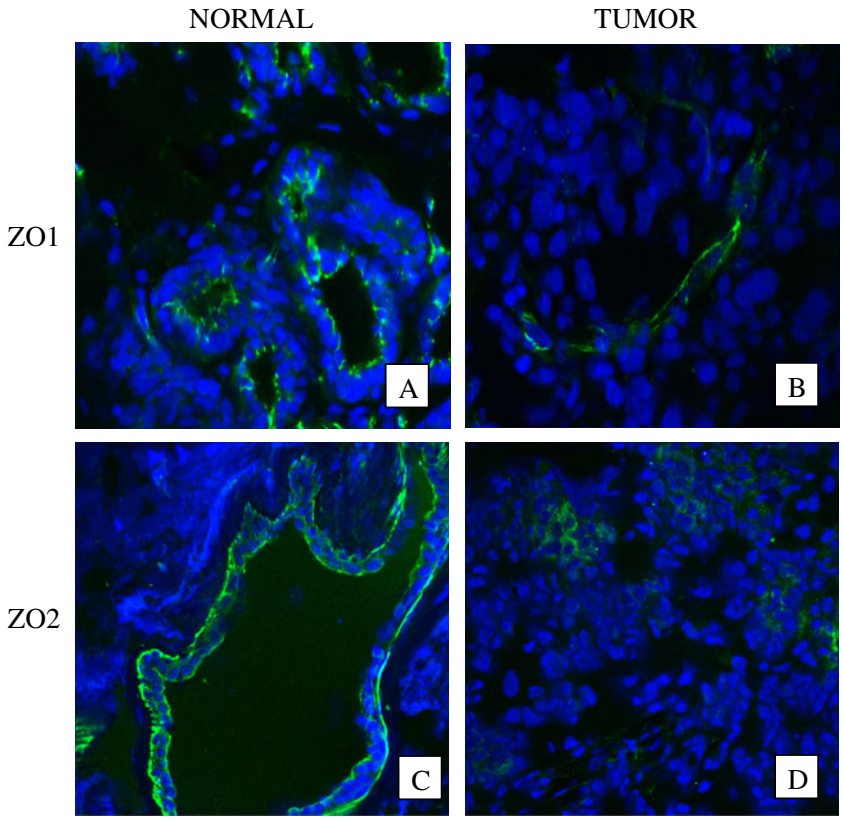


Fig. 3 The expression of ZO-1 and ZO-2 proteins in normal breast epithelium and invasive ductal breast carcinomas. Images were obatined using laser scanning confocal microscopy. **a** Characteristic ZO-1 positivity in normal breast epithelium. Shown is the immunohistochemical reaction localized close to the apical end of the cells using ZO-1 polyclonal antibody. The secondary antibody is IgG conjugated to Alexafluor 488. Cell nuclei were counter-stained with DAPI (Original magnification: 400×). **b** The expression of ZO-1 protein in invasive ductal breast carcinoma. Note the significant loss of this protein as compared with normal epithelial cells (Original magnification: 400×). **c** ZO-2 expression in benign breast epithelium. Intense ZO-2 positivity shown in the immunohistochemical reaction using polyclonal antibody (Original magnification: 400×). **d** ZO-2 expression in invasive ductal breast carcinoma. Note the membrane staining in some scattered tumor cells only, as compared with normal epithelial cells (Original magnification: 400×)

and percentage. CLDN5 showed strong positivity in the endothelial cells and some positivity in certain normal epithelial cells. In the tumors, intense CLDN5 was observed in the endothelial cells and reduced or no CLDN5 was detected in the tumor cells. The images of immunofluorescent results related to CLDNs 5, 10, 16, 17, 18, ZO-1 and ZO-2 are shown in Figs. 1, 2 and 3.

According to our data and by analysing this cohort of breast carcinomas, CLDN 5, 10, 16, 18, ZO-1 and ZO-2 proteins were downregulated in tumors in comparison with normal breast tissue (Figs. 1, 2 and 3).

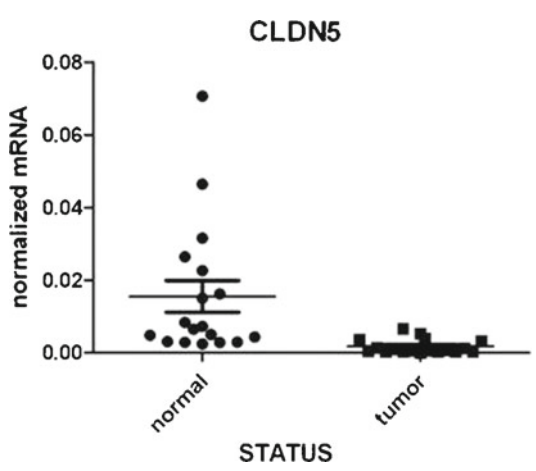
The expression of CLDN17 in concordance with the PCR results was not uniform. In some cases upregulation was observed, whereas in other cases this protein was found to be downregulated (Fig. 2). By comparing our own data with that of HPA our results showed only partial correlation, since the data presented in the Atlas were obtained by using paraffin embedded sections and certain antibodies were of different type (Figs. 4 and 5).

Discussion

Despite our knowledge that numerous events contribute to the evolution of breast carcinoma, it is widely accepted that the loss of cell-to-cell adhesion in epithelial cells is necessary in cancer progression. While some of these proteins are generally accepted to be downregulated in tumors as compared with normal tissues, there are studies demonstrating opposite results. Relationship between losses or gains of TJ proteins in different types of cancers is most probably the result of a complex mechanism yet to be understood [32-34].

Of the TJ components analysed in this study, we found ten TJ-associated genes and six proteins to be downregulated. The

Fig. 4 The expression of eleven significantly differentially expressed transcripts in normal tissues and breast carcinomas. By using GAPDH as reference gene, eleven significantly differentially expressed transcripts were found in tumors as compared with normal breast tissue. The expression of these transcripts in normal tissues is compared with the expression observed in breast carcinomas ▶



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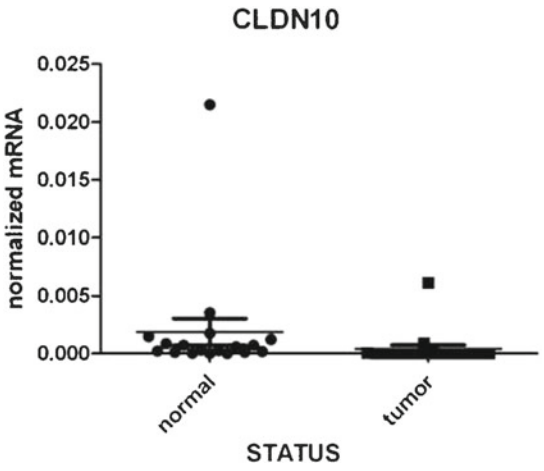

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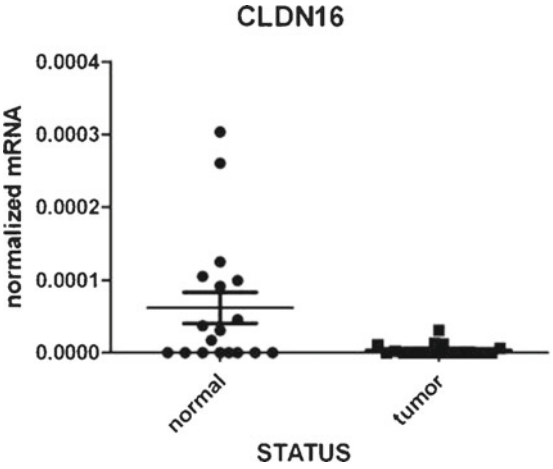

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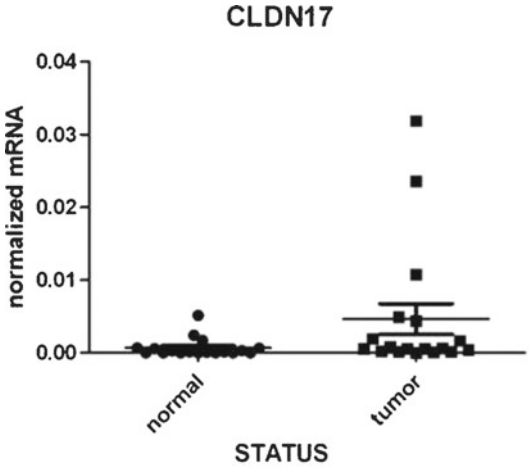

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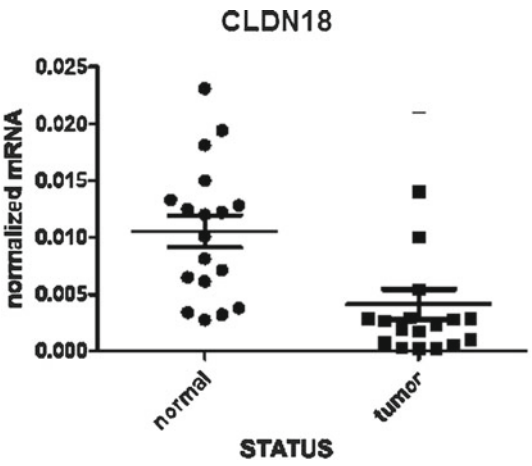
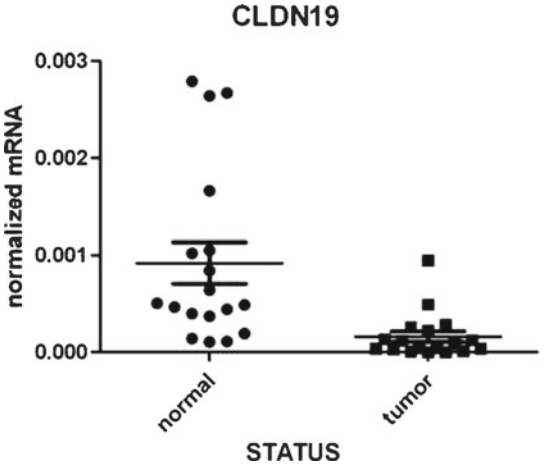


P value = P<0.0001



P value = 0,0001

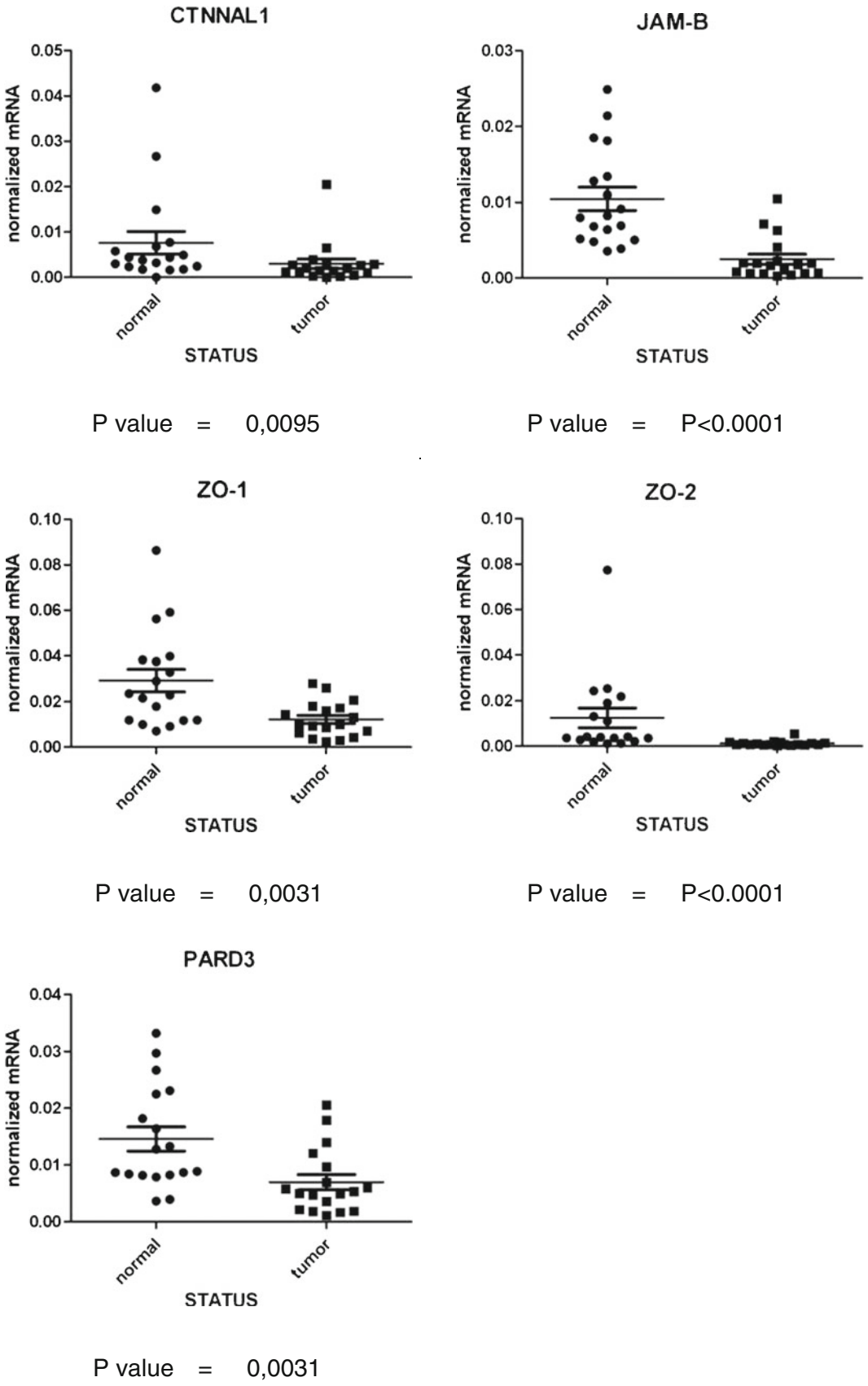


Fig. 4 (continued)

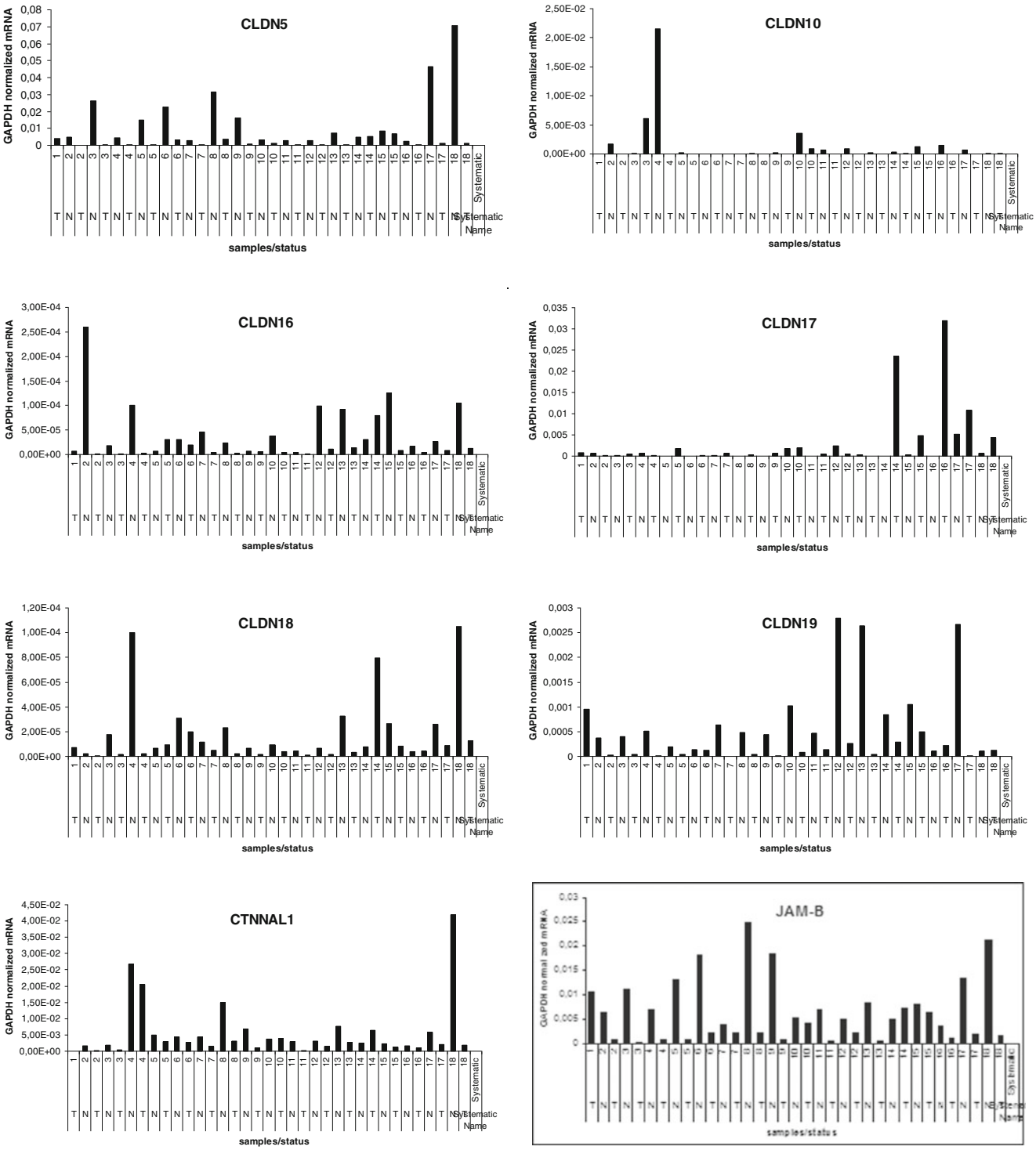
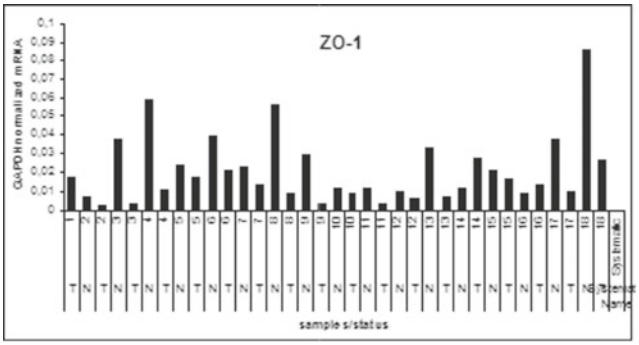
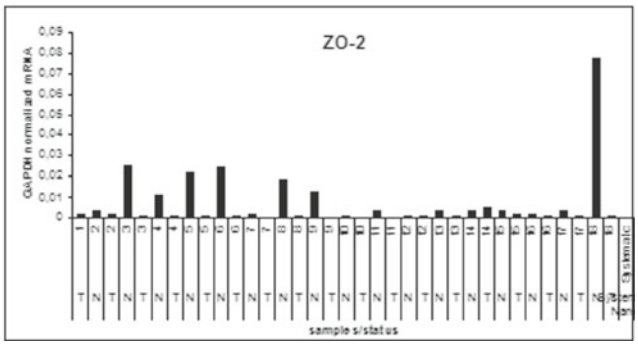


Fig. 5 The results of low-density PCR- array presented case by case. By using GAPDH as reference gene, eleven significantly differentially expressed transcripts were found in tumors as compared with normal breast tissue. These transcripts are presented case by case

expression of CLDN17 protein was not uniform. In some cases upregulation was observed, whereas in others the down-regulation of this protein was detected. Results of recent evaluations indicate that no clear role has been identified for CLDNs 1, 5, 11, 14, 15, 19, 20, 21, while for CLDNs 3, 4, 8, and 19, a tightening potential is indicated in different assays. CLDNs 2, 7, 10, 15, and 16 were identified as paracellular pore-forming claudins [1, 9-11, 15-20, 23, 35-39].

From the TJ components analysed and found by us to be differentially expressed in normal breast epithelia as compared





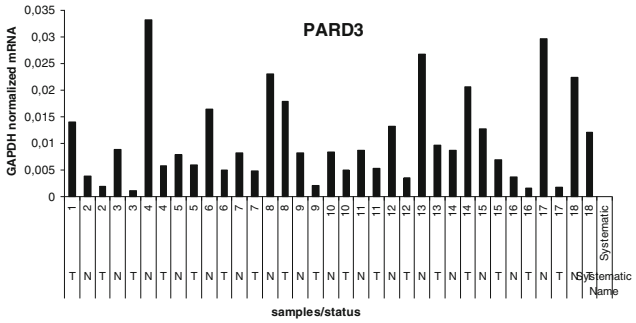


Fig. 5 (continued)

with tumor cells, there are no convincing literature data regarding CLDN 5, 10, 17, 18, 19, CTNNAL1, JAM-B, and PARD3 expressions in breast carcinomas, whereas CLDN16, ZO1, and ZO2 expressions have been discussed in current papers [1, 23].

Concerning CLDN5 expression in breast carcinomas, a recent study by Turunen et al. showed that increased expression of this protein is associated with aggressive behaviour in serous ovarian adenocarcinoma [40]. Our study found CLDN5 to be markedly downregulated in breast tumor tissue, while being highly expressed in endothelial cells.

In a recent study we found that lymph node metastases presented with a notable but not significant increase of claudin-5 expression in both ductal and lobular carcinoma groups [41].

Regarding CLDN10 mRNA, decreased expression was found in breast carcinoma when compared with normal breast tissue at protein level. Analysis of organs other than the breast revealed strong CLDN10 expression in normal gallbladder, with only weak reactions notable in normal intrahepatic bile ducts [42].

Little is known about the expression of CLDNs17, 18 and 19 in breast carcinoma. Using TLDA, we found a tendency for CLDN17 mRNA overexpression, whereas on the contrary, in four cases a downregulation was manifest in tumors as compared with normal breast epithelium, with the tendency confirmed at protein level as well. Further, the downregulation of CLDN18 was also detected in tumors as compared with normal breast tissues. Sanada et al. found that the expression of CLDN18 was reduced in several intestinal metaplasias of the stomach. These authors concluded that downregulation of CLDN18 may be involved in gastric cancers of intestinal phenotype, and may be an early event in gastric carcinogenesis [43]. According to our observation CLDN19 mRNA was downregulated in breast carcinomas. In mouse cell lines Hou et al. described that CLDN16- and CLDN19-depleted TJs had normal barrier function, but defective ion selectivity [44]. No literature data have been presented to date about the role of CLDN19 in the breast.

There are controversial data about expression of the PAR complex in breast carcinomas. At mRNA level, we found PARD3 to be downregulated in tumors compared with normal breast epithelial cells. PAR3 expression has been found to be reduced in oesophageal squamous cell carcinomas in association with lymph node metastases [45].

At mRNA level we found CTNNAL1 to be significantly downregulated in breast carcinomas as compared with normal breast epithelium. A recent report by Ding et al. compared the whole genome of peripheral blood, pimary tumor, brain metastasis and xenograft derived from a single patient, with the finding that two overlapping large deletions, encompassing CTNNA1, were present in all tumor samples supporting the importance of junctional structures in tumor progression [46]. There is an increasing number of published and occasionally controversial data about JAM-A expression in breast carcinomas. Studies have demonstrated that the attenuation of junctional adhesion molecules contributes to breast cancer cell

invasion [24, 25]. There are very few data on the expression of JAM-B and its contribution to breast cancer. In the present study, we found JAM-B mRNA to be downregulated in breast carcinomas when compared with normal breast tissue. Recently published data on CLDN16 demonstrated the expression levels of this protein to be significantly decreased in node positive as compared with node negative breast carcinomas and in patients who died of breast cancer or had generally poor prognosis [23]. We have found CLDN16 to be significantly downregulated in tumors.

The expression of ZO-1 protein has been widely studied, but there are only few data about the expression of ZO-2 in breast carcinomas. In a review published by Brennan et al. (2010) it is concluded that ZO proteins play important roles in migratory events associated with breast cancer progression [1]. We found that ZO-1 and ZO-2 are significantly downregulated at mRNA and protein levels in tumors as compared with normal breast epithelium.

Numerous studies have demonstrated the involvement of decreased or sometimes abnormal expression of cell adhesion/junctional proteins in the process of tumor progression. Very recently an interesting study confirmed that the claudin-low subtypes representing a breast carcinoma subtype with poor prognosis most closely resembles the mammary epithelial stem cell [30].

Literature data regarding the role and expression of TJs in various cancers are constantly expanding, but several questions remain to be answered concerning the mechanisms by which decreased or increased TJ associated genes and proteins contribute to the eventual neoplastic progression.

Conclusions

There have been no published data to date related to several TJ proteins and genes. In our study we found that a large number of TJ components were downregulated in breast carcinomas when compared with normal breast epithelium. We also found TJ genes and proteins with altered expression not yet associated with breast carcinomas.

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Authors' contributions A-MT and JK analysed the selected cases, evaluated the immunohistochemical results and wrote the article. AMSz helped in selecting the genes for TLDA and isolated RNA with A-MT. EJ carried out the immunohistochemical reactions. ZsS participated in the evaluation of the immunohistochemical reactions and in the writing of the manuscript in its final stage. LH, IAM, ZsB, IB and AZ performed surgery and provided samples. SF performed biopsy of specimens after surgery.

References

1. Brennan K, Offiah G, McSherry EA et al (2010) Tight junctions: a barrier to the initiation and progression of breast cancer? J Biomed Biotechnol. doi:10.1155/2010/460607
2. Gonzalez-Mariscal L, Betanzos A, Nava P et al (2003) Tight junction proteins. Progr Biophys Mol Biol 81:1–44
3. Gonzalez-Mariscal L, Lechuga S, Garay E (2007) Role of tight junctions in cell proliferation and cancer. Progr Histochem Cytochem 42:1–57
4. Lelievre SA (2010) Tissue polarity-dependent control of mammary epithelial homeostasis and cancer development: an epigenetic perspective. J Mammary Gland Biol Neoplasia 15:49–63
5. Alroy J (1979) Tight junctions adjacent to tumor stromal interface in human invasive transitional cell carcinomas. Virchows Arch 30:289–296
6. Martinez-Palomo A (1970) Ultrastructural modifications of intercellular junctions between tumor cells. In Vitro 6:15–20
7. Martinez-Palomo A (1970) Ultrastructural modifications of intercellular junctions in some epithelial tumors. Lab Investig 22:605–614
8. Hough CD, Sherman-Baust CA, Pizer ES et al (2000) Large-scale serial analysis of gene expression reveals genes differentially expressed in ovarian cancer. Cancer Res 60:6281–6287
9. Hoevel T, Macek R, Mundigl O et al (2002) Expression and targeting of the tight junction protein CLDN1 in CLDN1-negative human breast tumor cells. J Cell Physiol 191:60–68
10. Hoevel T, Macek R, Swisshelm K et al (2004) Reexpression of the TJ protein CLDN1 induces apoptosis in breast tumor spheroids. Int J Cancer 108:374–383
11. Kominsky SL, Argani P, Korz D et al (2003) Loss of the tight junction protein claudin-7 correlates with histological grade in both ductal carcinoma in situ and invasive ductal carcinoma of the breast. Oncogene 22:2021–2033
12. Kramer F, White K, Kubbies M et al (2000) Genomic organization of claudin-1 and its assessment in hereditary and sporadic breast cancer. Human Genet 107:249–256
13. Sobel G, Paska C, Szabo I et al (2005) Increased expression of claudins in cervical squamous intraepithelial neoplasia and invasive carcinoma. Human Pathol 36:162–169
14. Sobel G, Nemeth J, Kiss A et al (2006) Claudin 1 differentiates endometrioid and serous papillary endometrial adenocarcinoma. Gynecol Oncol 103:591–598
15. Blanchard AA, Skliris GP, Watson PH et al (2009) Claudins 1, 3, and 4 protein expression in ER negative breast cancer correlates with markers of the basal phenotype. Virchows Arch 454:647–656
16. Erin N, Wang N, Xin P et al (2009) Altered gene expression in breast cancer liver metastases. Int J Cancer 124:1503–1516
17. Hewitt KJ, Agarwal R, Morin PJ (2006) The claudin gene family: expression in normal and neoplastic tissues. BMC Cancer 6:186
18. Kim TH, Huh JH, Lee S et al (2008) Down-regulation of claudin-2 in breast carcinomas is associated with advanced disease. Histopathology 53:48–55
19. Kulka J, Szasz AM, Nemeth Z et al (2005) Expression of tight junction protein claudin-4 in basal-like breast carcinomas. Pathol Oncol Res 15:59–64
20. Lanigan F, McKiernan E, Brennan DJ et al (2009) Increased claudin-4 expression is associated with poor prognosis and high tumour grade in breast cancer. Int J Cancer 124:2088–2097

21. Osanai M, Murata M, Nishikiori N et al (2007) Occludin-mediated premature senescence is a fail-safe mechanism against tumorigenesis in breast carcinoma cells. Cancer Sci 98:1027–1034
22. Tokes AM, Kulka J, Paku S et al (2005) Claudin-1, −3 and −4 proteins and mRNA expression in benign and malignant breast lesions: a research study. Breast Cancer Res 7:296–305
23. Martin TA, Harrison GM, Watkins G et al (2008) Claudin-16 reduces the aggressive behavior of human breast cancer cells. J Cell Biochem 105:41–52
24. Naik MU, Naik TU, Suckow AT et al (2008) Attenuation of junctional adhesion molecule-A is a contributing factor for breast cancer cell invasion. Cancer Res 68:2194–2203
25. Naik UP, Naik MU (2008) Putting the brakes on cancer cell migration: JAM-A restrains integrin activation. Cell Adh Migr 2:249–251
26. Morohashi S, Kusumi T, Sato F et al (2007) Decreased expression of claudin-1 correlates with recurrence status in breast cancer. Int J Mol Med 20:139–143
27. Creighton CJ, Li X, Landis M et al (2009) Residual breast cancers after conventional therapy display mesenchymal as well as tumor-initiating features. PNAS 106:13820–13825
28. Herschkowitz JI, Simin K, Weigman VJ et al (2007) Identification of conserved gene expression features between murine mammary carcinoma models and human breast tumors. Genome Biol 8:R76
29. Prat A, Perou CM (2009) Mammary development meets cancer genomics. Nat Med 15:842–844
30. Prat A, Perou CM (2011) Deconstructing the molecular portraits of breast cancer. Mol Oncol 5:5–2331
31. Perou CM, Sorlie T, Eisen MB et al (2000) Molecular portraits of human breast tumours. Nature 406:747–752
32. Escudero-Esparza A, Jiang WG, Martin TA (2011) The Claudin family and its role in cancer and metastasis. Front Biosci 1:1069–1083
33. Turksen K, Troy TC (2011) Junctions gone bad: claudins and loss of the barrier in cancer. Biochim Biophys Acta 1:73–79
34. Dahiya N, Becker KG, Wood WH et al (2011) Claudin-7 is frequently overexpressed in ovarian cancer and promotes invasion. PLoS One 6:e22119
35. Amasheh S, Meiri N, Gitter AH et al (2002) Claudin-2 expression induces cation-selective channels in tight junctions of epithelial cells. J Cell Sci 115:4969–4976
36. Amasheh S, Schmidt T, Mahn M et al (2005) Contribution of claudin-5 to barrier properties in tight junctions of epithelial cells. Cell Tissue Res 321:89–96
37. Nguyen DA, Neville MC (1998) Tight junction regulation in the mammary gland. J Mammary Gland Biol Neoplasia 3:233–246
38. Schulzke JD, Fromm M (2009) Tight junctions: molecular structure meets function. Ann NY Acad Sci 1165:1–6
39. Tsukita S, Furuse M (2000) The structure and function of claudins, cell adhesion molecules at tight junctions. Ann NY Acad Sci 915:129–135
40. Turunen M, Talvensaari-Mattila A, Soini Y et al (2009) Claudin-5 overexpression correlates with aggressive behavior in serous ovarian adenocarcinoma. Anticancer Res 29:5185–5189
41. Szasz A, Tokes A, Micsinai MJ et al (2011) Prognostic significance of claudin expression changes in breast cancer with regional lymph node metastasis. Clin Exp Metastasis 28:55–63
42. Nemeth Z, Szasz AM, Tatrai P et al (2009) Claudin-1, −2, −3, −4, −7, −8, and −10 protein expression in biliary tract cancers. J Histochem Cytochem 57:113–121
43. Sanada Y, Oue N, Mitani Y et al (2006) Down-regulation of the claudin-18 gene, identified through serial analysis of gene expression data analysis, in gastric cancer with an intestinal phenotype. J Pathol 208:633–642
44. Hou J, Renigunta A, Gomes AS et al (2009) Claudin-16 and claudin-19 interaction is required for their assembly into tight junctions and for renal reabsorption of magnesium. PNAS 106:15350–15355
45. Zen K, Yasui K, Gen Y et al (2009) Defective expression of polarity protein PAR-3 gene (PARD3) in esophageal squamous cell carcinoma. Oncogene 28:2910–2918
46. Ding L, Ellis MJ, Li S et al (2010) Genome remodelling in a basal-like breast cancer metastasis and xenograft. Nature 464:999–1005