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Claudin expression in breast cancer: High or low, what to expect?

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Summary. The evaluation of claudins (CLDNs) expression pattern in tumours can be important to understand breast carcinogenesis. The study of CLDNs became more appealing since it was found that CLDN3 and CLDN4 are putative therapeutic targets for *Clostridium perfrigens* enterotoxin (CPE), as well as for monoclonal antibody-based therapy. Moreover, the recently characterized CLDN-low molecular subgroup of breast tumours increased the interest in these molecules. Based on these facts, our aim was to explore the pattern of expression of CLDNs among a large series of invasive breast carcinomas. We also analysed the correlation between the combinatorial expression of CLDN3/CLDN4 and classical prognostic factors and biological markers. In addition, we also compared the characteristics of tumours with low expression of CLDN3, CLDN4 and CLDN7, assessed by immunohistochemistry (IHC), and the ones from CLDN-low subgroup of tumours previously defined by genomic assays.

The combinatorial analysis of the expression of CLDN3/CLDN4 showed a significant association between high CLDN3/CLDN4 levels and triple-negative tumours, as well as with worse patient outcome. This combined analysis may provide useful information for breast carcinomas, since these two CLDN members are putative therapeutic targets. Comparing tumours with low expression of CLDN3, CLDN4 and CLDN7 with tumours previously referred to as CLDN-low by genomic assays, we demonstrated that the single IHC evaluation of these three specific CLDNs is insufficient to identify the CLDN-low molecular subtype of breast tumours. The analysis of several other molecular markers, such as EMT (epithelial-to-mesenchymal transition) and CSC (cancer stem cell) markers should probably be added to improve the identification of this subgroup of tumours by IHC, which probably are enriched in carcinomas with metaplastic differentiation.

Key words: Claudin-1, Claudin-3, Claudin-4, Claudin-7, Breast cancer molecular subtype

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Introduction

Tight junctions are part of the apical junctional complex in epithelial tissues, being essential for the tight sealing of the cellular sheets, controlling paracellular ion flux and, therefore, maintaining tissue homeostasis. These junctions also play a crucial role in the maintenance of cell polarity and have been hypothesized to be involved in the regulation of proliferation, differentiation and other cellular functions, due to their ability to recruit signalling proteins (Morin, 2005).

Tight junctions are composed of three major integral membrane proteins: occludin, claudins (CLDNs), and junctional adhesion molecules. Although the exact roles of these proteins are not completely clear, it seems that CLDNs form the backbone of the tight junction strands. The CLDN family comprises 27 members, ranging in size from 22 to 27 kD, and topologically categorized into the four transmembrane protein classes with the carboxyl-terminus in the cytoplasm and two extracellular loops (Mineta et al., 2011). Although the expression pattern of CLDNs is tissue specific, most tissues express multiple CLDNs that can interact in either a homotypic or heterotypic fashion to form the tight junction strand (Turksen and Troy, 2004). Accumulating data support the view that CLDN expression can reflect biochemical

and functional changes that can occur in both normal and neoplastic epithelium (Morin, 2005; Hewitt et al., 2006; Lanigan et al., 2009; Turksen, 2011). However, due to the lack of specific antibodies, as well as of specific probes on microarrays for every known CLDN, the full CLDN expression profile in normal and neoplastic tissues is still not well characterized.

Nonetheless, CLDN expression has been found to be altered in several cancers and its study in tumours is now growing. Although the exact roles of these proteins in tumorigenesis are still being uncovered, it is clear that they represent promising targets for cancer detection, diagnosis, and therapy. CLDN3 and CLDN4, for example, are frequently overexpressed in several neoplasias, including ovarian, pancreatic and prostate cancers (Morin, 2005). Additionally, it was found that CLDN3 and CLDN4 can function as putative therapeutic targets, since these are receptors for *Clostridium perfringens* enterotoxin (CPE), which has been shown to demonstrate anti-tumour effects (Kominsky et al., 2004). CPE binding triggers the formation of membrane pore complexes, leading to rapid cell death (Walther et al., 2011), and human tumours grown as xenografts could also successfully be treated by CPE, again on the condition of CLDN3 or CLDN4 expression (Michl et al., 2001; Kominsky et al., 2004; Santin et al., 2005). Importantly, these studies showed that no significant toxicity was encountered in mice upon CPE treatment. However, as CLDN3 and/or CLDN4 are expressed in several normal tissues, this may represent a problem in the use of CPE for systemic cancer therapy, and it remains to be seen whether this approach will be useful in the clinic. Approaches that would involve regional application of CPE would be preferable: it has been recently suggested that a nontoxic, but CLDN-specific, COOH-terminal CPE fragment (C-CPE) could be delivered locally and prevent CPE toxicity (Gao and McClane 2012). It has also been shown that CPE gene transfer can be employed for a targeted suicide gene therapy of CLDN3- and CLDN4-overexpressing tumors, leading to rapid and efficient tumour cell killing *in vitro* and *in vivo* (Walther et al., 2011). Lastly, since CLDNs are transmembrane proteins and typically have two relatively large extracellular loops, these proteins may also offer promising targets for antibody-based therapy: antibodies that specifically recognize different extracellular loops have been produced and shown to specifically bind CLDNs on the surface of the cell, providing a proof of principle for the approach. Monoclonal antibodies against CLDN4 individually (Saeki et al., 2009) or in combination with anti-CLDN3 (Kato-Nakano et al., 2010) have been shown to produce prominent results in cancer treatment.

Additionally, the recently characterized CLDN-low molecular subgroup of breast tumours also increased the interest in these molecules. CLDN-low tumours, as well as the other breast tumour subtypes, were categorized using cDNA microarrays and patterns of gene expression profiles (Hennessy et al., 2009; Prat et al., 2010; Taube et al., 2010). Since then, researchers have attempted to develop immunohistochemical surrogates to distinguish these intrinsic subtypes in order to explore the pathology archive material and expand the data available from these studies (Perou, 2011). However, regardless this effort, the CLDN-low subgroup was never detected using immunohistochemistry (IHC), although the characteristics were very well defined: low to absent expression of luminal differentiation markers, high enrichment for epithelial-to-mesenchymal transition (EMT) markers, immune response genes and cancer stem cell-like features. Clinically, the majority of CLDN-low tumours are poor prognosis, estrogen receptor (ER)-negative, progesterone receptor (PgR)-negative, and epidermal growth factor receptor 2 (HER2)-negative (triple negative) invasive ductal carcinomas, with a high frequency of metaplastic and medullary differentiation (Prat et al., 2010).

Thus, the first aim of this work was to expand the knowledge about the expression of specific CLDNs, namely CLDN1, CLDN3, CLDN4 and CLDN7, in a large series of invasive breast carcinomas, and to compare the features of tumours with high versus low expression of CLDNs with standard pathological variables, IHC-surrogate definitions for the intrinsic subtypes, breast cancer stem cell (BCSC) markers and patient outcome. We have found that the majority of breast tumours are negative for CLDNs, but that high expression of CLDN3/CLDN4 is associated with triple-negative tumours and predicts poor patient outcome. In addition, the low expression of CLDN3/CLDN4/CLDN7, assessed by IHC, does not seem to identify the features described in the CLDN-low molecular subtype, previously defined by genomic assays. According to our knowledge, this article is the first to evaluate the expression of CLDNs by IHC across a large panel of breast tumours and across the subtypes, and also makes an interesting observation that CLDN3/CLDN4^{high} tumours seem to do worse than others. Since CLDN3 and CLDN4 are putative therapeutic targets, this may provide useful information for breast cancer management in prognostic and predictive terms.

Material and methods

Patient selection

A series of 466 primary and sporadic invasive breast carcinomas were retrieved consecutively, without selection among the cases, from the Pathology Department, Hospital Xeral-Cíes, Vigo, Spain, diagnosed between 1978-1992. Patients' ages ranged from 28 to 92 years of age. The formalin-fixed paraffin-embedded histological sections were reviewed and the diagnoses confirmed. Tumours were characterised for clinical and pathological features, namely age, tumour size, lymph nodes status and histological grade (Table 1). Patient follow-up information was available for 455 cases, ranging from a minimum of one to a maximum of

120 months after diagnosis. The disease-free survival (DFS) interval was defined as the time from the diagnosis to the date of breast-cancer-derived relapse/metastasis, whereas overall survival (OS) was considered as the number of months from diagnosis to disease-related death. Information concerning the treatment in the adjuvant setting was not available, so its influence was not considered on survival analyses. This study was conducted under the national regulative law for the handling of biological specimens from tumour banks, the samples being exclusively available for research purposes in retrospective studies.

Tissue microarray construction and immunohistochemistry

Twelve TMA blocks were constructed with at least two tissue cores (0.6 mm in diameter) of each tumour sample. Tumours were immunostained for ER, PgR, HER2, EGFR, CK5, P-cadherin, CK14, Vimentin and Ki67 and classified into distinct molecular subtypes, as previously described (Sousa et al., 2010; Ricardo et al., 2011) (Table 1). To study CLDN expression in this tumour series, specific antibodies for CLDN1 (PAD:JAY.8, Invitrogen, Carlsbad, CA, USA), CLDN3 (Z23.JM, Invitrogen, Carlsbad, CA, USA), CLDN4 (Clone Ab15104, Abcam, Cambridge, MA) and CLDN7 (clone Ab27487, Abcam, Cambridge, MA) were used. The primary antibodies were detected using a secondary antibody with HRP polymer (Cytomation Envision System HRP, DAKO, Carpinteria, CA). For visualization of the reaction diaminobenzidine was used as chromogen, according to the manufacturer's instructions. Detailed conditions for each antibody can be found in Table 2. Positive and negative controls were included in all series for all the antibodies used. Paraffin sections of normal skin tissue were used as positive control for CLDN1, normal stomach for CLDN4, and a colon carcinoma for CLDN3 and CLDN7.

Immunohistochemical evaluation

The immunohistochemical expression data obtained for ER, PgR, HER2, EGFR, CK5, P-cadherin, CK14, Vimentin and Ki67 were used to reflect the classification of tumours, according to the different molecular breast cancer subtypes, as described previously by our group (Sousa et al., 2010) (Tables 1 and 3). E-cadherin expression, the BCSC phenotype $CD44^{+}CD24^{-/low}$, as well as ALDH1 expression, were also analysed in this tumour series, as with our previous work (Paredes et al., 2008; Ricardo et al., 2011) (Table 3). Membrane CLDN expression was analyzed by two independent observers (SR, RG). Both registered the intensity (negative, weak, moderate and strong) and the percentage of cells positive for CLDNs (0-10%, 10-25%, 25-50%, >50%) of the membrane expression pattern. Then, a score was generated (scale 0-12) based on these observations (detailed information can be accessed in Table 4). Breast carcinomas were considered $CLDN1^{low}$, $CLDN3^{low}$, $CLDN4^{low}$ and $CLDN7^{low}$ when the score was less than 3; when the score was equal to or higher than 3, tumours were classified as $CLDN1^{high}$, $CLDN3^{high}$, $CLDN4^{high}$ and $CLDN7^{high}$. Based on these results, combined scores for CLDNs were also calculated: cases were classified as $CLDN3/CLDN4^{low}$ when the expression of both CLDN3 and CLDN4 was considered lower than 3; cases were classified as $CLDN3/CLDN4^{high}$ when one of these CLDNs or both were considered to have high levels of

Table 1. Patient and tumour parameters of the invasive breast cancer series.

Variable (N=466)		Frequency	Percentage (%)
Age at Diagnosis	<50 years	115	26.2
	≥50 years	324	73.8
	Missing (n= 27)		
Tumour Size	T1:<2cm	101	24.8
	T2: 2-5 cm	244	59.8
	T3: >5cm	63	15.4
	Missing (n=58)		
Lymph Nodes	Positive	206	56.4
	Negative	159	43.6
	Missing(n=101)		
Histological Grade	Grade I	81	18.3
	Grade II	135	30.5
	Grade III	227	51.2
	Missing (n=23)		
Molecular Subtype	Luminal A	302	64.8
	Luminal B	41	8.8
	HER2-OE	33	7.1
	Basal-like	68	14.6
	Unclassified	22	4.7

Table 2. Specific antibodies and conditions used in the immunohistochemical reactions.

Antigen	Primary antibodies				Antigen retrieval	Detection method
	Clone/PAD	Origin	Incubation time (min)	Dilution		
CLDN1	PAD:JAY.8	Invitrogen	60	1:400	Citrate	HRP-Polymer
CLDN3	Z23.JM	Invitrogen	60	1:300	Citrate	HRP-Polymer
CLDN4	Ab15104	Abcam	60	1:300	Citrate	HRP-Polymer
CLDN7	Ab27487	Abcam	60	1:400	Citrate	HRP-Polymer

expression (≥3). Similarly, the cases were classified as CLDN3/CLDN4/CLDN7^{low} when the three CLDNs were considered to have low expression (<3); accordingly, cases were classified as CLDN3/CLDN4/CLDN7^{high} when one, two or the three CLDNs showed high levels of expression (≥3).

Statistical analysis

Correlations between CLDN1, CLDN3, CLDN4 and CLDN7 and the different molecular subtypes, the clinicopathological parameters, or the different molecular markers, were assessed by Pearson Correlation and Chi-squared tests. Survival curves were estimated using the Kaplan-Meier method and compared using the log-rank test. Univariable and multivariable survival analyses were assessed with Cox Proportional Hazard models (including tumour size, histological grade and lymph node involvement as classical independent prognostic factors in breast cancer), considering a statistically significant level of 5%. Statistical analyses were carried out using SPSS statistics 17.0 software (SPSS Inc., Chicago, IL, USA).

Results

Pattern of expression of CLDN1, CLDN3, CLDN4 and CLDN7 in invasive breast cancer

The staining pattern of the four CLDNs evaluated was predominantly membranous. A well-defined membrane staining pattern was observed for CLDN1 and CLDN3 positive cases, whereas CLDN4 staining sometimes showed a positive cytoplasmic expression, independently of the membrane staining. For CLDN7, the majority of the high expressing tumours presented a discontinuous membrane staining pattern. Within tumours, apart from neoplastic epithelial cells, no other positive cells were found for these markers, namely stromal cells and inflammatory cells. Concerning normal breast, CLDN1 and CLDN4 are expressed by luminal/epithelial cells, whereas CLDN3 and CLDN7 are not detected.

In our tumour series, we found 4.0% (18/451) of high expressing cases for CLDN1, 21.8% (98/450) for CLDN3, 24.8% (110/444) for CLDN4 and 16.9%

Table 3. Immunohistochemical results for the different breast cancer biomarkers (ER, PgR, HER2, EGFR, CK5, P-cadherin, CK14, vimentin, Ki67, E-cadherin, BCSC phenotype (CD44/CD24) and ALDH1).

Variable (N=466)		Frequency	Percentage (%)
ER	Positive	309	66.6
	Negative	155	33.4
	Missing (n=2)		
PgR	Positive	228	49.0
	Negative	237	51.0
	Missing (n=1)		
HER2	Positive	68	14.7
	Negative	394	85.3
	Missing (n=4)		
EGFR	Positive	22	4.7
	Negative	444	95.3
	Missing (n=0)		
CK5	Positive	66	14.2
	Negative	399	85.8
	Missing (n=1)		
P-cadherin	Positive	114	24.5
	Negative	352	75.5
	Missing (n=0)		
CK14	Positive	24	5.2
	Negative	441	94.8
	Missing (n=1)		
Vimentin	Positive	78	17.0
	Negative	380	83.0
	Missing (n=8)		
Ki67	High	29	6.5
	Low	415	93.5
	Missing (n=22)		
E-cadherin	Positive	429	94.9
	Negative	23	5.1
	Missing (n=14)		
BCSC phenotype	$CD44^+CD24^{-/low}$≥10%	209	45.3
	$CD44^+CD24^{-/low}$<10%	252	54.7
	Missing (n=5)		
ALDH1	Positive	33	7.1
	Negative	430	92.9
	Missing (n=3)		

Table 4. Score values considering the intensity and the percentage of positive tumor cells for CLDN membrane staining.

Extension/Intensity	None	Weak	Moderate	Strong
0-10%	0	1	2	3
10-25%	0	2	4	6
25-50%	0	3	6	9
≥50%	0	4	8	12

Table 5. Score frequencies of CLDN1, CLDN3, CLDN4 and CLDN7 in the invasive breast cancer series.

Variable (N=466)		Frequency	Percentage (%)
CLDN1	Low (score <3)	433	96.0
	High (score≥3)	18	4.0
	Missing (n=15)		
CLDN3	Low (score <3)	352	78.2
	High (score≥3)	98	21.8
	Missing (n=16)		
CLDN4	Low (score <3)	334	75.2
	High (score≥3)	110	24.8
	Missing (n=22)		
CLDN7	Low (score <3)	373	83.1
	High (score≥3)	76	16.9
	Missing (n=17)		

Table 6. Associations between the expression of CLDN1, CLDN3, CLDN4 and CLDN7 and the classical breast cancer prognostic factors, biological markers and molecular subtypes.

	CLDN1				CLDN3				CLDN4				CLDN7			
	n	High (score≥3)	Low (score<3)	p	N	High (score≥3)	Low (score<3)	P	n	High (score≥3)	Low (score<3)	p	n	High (score≥3)	Low (score<3)	p
Tumour Size	396	17	379		395	87	308		390	100	290		393	69	324	
T1: <2cm	99	3 (17.7)	96 (25.3)		100	12 (13.8)	88 (28.6)		99	11 (11.0)	88 (30.3)		99	5 (7.3)	94 (29.0)	
T2: 2-5 cm	237	10 (58.8)	227 (59.9)		233	54 (62.1)	179 (58.1)		229	63 (63.0)	166 (57.2)		232	47 (68.1)	185 (57.1)	
T3: >5cm	60	4 (23.5)	56 (14.8)	0.546	62	21 (24.1)	41 (13.3)	0.004**	62	26 (26.0)	36 (12.5)	≤0.001**	62	17 (24.6)	45 (13.9)	≤0.001**
Lymph Nodes	354	17	337		355	76	279		350	90	260		354	60	294	
Positive	198	11 (64.7)	187 (55.5)		200	55 (72.4)	145 (51.9)		197	54 (60.0)	143 (55.0)		199	44 (73.3)	155 (52.7)	
Negative	156	6 (35.3)	150 (44.5)	0.455	155	21 (27.6)	134 (48.1)	0.001**	153	36 (40.0)	117 (45.0)	0.410	155	16 (26.7)	139 (47.3)	0.003**
Histological Grade	428	18	410		428	94	334		423	106	317		426	74	352	
Grade I	78	1 (5.6)	77 (18.8)		77	7 (7.5)	70 (20.9)		76	10 (9.4)	66 (20.8)		78	9 (12.2)	69 (19.6)	
Grade II	128	1 (5.6)	127 (31.0)		130	30 (31.9)	100 (30.0)		126	27 (25.5)	99 (31.2)		128	26 (35.1)	102 (29.0)	
Grade III	222	16 (88.8)	206 (50.2)	0.006**	221	57 (60.6)	164 (49.1)	0.009**	221	69 (65.1)	152 (48.0)	0.004**	220	39 (52.7)	181 (51.4)	0.267
ER	449	18	431		448	98	350		442	110	332		447	75	372	
Positive	295	1 (5.6)	294 (68.2)		296	58 (59.2)	238 (68.0)		291	50 (45.5)	241 (72.6)		294	50 (66.7)	244 (65.6)	
Negative	154	17 (94.4)	137 (31.8)	≤0.001**	152	40 (40.8)	112 (32.0)	0.103	151	60 (54.5)	91 (27.4)	≤0.001**	153	25 (33.3)	128 (34.4)	0.858
PgR	450	18	432		449	97	352		443	110	333		448	76	372	
Positive	222	4 (22.2)	218 (50.5)		219	45 (46.4)	174 (49.4)		215	38 (34.5)	177 (53.1)		220	37 (48.7)	183 (49.2)	
Negative	228	14 (77.8)	214 (49.5)	0.019*	230	52 (53.6)	178 (50.6)	0.596	228	72 (65.5)	156 (46.9)	0.001**	228	39 (51.3)	189 (50.8)	0.935
HER2	447	18	429		446	97	349		440	109	331		445	76	369	
Positive	68	6 (33.3)	62 (14.5)		68	12 (12.4)	56 (16.0)		67	22 (20.2)	45 (13.6)		68	10 (13.2)	58 (15.7)	
Negative	379	12 (66.7)	367 (85.5)	0.029*	378	85 (87.6)	293 (84.0)	0.373	373	87 (79.8)	286 (86.4)	0.097	377	66 (86.8)	311 (84.3)	0.572
Ki67	435	18	417		434	95	339		429	109	320		433	73	360	
High	30	3 (16.7)	27 (6.5)		31	8 (8.4)	23 (6.8)		31	7 (6.4)	24 (7.5)		31	6 (8.2)	25 (6.9)	
Low	405	15 (83.3)	390 (93.5)	0.095	403	87 (91.6)	316 (93.2)	0.584	398	102 (93.6)	296 (92.5)	0.707	402	67 (91.8)	335 (93.1)	0.700
EGFR	451	18	433		450	98	352		444	110	334		449	76	373	
Positive	21	5 (27.8)	16 (3.7)		21	6 (6.1)	15 (4.3)		21	7 (6.4)	14 (4.2)		21	3 (3.9)	18 (4.8)	
Negative	430	13 (72.2)	417 (96.3)	≤0.001**	429	92 (93.9)	337 (95.7)	0.440	423	103 (93.6)	320 (95.8)	0.352	428	73 (96.1)	355 (95.2)	0.741
CK5	451	18	433		450	98	352		444	110	334		449	76	373	
Positive	66	14 (77.8)	52 (12.0)		65	16 (16.3)	49 (13.9)		65	28 (25.5)	37 (11.1)		65	10 (13.2)	55 (14.7)	
Negative	385	4 (22.2)	381 (88.0)	≤0.001**	385	82 (83.7)	303 (86.1)	0.549	379	82 (74.5)	297 (88.9)	≤0.001**	384	66 (86.8)	318 (85.3)	0.720
P-cadherin	451	18	433		450	98	352		444	110	334		449	76	373	
Positive	111	15 (83.3)	96 (22.2)		112	27 (27.6)	85 (24.1)		110	40 (36.4)	70 (21.0)		111	18 (23.7)	93 (24.9)	
Negative	340	3 (16.7)	337 (77.8)	≤0.001**	338	71 (72.4)	267 (75.9)	0.491	334	70 (63.6)	264 (79.0)	0.001**	338	58 (76.3)	280 (75.1)	0.818
CK14	451	18	433		450	98	352		444	110	334		449	76	373	
Positive	24	5 (27.8)	19 (4.4)		24	4 (4.1)	20 (5.7)		24	8 (7.3)	16 (4.8)		24	3 (3.9)	21 (5.6)	
Negative	427	13 (72.2)	414 (95.6)	≤0.001**	426	94 (95.9)	332 (94.3)	0.533	420	102 (92.7)	318 (95.2)	0.318	425	73 (96.1)	352 (94.4)	0.552
Vimentin	446	18	428		446	97	349		440	110	330		444	76	368	
Positive	77	9 (50.0)	68 (15.9)		76	13 (13.4)	63 (18.0)		75	29 (26.4)	46 (13.9)		76	7 (9.2)	69 (18.7)	
Negative	369	9 (50.0)	360 (84.1)	≤0.001**	370	84 (86.6)	286 (82.0)	0.281	365	81 (73.6)	284 (86.1)	0.003**	368	69 (90.8)	299 (81.3)	0.044*
E-cadherin	444	18	426		444	96	348		438	108	330		443	76	367	
Positive	423	17 (94.4)	406 (95.3)		421	94 (97.9)	327 (94.0)		416	105 (97.2)	311 (94.2)		421	75 (98.7)	346 (94.3)	
Negative	21	1 (5.6)	20 (4.7)	0.866	23	2 (2.1)	21 (6.0)	0.122	22	3 (2.8)	19 (5.8)	0.218	21	1 (1.3)	21 (5.7)	0.108
BCSC phenotype	448	18	430		448	98	350		441	110	331		446	76	370	
$CD44^{+}CD24^{-/low}$≥10	202	12 (66.7)	190 (44.2)		202	39 (39.8)	163 (46.6)		198	54 (49.1)	144 (43.5)		199	32 (42.1)	167 (45.1)	
$CD44^{+}CD24^{-/low}$<10%	246	6 (33.3)	240 (55.8)	0.060	246	59 (60.2)	187 (53.4)	0.233	243	56 (50.9)	187 (56.5)	0.307	247	44 (57.9)	203 (54.9)	0.628
ALDH1	448	18	430		447	98	349		441	109	332		446	76	370	
Positive	33	5 (27.8)	28 (6.5)		32	11 (11.2)	21 (6.0)		33	17 (15.6)	16 (4.8)		33	8 (10.5)	25 (6.7)	
Negative	415	13 (72.2)	402 (93.5)	0.001**	415	87 (88.8)	328 (94.0)	0.077	408	92 (84.4)	316 (95.2)	≤0.001**	413	68 (89.5)	345 (93.3)	0.253
Molecular Subtypes	451	18	433		450	98	352		444	110	334		449	76	373	
Luminal A	289	1 (5.6)	288 (66.5)		288	54 (55.1)	234 (66.5)		284	51 (46.4)	233 (69.8)		287	48 (63.2)	239 (64.1)	
Luminal B	40	4 (22.2)	36 (8.3)		41	10 (10.2)	31 (8.8)		40	9 (8.2)	31 (9.3)		41	7 (9.2)	34 (9.1)	
HER2-OE	33	2 (11.1)	31 (7.2)		33	4 (4.1)	29 (8.2)		33	13 (11.8)	20 (5.9)		33	5 (6.6)	28 (7.5)	
Basal-like	67	10 (55.5)	57 (13.2)		66	20 (20.4)	46 (13.1)		66	31 (28.2)	35 (10.5)		66	7 (9.2)	59 (15.8)	
Unclassified	22	1 (5.6)	21 (4.8)	≤0.001**	22	10 (10.2)	12 (3.4)	0.009**	21	6 (5.4)	15 (4.5)	≤0.001**	22	9 (11.8)	13 (3.5)	0.027*

* Significant correlation at the 0.05 level; ** Significant correlation at the 0.01 level.

(76/449) for CLDN7 (Table 5). Concerning the distribution of CLDNs expression within the distinct breast cancer molecular subtypes, we observed that, from the low number of CLDN1^{high} cases, the majority were basal-like (55.5%, 10/18) (p≤0.001). A high percentage of the triple-negative tumours (basal-like and unclassified) had high levels of expression of CLDN3 (34.1%, 30/88), whereas only 12.1% (4/33) of HER2-OE and 19.5% (64/329) of the luminal tumours (A and B) were classified as CLDN3^{high} (p=0.009) (Fig. 1). The evaluation of CLDN4 expression revealed that 47% (31/66) of basal-like carcinomas and 39.4% (13/33) of HER2-OE tumours showed high levels of this protein, whereas only a minority of luminal tumours (18.5%, 60/324) were CLDN4^{high} (p≤0.001). Regarding CLDN7, the greater part of the cases presented low expression levels and the majority of the high expressing tumours were luminal A (63.2%, 48/76) (p=0.027) (Table 6).

Association between CLDNs expression and classical prognostic factors and biological markers

When we analysed the few CLDN1^{high} cases, we demonstrated that the majority were high grade tumours (88.8%, 16/18) (p=0.006) and negative for hormonal receptors: 94.4% (17/18) were ER negative (p≤0.001) and 77.8% (14/18) were PgR negative (p=0.019). There was also an association with HER2 positive cases (p=0.029). When we analysed the putative association with basal markers, we observed a significant association between CLDN1^{high} expression and the expression of basal markers, such as EGFR (p≤0.001), CK5 (p≤0.001), P-cadherin (p≤0.001), CK14 (p≤0.001) and vimentin (p≤0.001) (Table 6). In addition, 66.7% (12/18; p=0.060) of CLDN1^{high} showed a high percentage of cells expressing the BCSC phenotype CD44^{+}CD24$^{-/low}$, although this was not statistically significant; however, the association with the breast cancer stem cell marker ALDH1 was highly significant (5/18, 27.8%; p=0.001). Using log-rank test to generate Kaplan-Meier curves, we found no significant differences in DFS and OS between patients harbouring CLDN1^{high} and CLDN1^{low} tumours, mostly due to the low number of cases in the CLDN1^{high} subgroup (data not shown).

Our results also showed that CLDN3^{high} expression was associated with large tumour size (T2 and T3) (86.2%, 75/87) (p=0.004), lymph node metastasis (72.4%, 55/76) (p=0.001) and tumours with histological grades II and III (92.5%, 87/94) (p=0.009). We did not find any association between CLDN3 expression and ER, PgR or HER2 staining. In the same way, we did not observe a statistically significant association between this CLDN and basal-like markers, such as EGFR, CK5, P-cadherin, CK14 or Vimentin (Table 6). Although not statistically significant, survival studies revealed that patients with CLDN3 positive tumours tend to have a worse DFS (p=0.096) and OS (p=0.120) (data not shown).

Table 7. Associations between the combined expression of CLDN3 and/or CLDN4 and the classical breast cancer prognostic factors, biological markers and molecular subtypes.

		CLDN3/CLDN4 expression		
	n	CLDN3^{high} and/or CLDN4^{high}	CLDN3^{low} and CLDN4^{low}	p
Tumour Size	389	200	189	
T1: <2cm	99	31 (15.5)	68 (36.0)	
T2: 2-5 cm	228	127 (63.5)	101 (53.4)	
T3: >5cm	62	42 (21.0)	20 (10.6)	≤0.001**
Lymph Nodes	350	180	170	
Positive	197	120 (66.7)	77 (45.3)	
Negative	153	60 (33.3)	93 (54.7)	≤0.001**
Histological Grade	421	216	205	
Grade I	75	25 (11.6)	50 (24.4)	
Grade II	126	63 (29.2)	63 (30.7)	
Grade III	220	128 (59.2)	92 (44.9)	0.001**
ER	440	225	215	
Positive	289	129 (57.3)	160 (74.4)	
Negative	151	96 (42.7)	55 (25.6)	≤0.001**
PgR	441	225	216	
Positive	213	99 (44.0)	114 (52.8)	
Negative	228	126 (56.0)	102 (47.2)	0.065
HER2	438	223	215	
Positive	67	37 (16.6)	30 (13.9)	
Negative	371	186 (83.4)	185 (86.1)	0.443
Ki67	428	223	205	
High	31	17 (7.6)	14 (6.8)	
Low	397	206 (92.4)	191 (93.2)	0.752
EGFR	442	226	216	
Positive	21	14 (6.2)	7 (3.2)	
Negative	421	212 (93.8)	209 (96.8)	0.144
CK5	442	226	216	
Positive	65	42 (18.6)	23 (10.7)	
Negative	377	184 (81.4)	193 (89.3)	0.019*
P-cadherin	442	226	216	
Positive	110	69 (30.5)	41 (19.0)	
Negative	332	157 (69.5)	175 (81.0)	0.005**
CK14	442	226	216	
Positive	24	12 (5.3)	12 (5.5)	
Negative	418	214 (94.7)	204 (94.5)	0.909
Vimentin	438	225	213	
Positive	75	39 (17.3)	36 (16.9)	
Negative	363	186 (82.7)	177 (83.1)	0.905
E-cadherin	436	223	213	
Positive	414	218 (97.7)	196 (92.0)	
Negative	22	5 (2.3)	17 (8.0)	0.006**
BCSC phenotype	440	226	214	
CD44^{+}CD24$^{-/low}$≥10%	198	90 (39.8)	108 (50.5)	
CD44^{+}CD24$^{-/low}$<10%	242	136 (60.2)	106 (49.5)	0.025*
ALDH1	439	224	215	
Positive	32	26 (11.6)	6 (2.8)	
Negative	407	198 (88.4)	209 (97.2)	≤0.001**
Molecular Subtypes	442	226	216	
Luminal A	282	127 (56.2)	155 (71.7)	
Luminal B	40	20 (8.9)	20 (9.3)	
HER2-OE	33	20 (8.9)	13 (6.0)	
Basal-like	66	45 (19.9)	21 (9.7)	
Unclassified	21	14 (6.2)	7 (3.3)	0.004**

*: significant correlation at the 0.05 level; **: significant correlation at the 0.01 level

High CLDN4 expression was correlated with large tumour size (26/62, 41.9%; p≤0.001), higher histological grade (65.1%, 69/106) (p=0.004) and triple-negative phenotype, meaning ER negative (54.5%, 60/110) (p≤0.001), PgR negative (65.5%, 72/110) (p≤0.001) and HER2 negative cases (79.8%, 87/109) (p=0.097). The high expression of CLDN4 was associated with the positivity for basal markers, such as CK5 (p≤0.001), P-cadherin (p=0.001), and vimentin (p=0.003) (Table 6). In addition, like CLDN1, CLDN4^{high} tumours showed a high percentage of cells expressing ALDH1 (17/109, 15.6%; p≤0.001), but no association was found with the BCSC phenotype evaluated by CD44/CD24 expression. Overall, CLDN4 expression showed a tendency to be associated with a worse patient DFS (p=0.118) and OS (p=0.071) (data not shown).

Concerning CLDN7 expression, we found a significant association between high levels and larger tumour sizes (64/69, 92.7%; p≤0.001) and positive lymph nodes (44/60, 73.3%; p=0.003). We also found that these tumours were frequently negative for vimentin expression (69/76, 90.8%; p=0.044). No association was found with the classical breast cancer markers ER, PgR and HER2, or with basal-like markers (Table 6). Finally, no statistical difference was observed concerning patient outcome (DFS and OS) (data not shown).

Combinatorial evaluation of CLDN3/CLDN4 expression pattern

Since CLDN3 and CLDN4 are highly homologous in their molecular structure (Lal-Nag and Morin, 2009), and each one individually was highly associated with more aggressive tumours, we decided to evaluate the combined expression of CLDN3 and CLDN4 in our series of breast carcinomas. Remarkably, we found a significant association between high levels of both these CLDNs and all the classical prognostic factors evaluated in this series, such as large tumour size (p≤0.001), lymph node metastasis (p≤0.001) and high histological grade (p≤0.001) (Table 7). A significant correlation was also found between CLDN3/CLDN4 high positivity and ER negative tumours (p≤0.001), but no association was observed with PgR, HER2 or Ki67. In contrast, we found a statistically significant correlation between the expression of the basal markers CK5 (p=0.019) and P-cadherin (p=0.005) and the expression of E-cadherin (p=0.006) with the high expression of CLDN3/CLDN4. We also found that these tumours show positivity for ALDH1 (81.3%, 26/32; p≤0.001), but less than 10% of tumour cells presenting the CD44^{+}CD24$^{-/low}$ phenotype (60.2%, 136/226; p=0.025) (Table 7). Furthermore, 67.8% (59/87) of the triple-negative tumours showed a high expression of CLDN3 and/or CLDN4. From these tumours, a significant percentage of 76.3% (45/59) were basal-like (Fig. 1) and 23.7% (14/59) cases were triple-negative non-basal tumours or unclassified (Table 7). When we examined the relationship between CLDN3/CLDN4 expression and patient survival, our findings showed that the expression of these markers was statistically correlated with worse DSF (p=0.012) and OS (p=0.003) (Fig. 2). Cox regression univariable analyses confirmed that patients with tumours expressing high levels of CLDN3 and/or CLDN4 have a hazard ratio (HR) of 1.451 (p=0.013) for DFS and HR=1.591 (p=0.003) for OS, when compared to patients with tumours with low expression of both proteins (Table 8). The same analysis performed for the single

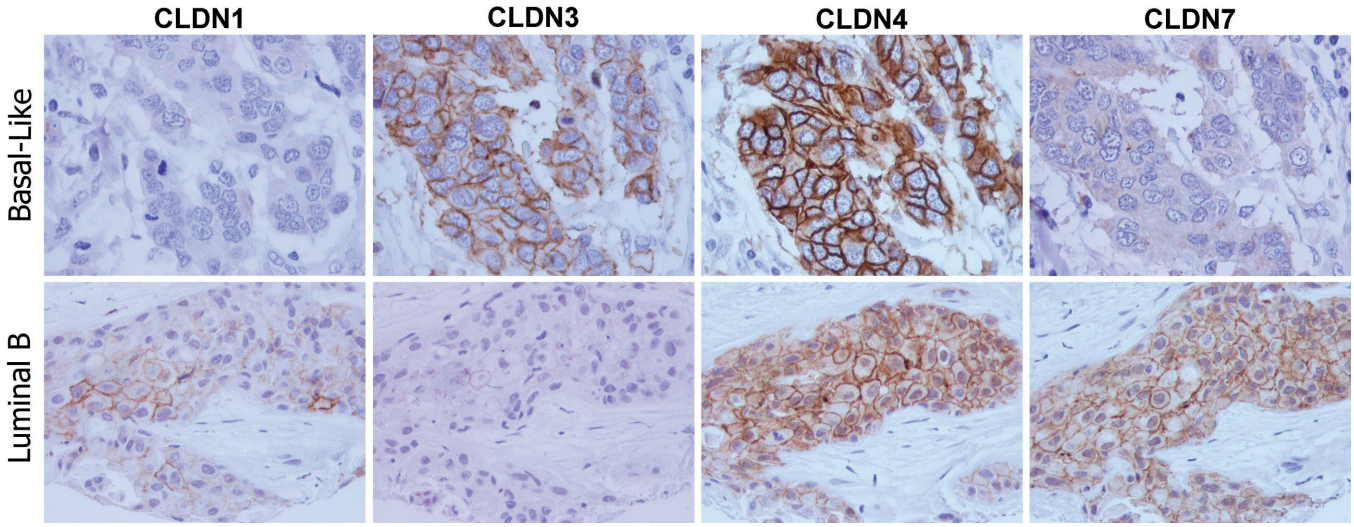

Fig. 1. Illustrations of CLDN expression pattern in two invasive breast carcinomas: a Basal-like carcinoma presenting negative expression for CLDN1 and CLDN7, and positivity for CLDN3 and CLDN4 (x 650); and, a Luminal B carcinoma presenting positivity for CLDN1, CLDN4 and CLDN7, and negativity for CLDN3 (x 400).

expression of each one of these CLDNs did not show a statistically valid difference between high and low expression groups (Table 8). Nevertheless, in multivariable analysis, with models including tumour size, histological grade and lymph node involvement, CLDN3/CLDN4 did not reach statistically significant levels to be considered an independent prognostic factor (data not shown).

Identification of tumours with low expression of CLDN3, CLDN4 and CLDN7

Taking into account the recently characterized CLDN-low molecular subtype of breast carcinomas, we hypothesized that tumours with low expression of CLDN3, CLDN4 and CLDN7, assessed through IHC, should show similar characteristics to the CLDN-low subgroup defined by genomic assays (Prat et al., 2010). Overall, we found 44.3% (195/440) of cases with low CLDN expression and 55.7% (245/440) of tumours with high expression of CLDN3, CLDN4 and CLDN7. Unexpectedly, a significant association between low levels of these three CLDNs and T1 tumours [64.6% (64/99), p<0.001], negative lymph node status [54.9% (84/153), p<0.001] and grade I tumours [61.3% (46/75), p<0.001] was found (Table 9). Low CLDN expression was significantly associated with positivity for ER [74.2% (144/194), p=0.001], and negativity for basal markers EGFR [97.5% (190/195), p=0.053], CK5 [90.3% (176/195), p=0.008] and P-cadherin [81.5% (159/195), p=0.005]. In addition, from all E-cadherin negative cases, 77.3% (17/22) were also negative for all three CLDNs (p=0.001). No significant association was found between low CLDN expression and BCSC phenotype [49.7% (96/193) presented $CD44^+CD24^{-/low}\geq 10\%$ and 50.3% (97/193) showed a $CD44^+CD24^{-/low}<10\%$ phenotype (p=0.075)] and, from all ALDH1 positive cases, only 12.5% (4/32) presented low

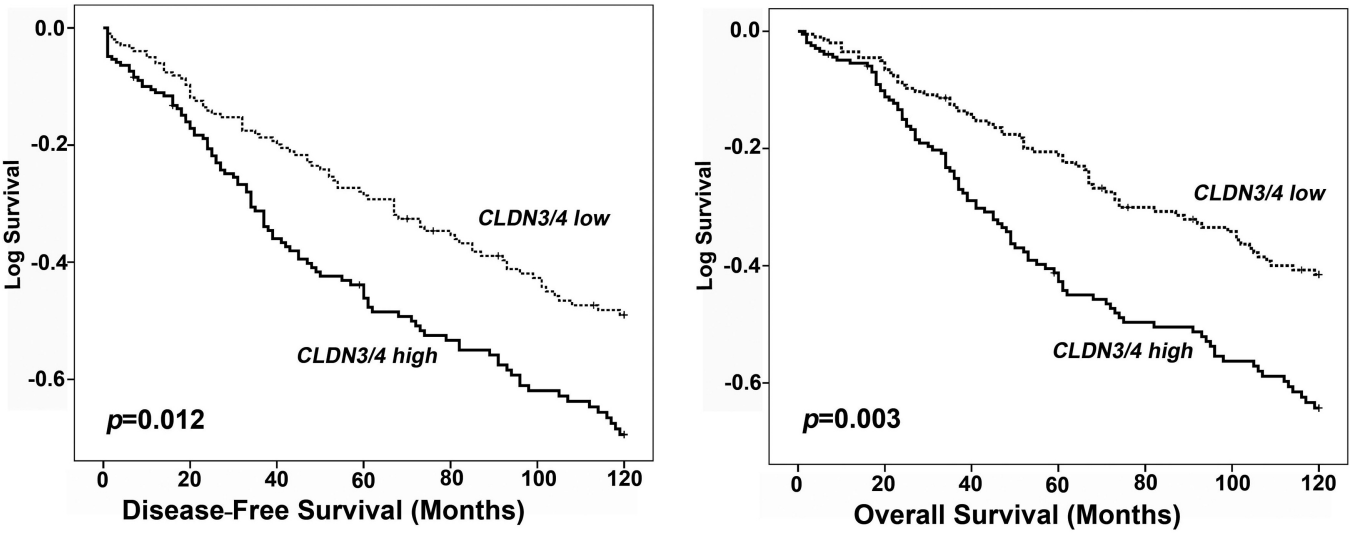

Fig. 2. Kaplan-Meier survival curves for all breast cancer patients harbouring tumours expressing high and low levels of CLDN3/CLDN4: disease-free survival (p=0.012) **(a)** and overall survival (p=0.003) **(b)**.

Table 8. Univariable analysis of Disease-free Survival (DFS) and Overall Survival (OS) in a large series of patients diagnosed with invasive breast cancer.

Variable	Disease-free Survival			Overall Survival		
	HR	(95% CI)	p*	HR	(95% CI)	p*
CLDN1 *(high vs low, ref)*	0.956	0.449-2.054	0.907	1.100	0.516-2.343	0.806
CLDN3 *(high vs low, ref)*	1.319	0.950-1.832	0.098	1.312	0.930-1.849	0.122
CLDN4 *(high vs low, ref)*	1.291	0.935-1.783	0.121	1.631	0.972-1.906	0.073
CLDN7 *(high vs low, ref)*	1.037	0.712-1.511	0.848	1.015	0.684-1.505	0.943
CLDN3/CLDN4 *(high vs low, ref)*	1.451	1.083-1.945	0.013	1.591	1.169-2.166	0.003
CLDN3/CLDN4/CLDN7 *(high vs low, ref)*	1.354	1.007-1.820	0.045	1.545	1.128-2.116	0.007

* p values were calculated by the log-rank test.

Table 9. Associations between the combined extression of CLDN3, CLDN4 and/or CLDN7 and the classical breast cancer prognostic factors, biological markers and molecular subtypes.

	CLDN3/CLDN4/CLDN7 expression			
	n	CLDN (3, 4 and 7)^{low}	CLDN (3, 4 and/or 7)^{high}	p
Tumour Size	387	171	216	
T1: <2cm	99	64 (37.4)	35 (16.2)	
T2: 2-5 cm	226	89 (52.0)	137 (63.4)	
T3: >5cm	62	18 (10.6)	44 (20.4)	<0.001**
Lymph Nodes	350	155	195	
Positive	197	71 (45.8)	126 (64.6)	
Negative	153	84 (54.2)	69 (35.4)	<0.001**
Histological Grade	419	185	234	
Grade I	75	46 (24.8)	29 (12.4)	
Grade II	126	59 (31.9)	67 (28.6)	
Grade III	218	80 (43.3)	138 (58.9)	0.001**
ER	438	194	244	
Positive	287	144 (74.2)	143 (58.6)	
Negative	151	50 (25.8)	101 (41.4)	0.001**
PgR	439	195	244	
Positive	213	103 (52.8)	110 (45.1)	
Negative	226	92 (47.2)	134 (54.9)	0.107
HER2	436	194	242	
Positive	67	30 (15.5)	37 (15.3)	
Negative	369	164 (84.5)	205 (84.7)	0.960
Ki67	426	185	241	
High	31	13 (7.0)	18 (7.5)	
Low	395	172 (93.0)	223 (92.5)	0.862
EGFR	440	195	245	
Positive	21	5 (2.5)	16 (6.5)	
Negative	419	190 (97.5)	229 (93.5)	0.053
CK5	440	195	245	
Positive	65	19 (9.7)	46 (18.8)	
Negative	375	176 (90.3)	199 (81.2)	0.008**
P-cadherin	440	195	245	
Positive	110	36 (18.5)	74 (30.2)	
Negative	330	159 (81.5)	171 (69.8)	0.005**
CK14	440	195	245	
Positive	24	10 (5.1)	14 (5.7)	
Negative	416	185 (94.9)	231 (94.3)	0.788
Vimentin	436	192	244	
Positive	75	34 (17.7)	41 (16.8)	
Negative	361	158 (82.3)	203 (83.2)	0.804
E-cadherin	434	192	242	
Positive	412	175 (91.1)	237 (97.9)	
Negative	22	17 (8.9)	5 (2.1)	0.001**
BCSC phenotype	438	193	245	
$CD44^+CD24^{-/low}$≥10%	197	96 (49.7)	101 (41.2)	
$CD44^+CD24^{-/low}$<10%	241	97 (50.3)	144 (58.8)	0.075
ALDH1	437	194	243	
Positive	32	4 (2.1)	28 (11.5)	
Negative	405	190 (97.9)	215 (88.5)	<0.001**
Molecular Subtypes	440	195	245	
Luminal B	280	139 (71.3)	141 (57.5)	
Luminal A	40	20 (10.3)	20 (8.2)	
HER2-OE	33	13 (6.7)	20 (8.2)	
Basal-like	66	17 (8.7)	49 (20.0)	
Unclassified	21	6 (3.1)	15 (6.1)	p=0.004**

**: significant correlation at the 0.01 level.

expression of CLDN3, CLDN4 and CLDN7 (p<0.001). Considering all cases, Cox regression univariable analyses showed that tumours with high expression levels of CLDN3, CLDN4 and/or CLDN7 had a significantly higher hazard ratio for recurrence and death, when compared with tumours with low expression of CLDNs (HR=1.354 (DFS) (p=0.045); and HR=1.545 (OS) (p=0.007)) (Table 8). These same findings were confirmed by Kaplan-Meier survival curves. In multivariable analysis, with models including tumour size, histological grade and lymph node involvement, the combinatorial evaluation of these three CLDNs did not reach a statistically significant level, and thus cannot be considered an independent factor to predict patient survival (data not shown).

Since there was a big discrepancy between our findings and the ones found by Prat and collaborators, which described the CLDN-low subtype as enriched for poor prognosis triple-negative invasive ductal carcinomas, with a high frequency of metaplastic and medullary differentiation displaying EMT features, immune system responses, and stem cell-associated biological traits, we still decided to observe the characteristics of the tumours showing low expression levels of CLDN3/CLDN4/CLDN7, negative expression for E-cadherin and positive expression for Vimentin. We only found 5 cases with these characteristics within our series. Interestingly, two were metaplastic breast carcinomas (MBC) and the other three were invasive ductal carcinomas-not otherwise specified (IDC-NOS). The MBCs were grade III tumours, classified as triple-negative and enriched for the BCSC phenotype defined by CD44 and CD24. One of the IDC-NOS also presented the same characteristics as both MBCs (grade III, triple-negative and BCSC phenotype). However, the other 2 IDC-NOS were ER positive tumours, classified as grade II carcinomas; even so, these were also enriched for the BCSC phenotype ($CD44^+CD24^{-/low}$≥10%).

Discussion

CLDNs play a central role in tight junction cellular homeostasis, being important in the maintenance of cell polarity and regulation of paracellular permeability (Mineta et al., 2011). Several data suggest that the CLDN gene family is controlled by classical pathways important in embryogenesis and development, which can also be involved in neoplastic transformation (Swisshelm et al., 2005). The exact patterns of expression of CLDNs in different cancers and normal tissues are not well known. However, some studies indicate that, depending upon the type of neoplasia, CLDN proteins may be diminished, elevated or mislocated in tumour cells compared to normal adjacent cells (Swisshelm et al., 2005).

IHC has been a useful tool to study CLDN expression patterns within tumours and normal tissues. However, to date, there is no consensus concerning the IHC evaluation of CLDN expression in tumours. Some

authors evaluated membrane and cytoplasmic staining (Sauer et al., 2005; Soini et al., 2006; Morohashi et al., 2007; Blanchard et al., 2009), while others only considered the membrane staining pattern as we did (Soini, 2004; Tokes et al., 2005; Park et al., 2007; Kulka et al., 2009; Szasz et al., 2011a). Furthermore, while some of the studies considered only the percentage of stained cells, others considered the percentage and the intensity of the staining. In addition, all these previous works considered dissimilar cut-off values to distinguish positive and negative cases. This ambiguous data analysis makes it difficult to compare the results between the studies. Here, we evaluated the membrane staining, with a combined score, evaluating the percentage of positive cells and intensity, which we considered the better method to access an accurate estimation of this type of staining pattern (Blanchard et al., 2009).

The first aim of this study was to investigate the pattern of expression of CLDNs among invasive breast tumours. Overall, CLDN4 was the one more frequently expressed among invasive breast cancer (24.8%), followed by CLDN3 (21.8%) and CLDN7 (16.9%), being CLDN1 (4.0%) the less frequent. Indeed, the percentage of negative cases for each CLDN was always higher than the percentage of positive cases. In our view, this fact is mainly related with the evaluation system that we have used in this work, where only membrane expression was considered to classify a case as positive for each CLDN. In the few publications where only membrane expression is considered, the frequencies are nearly the same as the ones found by us (Szasz et al., 2011a). However, the majority of the studies evaluating CLDNs by IHC considered cytoplasmic expression to classify a case as positive, which immediately increases the number of positive cases (Morohashi et al., 2007). Nevertheless, in all studies, a significantly higher number of tumours are positive for CLDN3 and CLDN4, compared to CLDN7 and CLDN1 (Morin, 2005; Hewitt et al., 2006), as also found in our breast cancer series.

Previous studies have already shown a low frequency of CLDN1 positivity in breast cancers (Blanchard et al., 2009). It has been suggested that partial or total loss of CLDN1 expression correlates with increased malignant potential, invasiveness and with recurrence of disease (Tokes et al., 2005; Morohashi et al., 2007), whereas its re-expression induces apoptosis (Hoevel et al., 2004). Despite the low number of positive cases, our results support the previous study by Blanchard et al. (2009), showing that the frequency of CLDN1 positivity was significantly higher in ER negative breast cancers and associated with basal-like molecular subtype. However, no association with patient OS or DFS was found, corroborating previous studies by these same authors (Blanchard et al., 2009). We also described, for the first time, the interesting association between the expression of this CLDN and the presence of the BCSC phenotype.

CLDN7 failed to correlate with the majority of the markers evaluated in this series of breast tumours. Similar to earlier studies (Park et al., 2007; Szasz et al., 2011a), we also found no association of CLDN7 with patient survival. However, Blackman et al. (2005) found that CLDN7 was expressed constitutively in invasive cells, regardless of tumour type, grade or regional lymph node metastases. Moreover, Szasz et al., (Szasz et al., 2011a) demonstrated that the expression of this tight junction protein was decreased in regional lymph node metastasis in lobular and ductal carcinomas. Conversely, Park et al. (2007) showed an increase of CLDN7 expression in metastatic lobular and ductal carcinomas when compared to the primary corresponding tumours. We found an association between the expression of this protein and large tumours and positive lymph nodes.

Considering CLDN4, Lanigan et al. (2009) already demonstrated that its overexpression was predictive of shorter survival in breast carcinomas. Some studies have shown that CLDN4 was highly upregulated in the mammary gland during pubertal development, a time of rapid cellular growth and invasion (McBryan et al., 2007). Although this parallel between normal tissue growth and breast cancer could be useful for the comprehension of carcinogenesis, the exact mechanism by which CLDN4 is exerting this effect is still unclear. In the present work, we did not find a statistically significant difference between CLDN4 high and low expression in patient prognosis survival curves. Similar results were achieved for CLDN3; however, due to the significant association found between CLDN3 and positivity for tumour cells in axillary lymph nodes, its expression in the primary tumour gained a putative predictive potential for axillary lymph node involvement. Indeed, although not statistically significant, survival studies revealed that patients with CLDN3 positive tumours tend to have a worse DFS. However, when CLDN3 and CLDN4 were analysed together, the association between tumours expressing high levels of both CLDNs with worse DFS and OS become statistically significant. Since these two CLDN members are phylogenetically closely related (Lal-Nag and Morin, 2009), we analysed them in combination and, to the best of our knowledge, this is the first study analysing the association of the combined expression of CLDN3 and CLDN4 with prognostic markers and patient outcome. Our results demonstrated that CLDN3/CLDN4 expression occur predominantly in larger tumours, with lymph node metastasis and higher histological grade. However, due to these significant statistical associations, this parameter was not an independent prognostic factor in breast cancer, when multivariable analysis was performed. Moreover, these tumours exhibit hormonal receptor negativity, corroborating earlier studies (Blanchard et al., 2009). The greater percentage of the CLDN3/CLDN4 high expressing tumours also correlated with positivity for basal markers, such as CK5 and P-cadherin, being enriched in triple-negative tumours (basal-like or non-basal-like tumours), in agreement with former studies

(Blanchard et al., 2009; Kulka et al., 2009). CLDN3 and/or CLDN4 are also associated with increased tumour grade in prostatic adenocarcinomas (Long et al., 2001), with advanced tumour stage in urothelial carcinoma of the upper urinary tract (Nakanishi et al., 2008) and with poor patient outcome in renal cell carcinoma (Lechpammer et al., 2008), endometrial cancer (Konecny et al., 2008) and gastric adenocarcinomas (Resnick et al., 2005). Very recently, an immunohistochemical protein profile consisting of CLDN4 and E-cadherin was also able to predict outcome in a breast cancer series in a very effective way. This combination of proteins resulted in a CLDN4 and E-cadherin score (CURIO), which was able to accurately predict relapse-free survival. The multivariate analysis, including clinicopathological variables and the CURIO, showed that the latter kept its predictive power. Furthermore, the CURIO was able to further refine prognosis, separating good versus poor prognosis subgroups in luminal A, luminal B, and triple-negative breast cancer intrinsic subtypes (Szasz et al., 2011b).

Interestingly, CLDN3 and CLDN4 are receptors for CPE, which can kill cells expressing these proteins. This toxin has been demonstrated to be effective in treating breast tumour xenografts in mice (Kominsky et al., 2004) and intracranial treatment was found to inhibit tumour growth and increase survival of breast cancer brain metastasis (Kominsky et al., 2007). Moreover, in other tumour types, CPE proved to be effective in tumour cells which are highly resistant to chemotherapy (Santin et al., 2005, 2007). This toxin is also effective in diminishing the barrier function of epithelial cells expressing CLDN3 and CLDN4 (Sonoda et al., 1999), which may prove useful in increasing uptake and effectiveness of cytotoxic drugs. Recently, a monoclonal antibody, KM3907 (IgG2a), which recognizes CLDN3 and CLDN4, but not CLDN5, CLDN6 and CLDN9, was successfully isolated. It has been demonstrated that this specific dual-targeting monoclonal antibody therapy induces an antibody-dependent cellular *in vitro* cytotoxicity, also inhibiting *in vivo* tumour formation in SCID mice (Kato-Nakano et al., 2010). Besides this, other authors already tested the anti-CLDN4 monoclonal antibody (mAb) single therapy and evaluated its anti-tumor efficacy, which proved to be effective for the treatment of pancreatic and ovarian cancer (Nichols et al., 2004; Suzuki et al., 2009).

The other aim of this study was to analyse whether tumours with low expression of CLDN3, CLDN4 and CLDN7, by IHC, showed similar features to the ones defined as CLDN-low by genomic assays (Prat et al., 2010). Through this analysis, we could observe that, using IHC, the subset of tumours with low CLDNs expression presented different features when compared with the characteristics of CLDN-low subtype, previously described by Prat et al. (2010). Actually, this CLDN-low subtype was enriched for poor prognosis triple-negative carcinomas, with a high frequency of metaplastic and medullary differentiation, with EMT features and BCSC phenotype. At the transcriptomic level, these were characterized by low levels of E-cadherin expression and high levels of vimentin, Snail-1, Snail-2, TWIST1, TWIST2, ZEB1 and ZEB2. Moreover, in the Prat et al. description, >55% of the so-called CLDN-low tumours express E-cadherin and/or CLDN3 moderate/strong expression (Prat et al., 2010). In our work, the low CLDN expression pattern, assessed by IHC, was demonstrated to be insufficient to discriminate the CLDN-low molecular subtype. However, although this needs to be proven with a large number of tumours, it seems that, when E-cadherin and Vimentin are added to CLDNs, we are able to discriminate the CLDN-low subtype, which is more representative of tumours with metaplastic differentiation and enriched for the BCSC phenotype. Thus, using these IHC-surrogate markers (CLDN3, CLDN4, CLDN7, E-cadherin, vimentin, CD44 and CD24) in a series of metaplastic breast carcinomas, we would probably identify a group of breast carcinomas with similar characteristics to the CLDN-low subgroup described by gene expression profiling.

In conclusion, in this study, we evaluated CLDN expression based on a composite method, measuring the staining intensity and the proportion of positive tumour cells. This measurement permitted a consistent assessment of the CLDN1, CLDN3, CLDN4 and CLDN7 patterns of expression among invasive breast carcinomas. We observed that the expression of these four members of the CLDN family varies among invasive breast carcinomas, CLDN3 and CLDN4 being the ones more frequently expressed. In addition, the combinatorial analysis of the expression of CLDN3/CLDN4 showed a significant correlation between high expression levels and triple negative tumours, as well as with worse patient outcome. This combined analysis may provide useful information for breast cancer patient prognosis, since these two CLDN members are putative therapeutic targets. Finally, we also demonstrated that the IHC evaluation of CLDN3, CLDN4 and CLDN7 is insufficient to identify the CLDN-low molecular subtype defined by genomic assays.

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