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Loss of tight junction plaque molecules in breast cancer tissues is associated with a poor prognosis in patients with breast cancer

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Abstract

The aim of this study was to investigate the expression of Zonula Occludens (ZO) proteins ZO-1, ZO-2 and ZO-3, and MUPP-1 (multi-PDZ domain protein 1), peripheral/plaque proteins that function in maintaining tight junction integrity and in transducing regulatory signalling events in patients with primary breast cancer. Breast cancer primary tumours ($n = 114$) and background tissues ($n = 30$) were processed for quantitative-polymerase chain reaction (PCR) analysis, Western blotting and immunostaining. Standardised transcript levels of ZO-1 and MUPP-1 were significantly lower in patients with metastatic disease compared with those remaining disease-free (DF) (median follow-up 72.2 months). Immunohistochemistry confirmed these results, with decreased levels in ZO-1 staining. For both ZO-1 and ZO-3, staining was confined to the intercellular regions in normal tissue, whereas in tumour tissues staining was diffuse and cytosolic. Q-PCR revealed a reduction in the levels of ZO-1 and MUPP-1 in patients with disease recurrence. Prognostic indicators of breast cancer were also inversely correlated with ZO-1 expression. We conclude that low levels of tight junction plaque molecules, such as ZO-1 and MUPP-1, in breast cancer are associated with poor patients prognosis.

Keywords: ZO-1; ZO-2; ZO-3; Tight junction; MUPP-1; Breast cancer; Prognosis; PCR

1. Introduction

Tight junctions are directly involved in paracellular sealing and in membrane domain differentiation [1,2]. The Tight junction is a region where the plasma membrane of adjacent cells forms a series of contacts that appear to completely occlude the extracellular space thereby creating an intercellular barrier and intramembrane diffusion fence [3]. Tight junctions in endothelial cells function as a barrier through which molecules and inflammatory cells can pass and in epithelial cells the tight junction functions in an adhesive manner and can prevent cell dissociation [4,5]. An important step in the formation of cancer metastases is the interaction with and penetration of the vascular endothelium by dissociated cancer cells. Tight junctions are therefore the first barrier that cancer cells must overcome in order to metastasise. We have previously demonstrated that tight junctions of vascular endothelium function *in vivo* as a barrier between blood and tissues against metastatic cancer cells [6].

The tight junction structure can be separated into three regions of molecules: (i) transmembrane region includes those molecules that mechanically confer adhesiveness to the cell by (usually) homotypic bonding to the same molecule on adjacent cells. These include Occludin, Claudins (−1 to −26) and junctional adhesion molecules (JAM) −1, −2 and −3. (ii) The plaque or

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peripheral region of the tight junction includes those molecules that anchor the transmembrane molecules to the tight junction structure and link them to the cell cytoskeleton and signalling pathways; essentially controlling the tight junction structure and function. The Zonula Occludens (ZO) family of MAGUK proteins, including MUPP-1 falls into this category. (iii) The third region is composed of associated molecules, that is, those molecules that often have other roles in the cell, but are known associates of the tight junction, where they function as part of the signalling/control mechanism for the structure (such as AF6, α-catenin and ponsin).

Cell adhesion to adjacent cells and the extracellular matrix is key not only to the organisation of epithelium into a tissue, but also to the regulation of cellular processes such as gene expression, differentiation, motility and growth [7]. Cell adhesion molecules, transmembrane receptors and cytoskeletal factors, all of which are organised into multimolecular complexes, and the activation of signalling pathways, mediate these regulatory functions. Studies suggest that some of the cell adhesion and cytoskeletal proteins may serve an additional and important function, namely, suppression of the malignant phenotype of cells during tumorigenesis [7].

Early studies demonstrated a correlation between the reduction of tight junctions and tumour differentiation and experimental evidence has emerged to place tight junctions on the frontline as the structure that cancer cells must overcome in order to metastasise [8–11]. Although a considerable body of work exists on tight junctions and their role in a number of diseases, it is only in the last few years that their possible role in tumorigenesis has been studied. To date, most of the work has concentrated on cell lines and, to a limited degree, on colorectal and pancreatic cancers, with only a few studies carried out on breast cancer tissues – which have concentrated on Claudin-1 (SEMP-1), claudin-7 and ZO-1 expression.

It has been shown that Claudin-1 appears to be absent in some breast cancer cell lines, that also have little/no Occludin or ZO-1 [12]. Paraffin-embedded breast cancer samples immunohistochemically-stained for ZO-1 have been examined. It has been found that staining is reduced or lost in 69% of the cancers examined and that ZO-1 staining has a positive correlation with tumour differentiation. Genetic changes in the ZO-1 gene may account for these changes [13]. Recently, it has been demonstrated that loss of claudin-7 correlates with histological grade in both ductal carcinoma *in situ* and invasive ductal carcinoma of the breast [14]. Such findings suggest that the loss of claudin-7 may aid in tumour cell dissemination and augment the cell's metastatic potential.

Such (albeit) limited evidence, along with work carried out on other types of cancer, indicates that tight junctions may play a key role in breast cancer cell tumorigenesis. In this report, we have examined the role of tight junction molecules in human breast cancer and examined the correlations between levels of plaque proteins, ZO-1, -2, -3 and MUPP-1, and patient prognosis in primary breast cancer tumours.

2. Patients and methods

2.1. Tissue collection and preparation

Breast tissue samples (tumour and matched background samples, Table 1) were collected and immediately frozen in liquid nitrogen before processing; a portion of each sample was used for quantitative-polymerase chain reaction (PCR) analysis, a portion for immunohistochemical analysis and another portion for routine histological examination. The clinical details of the cohort have been documented elsewhere in [15].

RNA was isolated from tissue samples using standard RNA-zol procedures. For reverse transcription (RT)-PCR, cDNA was synthesised in a 20 μl reaction mixture using 1 μg RNA, as described in the protocol (AB Gene Reverse Transcription System, Surrey, UK).

2.2. Quantitative-PCR

The quantitative real-time PCR analysis using the Amplofluor™ Uniprimer™ system (Intergen Company Oxford, UK) and Thermo-Start® (ABgene, Epsom, Surrey, UK), was done as we previously described in [15,16]. Tight junction specific primer pairs were designed by the authors using a Beacon Designer software and manufactured by Invitrogen (Invitrogen Life Technologies, Paisley, Scotland, UK), each amplifying a region that spans at least one intron (Table 2), generating an approximately 100 base pair product from both the control plasmid and cDNA. Using the Icycler IQ system (Bio-Rad), which incorporates a gradient thermocycler and a 96-channel optical unit, the plasmid standards and breast cancer cDNA were simultaneously assayed in duplicated reactions using a standard hot-start Q-PCR master mix. Q-PCR conditions were as follows: Enzyme activation 95 °C for 12 min, 1 cycle, followed by 50 cycles of Denaturing: 95 °C for 15 s; Annealing: 55 °C for 40 s; Extension: 72 °C for 25 s. Using purified plasmids as internal standards, the level of each tight junction molecule cDNA (copies/50 ng RNA) in the breast cancer samples were calculated. Q-PCR for β-actin was also performed on the same samples, to correct for any residual differences in the initial level of RNA in the specimens (in addition to spectrophotometry). The products of Q-PCR were verified on agarose gels.

Table 1
Clinical information for the patient samples analysed

Tissue type	Background	Tumour			
n = :	30	124			
Tumour grade	Grade 1	Grade 2	Grade 3		
n = :	24	42	58		
NPI	NPI 1	NPI 2	NPI 3	Unknown	
n = :	68	38	16	2	
TNM	TNM-1	TNM-2	TNM-3	TNM-4	Unknown
n = :	70	40	7	4	3
Histology	Ductal	Lobular	Other		
n = :	94	14	16		
Outcome	1: Alive & well	2: Metastatic disease	3: Local recurrence	4: Death from Breast cancer	234: All poor outcomes
n = :	85	7	5	15	27

NPI, Nottingham prognostic index.

Table 2
Quantitative-PCR primers pairs for analysis of tight junction plaque protein molecules

Molecule	Primer sequence	
ZO-1	ZO1F1	CCACATACAGATACGAGTCCTC
	ZO1ZR	ACTGAACCTGACCGTACAGTAACTGCGTGAA-TATTGCT
ZO-2	ZO2F1	CAAAAGAGGATTTGGAATTG
	ZO2ZR	ACTGAACCTGACCGTACAGAGCACATCAGAA-ATGACAA
ZO-3	ZO3F1	CTGACATGGAGGAGCTGA
	ZO3ZR	ACTGAACCTGACCGTACAGCTTAGCTTCCCTT-CTGACT
MUPP-1	MUPPF1	GGGATGTAGCAAATGAAGAC
	MUPPZR	ACTGAACCTGACCGTACATTGAAGTTGCTGAA-GTTGC

PCR, polymerase chain reaction.

2.3. Immunohistochemistry

Tissue samples from frozen sections were prepared as described previously in [15]. Sections were stained using ZO-1, ZO-2 and ZO-3 antibodies (PharMingen International). Primary antibodies were used (1:400) for 45 min and then washed in buffer. The secondary antibodies (biotinylated) were added (in horse serum/buffer solution) for 30 min, followed by numerous washings. Avidin/Biotin complex was added for 30 min, again followed with washes. Diaminobenzadine was used as a chromogen to visualise the antibody/antigen complex. Sections were counterstained in Mayers haematoxylin for 1 min, dehydrated, cleared and mounted in distyrene-tricresylphosphate-xylene (DPX) and screened using a 100× objective.

2.4. Western blotting

Total cell lysates were prepared as follows: 10 μm tissue sections were lysed in HCMF buffer containing 240 mg/ml Tris, 430 mg/ml NaCl, 250 mg sodium deoxycholate, 0.5% sodium dodecyl sulphate (SDS), 0.5% Triton X-100, 2 mM $CaCl_2$, 100 μg/ml phenylmethylsulphonyl fluoride, 1 mg/ml leupeptin, 1 mg/ml aprotinin and 10 mM sodium orthovanadate for 40 min. Protein concentration was quantified using a fluorescamine-based method as we previously reported in [17,18] and was made the same using the lysis buffer. Sample buffer was added and the protein boiled at 100 °C for 5 min before clarification at 13 000*g* for 10 min. Equal amounts of protein from each tissue sample were added onto an 8% polyacrylamide gel. Following electrophoresis, proteins were blotted onto nitrocellulose sheets and blocked in 10% skimmed milk for 60 min before probing with specific primary antibodies (ZO-1, ZO-2, ZO-3, 1:500 dilution, purchased from Insight Biotechnology Ltd., Middlesex, UK), and then with peroxidase-conjugated secondary antibody (1:1000). Protein bands were visualised with an enhanced chemiluminescent (ECL) system (Insight Biotechnology Ltd., Middlesex, UK). The band intensities on photographic film were analysed with densitometry software (Optimas 6.0, Optimas UK Ltd.) and shown as relative values [18].

2.5. Statistical analysis

Statistical analysis was performed by MINITAB version 13 (Minitab Inc. State College, PA, USA) using a two-sample Student *t*-tests.

3. Results

3.1. Distribution and expression of tight junction molecules in tumour and background cancer tissues

We firstly carried out an immunohistological study to assess the location, distribution and the degree of staining of the tight junction plaque proteins, ZO-1, ZO-2

and ZO-3, using matched tumour and background tissues. In normal mammary tissues, the location of these molecules appeared as strong staining at the apical part of the epithelial cells and in blood vessels in the luminal face of the structure (Fig. 1). However, the staining was reduced for all three proteins in the tumour sections when compared with their paired background sections. In addition, the distribution of the molecules was also altered in the tumour tissues compared with the background tissues. There was also a reduction of ZO-1 in the tight junction structures in tumour tissues, which could be clearly seen in the background tissue.

When the tight junction molecules were examined at mRNA levels using quantitative PCR, the levels of ZO-1, ZO-3 and MUPP-1 were lower in tumour tissues compared with normal tissues, although these were not statistically significantly different (Table 3). ZO-2 levels were slightly elevated in the tumour tissues compared with the controls. The mRNA results correlated with the Western blotting analysis, where both ZO-1 and -3 levels were reduced (Fig. 2).

3.2. Expression of tight junction molecules, NPI status and TNM staging

The Nottingham prognostic index (NPI) was used to assess patient prognosis (Tables 1 and 4). Significantly lower levels of ZO-1 were seen in patients with poorer prognoses, i.e., NPI-3 tumours (Table 4). There were reduced levels of ZO-3 in NPI-3 tumours compared with NPI-1 tumours. However, this was not significant. Conversely, ZO-2 and MUPP-1 levels increased with increased NPI status, although again the results were not statistically significant.

The levels of the molecules were also analysed according to the TNM status of the tumours (Table 4). Levels of ZO-1 were significantly reduced in TNM2 and TNM3 tumours (TNM1 *vs.* TNM3 $P = 0.006$;

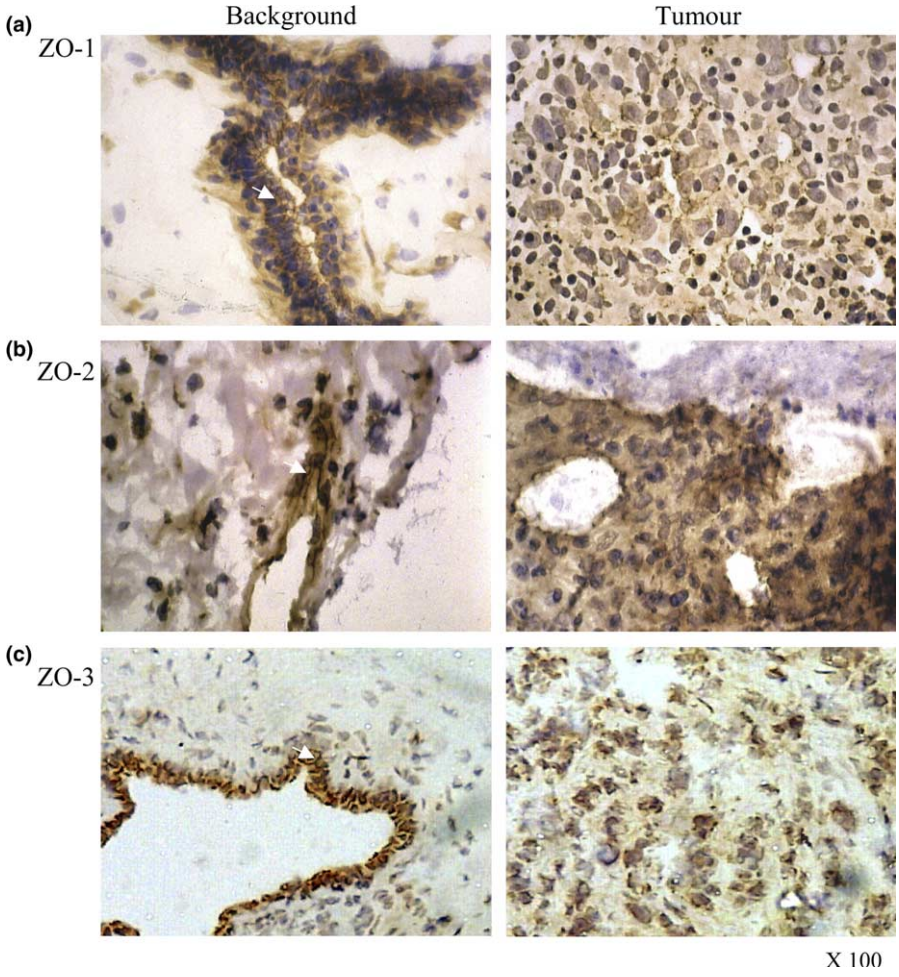

Fig. 1. Immunohistochemical staining (100×) of the tight junction plaque/peripheral proteins. (a) ZO-1, (b) ZO-2 and (c) ZO-3 in human breast cancer tissues. The panel on the left shows intense and clear staining patterns for all three molecules in normal background tissue (indicated by arrows). The panel on the right shows reduced staining for ZO-1 and ZO-3 in tumour sections; there was reduced or absent tight junction patterning for all 3 molecules in tumour tissues.

Table 3
RNA transcript levels of the 4 tight junction molecules in human primary breast tumours, comparing tumour and background sections

	Tumour		Background		*P* value
	Mean ± SD	*N*	Mean ± SD	*N*	
ZO-1	230.4 ± 60.1	111	109 ± 53.6	30	0.13
ZO-2	629 ± 138	115	878 ± 416	31	0.57
ZO-3	258 ± 134	101	117.2 ± 57.8	29	0.33
MUPP-1	1.111 ± 0.73	125	0.1498 ± 0.0951	32	0.19

SD, standard deviation.

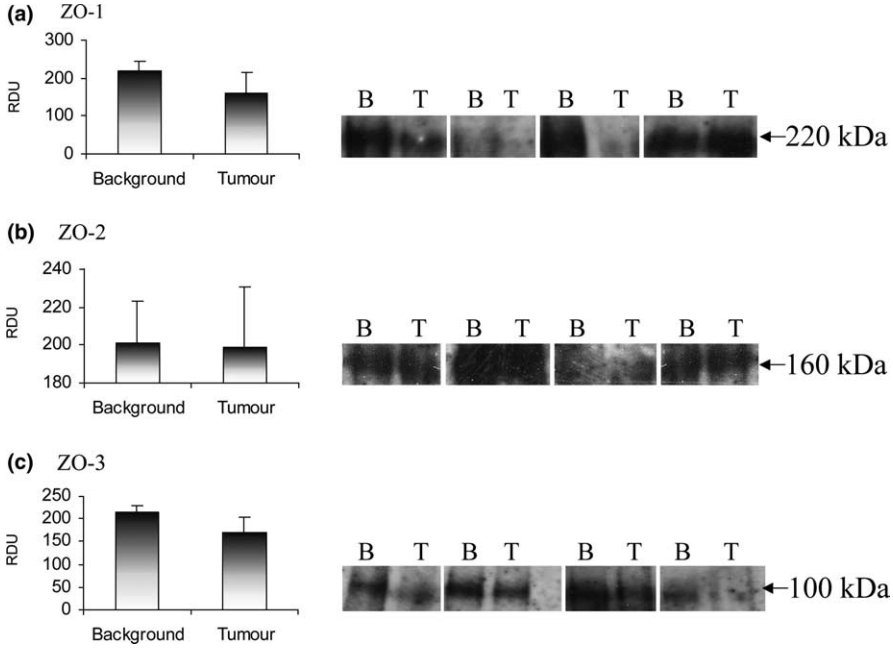

Fig. 2. Western blotting of proteins extracted from paired normal and tumour breast tissues with: (a) ZO-1, (b) ZO-2 and (c) ZO-3 and densitometric measurement to compare levels (left panel). Total protein levels of ZO-1 and ZO-3 were reduced in tumour sections compared with normal/background. There was little difference in the protein levels for ZO-2. RDU, relative densitometric units. 8 from 10 were reduced.

TNM2 *vs.* TNM3 $P = 0.04$) and levels of ZO-3 were also reduced with increasing TNM status, again a non-significant result. Data also indicated that MUPP-1 was reduced in patients with TNM2 status compared with TNM1 tumours. ZO-2 expression showed an overall increase with increased TNM stage (TNM3).

3.3. Tight junction expression in different tumour grades and histological types

ZO-1, ZO-2, ZO-3 and MUPP-1 levels decreased in higher grade tumours (Fig. 3). However, the differences were not statistically different. Interestingly, the

Table 4
Analyses of mRNA from samples showing levels of the four tight junction molecules tested and tumour prognosis by nottingham prognostic indicator (NPI) and nodal involvement (TNM)

	Plaque protein			
	ZO-1	ZO-2	ZO-3	MUPP-1
NPI1	247.4 ± 81.9	407.9 ± 98.2	62.7 ± 22.5	0.036 ± 0.009
NPI 2	204.0 ± 123.0	827.0 ± 350.0	69.1 ± 38.8	0.078 ± 0.037
NPI 3	51.9 ± 28.7*	1180.0 ± 531	51.2 ± 26.3	0.043 ± 0.028
TNM 1	278.6 ± 93.6	661 ± 176	72.5 ± 26.4	0.055 ± 0.012
TNM 2	195.4 ± 85.0	349.6 ± 94.0	70.2 ± 30.4	0.023 ± 0.009***
TNM 3	13.22 ± 9.03**	2762 ± 1720	30.0 ± 27.1	0.187 ± 0.176

* NPI 1 *vs.* NPI 3, $P = 0.028$.
** TNM 1 *vs.* TNM 3, $P = 0.006$; TNM 2 *vs.* TNM 3, $P = 0.04$.
*** TNM 2 *vs.* TNM 1, $P = 0.042$.

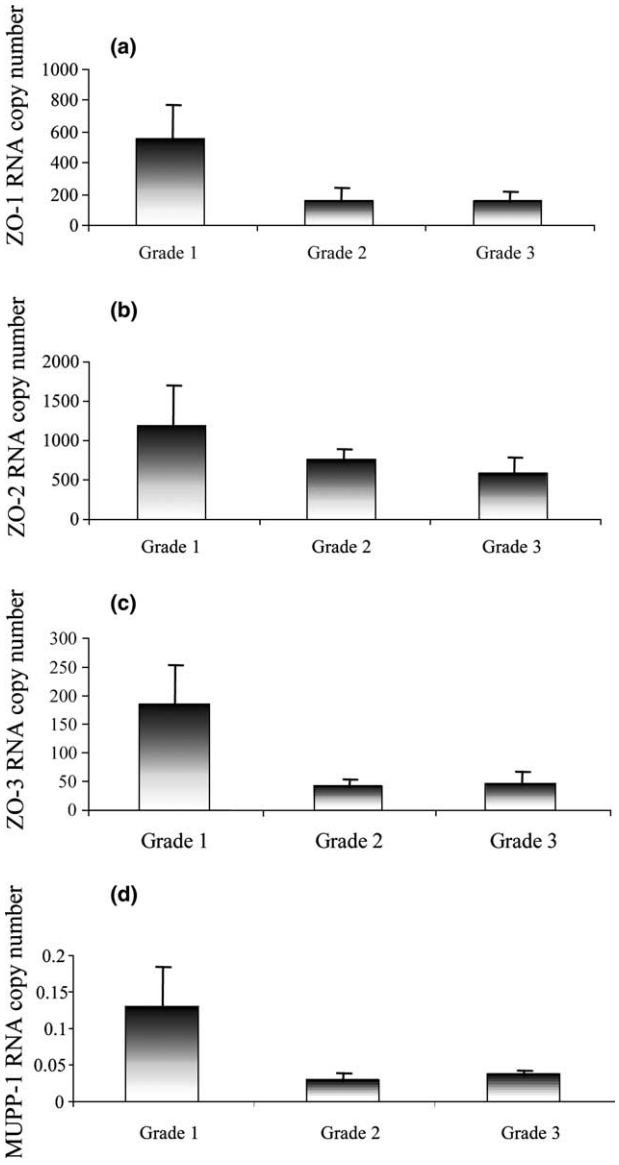

Fig. 3. Comparison of grade and RNA transcript level of the plaque/peripheral tight junction molecules ZO-1 (a), ZO-2 (b), ZO-3 (c) and MUPP-1 (d). All 4 molecules were reduced with increasing grade of tumour, although this did not reach statistical significance.

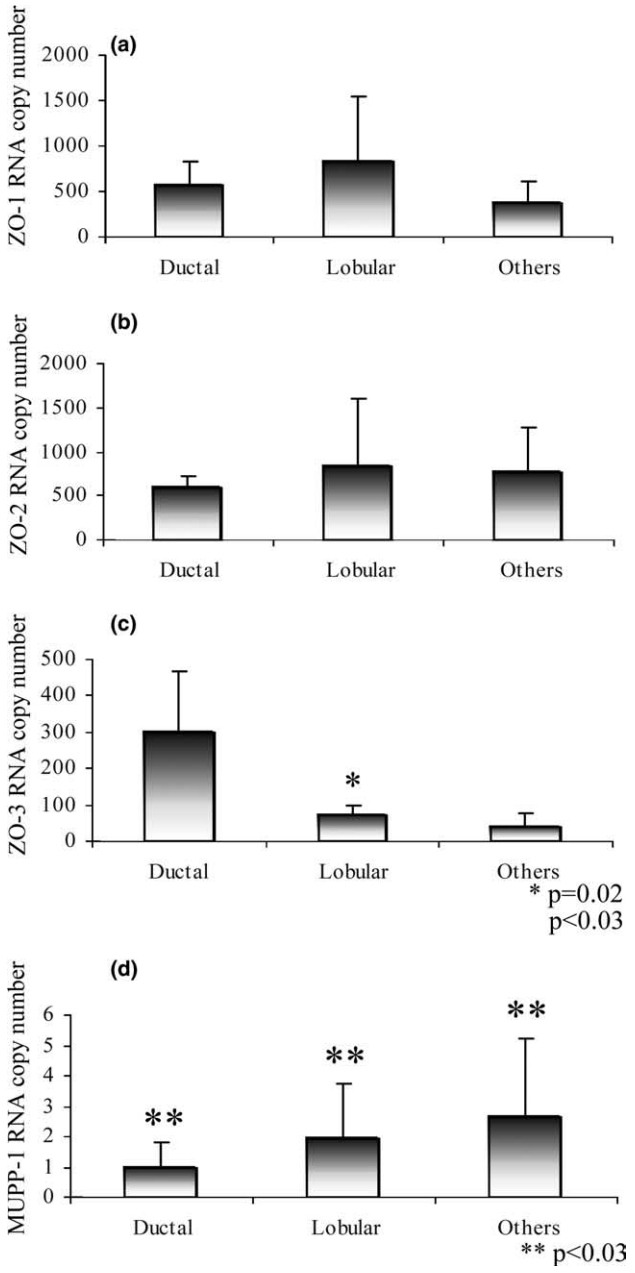

Fig. 4. Comparison according to the histology of primary breast tumours and RNA transcript level of the plaque/peripheral tight junction molecules ZO-1 (a), ZO-2 (b), ZO-3 (c) and MUPP-1 (d). Both ZO-1 and ZO-2 were increased in lobular carcinomas compared with the other types. ZO-3 showed significantly reduced levels in lobular carcinomas, and MUPP-1 was significantly reduced in ductal carcinomas compared with the other types.

tight junction molecules, ZO-1 and ZO-2, showed elevated levels in lobular carcinomas compared with ductal carcinomas (ZO-1: ductal 573 ± 250, lobular 836 ± 708; ZO-2: ductal 595 ± 133, lobular 825 ± 781) (Fig. 4). MUPP-1 was significantly increased in both lobular and all other types of breast cancer, compared with ductal type (ductal 0.938 ± 0.893 *vs.* lobular 1.93 ± 1.79, $P = 0.016$; ductal *vs.* all other types 2.64 ± 2.6, $P = 0.035$). Conversely, ZO-3 showed significantly reduced levels in lobular compared with ductal cancer (ductal 300 ± 166; lobular 69.7 ± 27.4, $P = 0.02$) and was also reduced in other cancer types (38.3 ± 37).

3.4. Expression of tight junction molecules and clinical outcome

Table 5 shows data comparing patients with different clinical outcomes after a median follow-up of 72.2 months. Those patients with metastatic disease had significantly reduced levels of ZO-1 and MUPP-1 compared with patients who remained disease-free (ZO-1

Table 5
Analyses of mRNA from samples showing levels of the four tight junction molecules tested and patient outcome

	Plaque protein			
	ZO-1	ZO-2	ZO-3	MUPP-1
(1) Disease-free	273.0 ± 891.5	631.0 ± 171.0	65.0 ± 19.6	0.056 ± 0.017
(2) Metastatic disease	19.3 ± 15.0*	517.0 ± 431.0	163.0 ± 161.0	0.0053 ± 0.005*
(3) Local recurrence	165.0 ± 139.0	456.0 ± 108.0	0.795 ± 0.505*	0.0289 ± 0.020
(4) With mortality	211.0 ± 120.0	829.0 ± 413.0	114.6 ± 52.9	0.0622 ± 0.031

* $P < 0.005$.

273 ± 891.5 *vs.* 19.3 ± 15.0; $P = 0.003$; MUPP-1 0.056 ± 0.017 *vs.* 0.0053 ± 0.005; $P = 0.003$, for disease-free *vs.* metastatic disease groups, respectively).

4. Discussion

From this study, we have shown that ZO-1 and MUPP-1 are closely associated with a poor prognosis in patients with breast cancer. Both these tight junction plaque/peripheral proteins are reduced in tumours with high differentiation grades, with increased TNM and NPI status and with metastatic disease. Both ZO-2 and ZO-3 were also reduced with increased tumour grade, and ZO-3 was also reduced with NPI and TNM status. Conversely, ZO-3 was the only molecule to show reduced expression in lobular cancers compared with ductal carcinomas.

The ZO family are peripheral membrane proteins that are localised in the immediate vicinity of the plasma membrane of tight junctions in epithelial and endothelial cells [19]. ZO proteins (MAGUK's) have in common domains with varying degrees of homology to the enzyme guanylate kinase (GUK); GUK in the presence of adenosine triphosphate (ATP) catalyses the phosphorylation of guanidine monophosphate (GMP) and guanidine diphosphate (GDP), suggesting that the Dlg protein and its homologues may be involved in signal transduction pathways [7]. Hence, ZO proteins are referred to as members of the MAGUK family. ZO-1, -2 and -3 constitute the 'undercoat' structure of the tight junction, together with other peripheral proteins, such as cingulin, 7H6 antigen and symplekin [20]. ZO-2 and -3 interact with F-actin; ZO-1 and ZO-3 form complexes [21]. MAGUKs may play a vital role in cellular functions preventing tumorigenesis, as indicated by neoplastic phenotypes in *Drosophila.* Mutations in *dlg-1* cause neoplastic overgrowth of imaginal discs in larval *Drosophila* and this suggests that *dlg* is a tumour suppressor gene. Structural similarities to ZO-1 and -2 suggest that these tight junction genes may also serve a similar function in mammals. Moreover, ZO-2 exhibits a 23-amino acid truncation at the N-terminus, which may play a role in limiting tumour development in pancreatic cells [7]. MUPP1 (multi-PDZ domain protein 1) is also a binding partner for the transmembrane tight junction proteins in the Claudin family at the COOH termini [22]. MUPP1 is not well characterised, but has 13 PDZ domains and is exclusively concentrated at tight junctions of epithelial cells via its binding to Claudins and JAM. Thus, it may play a role as a multivalent scaffold protein recruiting various proteins to the tight junction [22].

This study is the first to quantitatively analyse the tight junction proteins at the mRNA level; our results support previous findings, that loss of ZO-1 expression is associated with increased tumour differentiation and that this loss is associated with an increased metastatic status. Hoover *et al.* [13] examined the expression and subcellular localisation of ZO-1 in paraffin-embedded breast cancer samples. Normal tissue showed intense staining for ZO-1 at the position of the epithelial tight junctions, but this was lost or reduced in 69% of breast cancers analysed ($n = 48$). In infiltrating ductal carcinomas ($n = 38$), there was a reduction in staining in 42% of well differentiated, in 83% of moderately differentiated and in 93% of poorly differentiated tumours. ZO-1 was positively correlated with tumour differentiation, and, more specifically, with the glandular differentiation of tumours. The ZO-1 gene, *tjp-1*, was mapped relative to other markers flanking the gene. There was a loss of heterozygosity in 23% of the informative tumours ($n = 13$). Loss of a *tjp-1*-linked marker suggests that genetic loss may, in some cases, be responsible for a reduction in ZO-1 in breast cancer.

Sommers *et al.* [23] examined 18 breast cancer cell lines for, amongst others proteins, ZO-1. The most poorly-differentiated, fibroblastic cell lines were ZO-1-negative and were highly invasive. Bell and colleagues investigated a sub-set of breast carcinomas showing unexplained overexpression of HER-2/neu protein without HER-2/neu gene amplification [24]. ZO-1 can upregulate HER-2/neu expression *in vitro* by sequestering a repressor of the *Her-2/neu* gene promoter. The authors investigated the expression of ZO-1 in a series of breast cancers: one group contained those invasive cancers scoring for HER-2/neu status (negative (12), 2+ (13) and 3+ (10)) and were analysed by immunohistochemistry: ZO-1 expression did not correlate with HER-2/neu expression in breast carcinomas, and so other causes of HER-2/neu overexpression should be sought. Interestingly, the authors report that ZO-1

immunohistochemically-stained ductal carcinoma *in situ* were positive for ZO1 in 18/20 cases, with 4/18 being negative for ZO-1 amongst the invasive tumours.

In non-epithelial cells, ZO-1 works as a cross-linker between cadherin/catenin complexes and the actin-based cytoskeleton through direct interaction with α-catenin and actin filaments at its N- and C-terminal halves, respectively, and ZO-1 is a functional component of the cadherin-based cell adhesion system [20], i.e. adherens junctions. In MDCK-Galpha (12)-expressing cells, ZO-1 and Galpha (12) co-localise [25]. Galpha (12) binds to the GST-ZO-1-SH3 domain. ZO-1 interacts directly with Galpha (12); there is an indication that Galpha (12) may regulate the barrier function of MDCK cells. Multiple signalling proteins regulate junctional complexes and several (including G proteins) interact with ZO-1. We may deduce that reduced expression of ZO-1 in human breast cancers will reduce the structural integrity of both tight junctions and adherens junctions, leading to loss of cell–cell adhesion.

ZO-2 expression in our study displayed opposite trends to ZO-1. Levels were increased with increasing NPI and TNM status i.e., NPI3 and TNM3, although this protein was also reduced with increasing grade. Most noticeably, reduced ZO-2 expression was not associated with a poor patient prognosis. ZO-3 directly interacts with ZO-1 and the cytoplasmic domain of occludin, but not with ZO-2. Increased nuclear staining of ZO-2 is observed in epithelial cells that are subjected to environmental stress conditions. The phosphorylation level of ZO-2 is significantly higher in cells that either lack, or have disassembled tight junctions [26], due to the action of both protein kinase C (PKC) and cyclio adenosine monophosphate (cAMP)-dependent protein kinase. Thus, the phosphorylated state of ZO-2 restrains its capacity to operate at the junctional complex. ZO-2 can be expressed in two isoforms, ZO-2A and ZO-2C, in normal epithelia. ZO-2A is absent in pancreatic adenocarcinoma of the ductal type, with none of the common mechanisms of gene inactivation being responsible [27]. ZO-2 was found to be deregulated in breast adenocarcinoma, but not in colon or prostate adenocarcinoma, both of which are considered to be of acinar rather than ductal types. Adenovirus type 9 (Ad9) is distinct among the human adenoviruses as it elicits solely mammary tumours in mammals and its primary oncogenic determinant is the E4 region-encoded open reading frame 1 (ORF1) (E4-ORF1) protein. Glaunsinger *et al*. [28] have shown that ZO-2 is a cellular target for tumorigenic Ad9 E4-ORF1 via its first PDZ domain. This interaction results in the aberrant sequestration of ZO-2 within the cytoplasm. So, although levels of ZO-2 may be elevated in breast cancer, it may be suggested that this does not contribute directly to any negative progression of cancer, and may be a consequence of the reduction of ZO-1 leading to loss of tight junction formation, and re-distribution of ZO-2 within the cell.

In this study, we describe the reduction of MUPP-1 expression in patients with a poor prognosis and with increasing tumour grade. MUPP-1, like ZO-1, may function as a cross-linker between Claudin-based tight junction strands and JAM oligomers in tight junctions. This may indicate that other integral membrane proteins can be recruited to the Claudin-based tight junction through MUPP-1. It is interesting to note that viral oncogene products bind to MUPP-1. It has been proposed that MUPP-1 is involved in the formation of macromolecular complexes beneath the plasma membranes at tight junctions which may play an important role in the regulation of the growth and/or differentiation of epithelial cells [22].

Cancer metastasis proceeds by a series of steps, among which the capacity of cancer cells to invade surrounding normal tissues is of central importance in the dissemination of disease [11]. The interaction between cancer cells and mesothelial cells lining the cavity is crucial to achieve the complex sequence of cancer cell dissemination into the body cavity. In the process of sub-mesothelial invasion of cancer cells, tight junctions of mesothelial cells may function as a defence against the invasion of cancer cells, because the tight junctions are known to work as a barrier to the paracellular passage of cells and substances between epithelial or endothelial cells [11]. Metastasis is the primary cause of fatality in breast cancer patients. Although there are believed to be numerous events contributing to the process of metastasis, it is widely accepted that the loss of cell–cell adhesion in the neoplastic epithelium is necessary for invasion of surrounding stromal elements and subsequent metastatic events [14]. Regulation of vascular permeability is one of the most important functions of endothelial cells, and endothelial cells from different organ sites show different degrees of permeability [29]. Brain microvessel endothelial cells forming the blood-brain barrier are joined together by intracellular tight junctions, possess few pinocytic vesicles and have extremely limited permeability for macro-molecular diffusion. Tumour blood vessels are more permeable to macro-molecular diffusion than normal tissue vessels. However, the cause and mechanism of hyperpermeability of human vessels is not clear [29], although tumour-cell-conditioned medium increases endothelial cell permeability irreversibly.

From the work described here, we can conclude that the expression of tight junction plaque/peripheral proteins may be correlated with a poor prognosis of breast cancer patients. ZO-1 appears to be the most useful indicator of the 4 molecules investigated, as it was positively correlated with increasing grade and tumour status. ZO-1 correlated with poor patient outcome, particularly those with metastatic disease. Like ZO-1, MUPP-1 was also correlated with metastatic disease, which agrees with its suggested role as a binding partner for the transmembrane tight junction molecules. It is also evident

that as ZO-1 is an such essential component for the maintainance of the integrity of tight junction structure, its loss of expression in breast cancer will contribute to the disruption and loss of other components of the tight junction, such as occludin and claudins.

Conflict of interest statement

There are no conflicts of interest associated with this manuscript.

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