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Review

Loss of tight junction barrier function and its role in cancer metastasis

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ABSTRACT

As the most apical structure between epithelial and endothelial cells, tight junctions (TJ) are well known as functioning as a control for the paracellular diffusion of ions and certain molecules. It has however, become increasingly apparent that the TJ has a vital role in maintaining cell to cell integrity and that the loss of cohesion of the structure can lead to invasion and thus metastasis of cancer cells. This article will present data showing how modulation of expression of TJ molecules results in key changes in TJ barrier function leading to the successful metastasis of a number of different cancer types.

Contents

Abbreviations: BBB, blood brain barrier; BE, Barrett's esophagus; CAR, coxsackie adenovirus receptor; CIN/CIS, cervical intra-epithelial neoplasia/carcinoma in situ; CP, chronic pancreatitis; CPE, *Clostridium perfringens* enterotoxin; DGC, diffuse-type gastric cancer; DMSO, dimethyl sulfoxide; DCIS, ductal carcinoma in situ; EMT, epithelial-mesenchymal transition; EGFR, epidermal growth factor receptor; ELF3, E74-like factor 3; GLA, gamma linolenic acid; HGF/SF, hepatocyte growth factor/scatter factor; I, iodine; IDC, invasive ductal carcinoma; IGC, intestinal-type gastric cancer; IHC, immunohistochemistry; JAM, junctional adhesion molecule; LCIS, lobular carcinoma in situ; MAGI, membrane-associated guanylate kinase, WW and PDZ domains-containing; MAGUK, membrane-associated guanylate kinase homologs; MAPK, mitogen-activated protein kinase; MEK-2, MAPK kinase of ERK kinase MMP-2, matric metalloproteinase-2; MT, malotilate; MT1-MMP, Membrane-type 1 matrixmetallo-proteinase; MUPP-1, multi-PDZ domain protein 1; NF1, neurofibrolarosis type 1; Par, protease-activated receptors; PCP, paracellular permeability; PDZ, post synaptic density protein (PSD95), *Drosophila* disc large tumor suppressor (DlgA), and zonula occludens-1 protein (zo-1); PKC, protein kinase C; PMA, paramethoxyamphetamine; PPAC, progressive pseudorheumatoid, of childhood; PSA, prostate specific antigen; Q-PCR, quantitative polymerase chain reaction; RT-PCR, reverse-transcriptase-polymerase chain reaction; RACE, remote analysis computation for gene expression data; SCC, squamous cell cancer; SCE, specialized columnar epithelium; SqE, squamous epithelium; TCF/LEF, T-cell factor/; TER, trans-epithelial/endothelial resistance; TGF, transforming growth factor; TJ, Tight Junction/s; TNM, Tumor nodal status; VEGf, vascular endothelial growth factor

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1. Introduction

Tight junctions (TJ) govern the permeability of epithelial and endothelial cells and are the most topical structures of these cell types [1–3]. It is a region where the plasma membrane of adjacent cells forms a series of contacts that appear to completely occlude the extracellular space thus creating an intercellular barrier and intramembrane diffusion fence [4].

TJ in endothelial cells function as a barrier through which molecules and inflammatory cells can pass. In epithelial cells the TJ functions in an adhesive manner and can prevent cell dissociation [5]. An important step in the formation of cancer metastases is interaction and penetration of the vascular endothelium by dissociated cancer cells. TJ are therefore the first barrier that cancer cells must overcome in order to metastasize. We have previously demonstrated that TJ of vascular endothelium in vivo function as a barrier between blood and tissues against metastatic cancer cells [6].

Early studies have demonstrated a correlation between the reduction of TJ and tumor differentiation and experimental evidence has emerged to place TJ in the frontline as the structure that cancer cells must overcome in order to metastasize [6–9]. Although a considerable body of work exists on TJ and their role in a number of diseases, following the early work of Martinez-Paloma [10] and others [11,12] it is only in the last few years that there has been an upsurge in studies investigating their possible role in tumorigenesis. To date, most of the work has been concentrated on cell lines and to a limited degree on colorectal [13–16] and pancreatic cancers [15–17] and an increasing number of studies carried out on breast cancer [9,18–24] all of which will be discussed later.

The expression of TJ proteins may be modulated by growth factors, cytokines, regulatory mechanisms or promoter methylation. Regulatory mechanisms may be via the suggested pathway of the epithelial–mesenchymal-transition (EMT) as the process of acquisition of an invasive phenotype by tumors of epithelial origin can be regarded as a pathological version EMT [25,26]. TJ determine epithelial cell polarity and disappear during EMT. Snail and Slug are factors thought to be responsible for this loss [27]. Regulation also occurs via the Rho GTPase family, which is able to regulate TJ assembly [28]. Thus the TJ can be regulated in response to physiological and tissue-specific requirements [4]. TJ are able to rapidly change their permeability and functional properties in response to stimuli, permitting dynamic fluxes of ions and solutes in addition to the passage of whole cells [29,30].

This article will review the recent progress in elucidating the role of TJ in the invasion and metastasis of cancer via changes barrier function due to modulations in expression of TJ proteins and alterations in the structure of the TJ itself. It is apparent that changes in the function and regulation of TJ in cancer is not just a by-product of cancer progression but is integral to its formation and persistence, eventually enabling metastasis and secondary disease. As such, this area of research is of fundamental importance in the effort to understand and alleviate this terrible disease.

The changes in both tumor and endothelial cells are necessary for successful growth and spread of cancer cells and that these changes are somewhat similar. A change in cancer cells by up-regulation or down-regulation of relevant TJ proteins results in loss of cell–cell association, cell contact inhibition, leading to uncontrolled growth, loss of adhesion to and degradation of the basement. These must be a concurrent loss of cell–cell association in the endothelium and modulation of TJ proteins involved in facilitating the passage of the cancer cells through this barrier.

2. TJ molecular structure

Briefly, the TJ has a characteristic structure, appearing as discrete sites of fusion between the outer plasma membrane of adjacent cells. When visualized using freeze-fracture, they appear as continuous intramembrane particle strands in the protoplasmic face with complimentary grooves in the extracellular face when adjacent cells are viewed in ultra-thin section electron microscopy [30]. These completely circumscribe the apices of the cells as a network of intramembrane fibrils [4] appearing as what is generally described as a series of “kissing” points.

The TJ structure is representative of the conglomerate of molecules that constitute, associate with or regulate TJ [31]. Although a number of proteins were identified in the mid 1989s, the list of additional molecules has expanded considerably over recent years (Table 1). The molecular components of the TJ have been extensively investigated [3,32] and it is apparent that the junctions could be reasonably separated into 3 regions: (i) the integral transmembrane proteins — occludin, claudins and junctional adhesion molecules (JAM), together with other CTX family members; (ii) the peripheral or plaque anchoring proteins, often containing PDZ motifs — zonula occludens (ZO)-1, -2, -3, MAGI-1 etc.; and (iii) TJ-associated/regulatory proteins — α-catenin, cingulin etc.

The integral transmembrane proteins are the essential adhesion proteins responsible for correct assembly of the TJ structure and controlling TJ functions via homotypic and heterotypic interactions. Successful assembly and maintenance of the TJ is accomplished by anchorage of the transmembrane proteins by the peripheral or plaque proteins such as ZO-1 which act as a scaffold to bind the raft of TJ molecules together and provide the link to the actin cytoskeleton and the signalling mechanism of the cell. This is in conjunction with the associated/regulatory proteins.

3. TJ functions

Although cell adhesion to adjacent cells and the extracellular matrix is key to the organization of epithelium into a tissue, it is vital to the regulation of cellular processes such as differentiation, gene expression, motility and growth [33]. These regulatory functions are mediated by cell adhesion molecules, transmembrane receptors and cytoskeletal proteins all of which are organized into multimolecular complexes and the activation of signalling pathways.

The main functions ascribed to the TJ:

(1) The TJ seals the intercellular space and is responsible for the separation of apical and basolateral fluid compartments of epithelia and endothelia. The TJ functions as a diffusion barrier to plasma membrane lipids and proteins, which helps to define apical and basolateral membrane domains of these polarized epithelial and endothelial cells. Therefore the TJ is crucial for the epithelium to generate chemical and electrical gradients across the cell monolayer that is necessary for vectorial transport processes such as absorption and secretion.
(2) TJ molecules act as intermediates and transducers in cell signalling, thus playing a role in the processes of polarity, cell differentiation, cell growth and proliferation.
(3) TJ proteins act as cell–cell adhesion molecules.
(4) The TJ functions as a barrier to cell migration.

Increasing, evidence suggests that the suppression of the malignant phenotype of cells in tumorigenesis is an additional and important function of the TJ [33]. While the barrier and fence functions of TJ have been well appreciated, it is only relatively recently that concept of the TJ as a complex, multiprotein structure with roles in other cellular processes such as cell polarity, proliferation and differentiation has been recognized [34]. Moreover, it is becoming increasingly clear that the development of human cancer is frequently associated with the failure of epithelial cells to form TJ and to establish correct apicobasal polarity [35].

Table 1
Proteins involved in TJ structure, function and regulation

Integral transmembrane proteins	Peripheral plaque proteins	Associated proteins
Occludin	Zonula occludens-1 (ZO-1)	Cingulin, 7H6,
Claudins 1–24	ZO-2	Symplekin, ZONAB
Junctional adhesion	ZO-3	Rab-13, 19B1, ponsin
molecules (A–C, 4) and	MAGI-1, -2, -3	Rab 3B, PKC, l-afadin
other CTX proteins such	MUPP-1	c-src, Gαi-2, Gαi-12,
as Coxsackie adenovirus	PAR-3/ASIP	α-catenin, Pals, PATJ
receptor (CAR)	PAR-6	PKA, JEAP, Pilt, PTEN,
	AF-6/s-afadin	ZAK, SCRIB, ITCH,
	CASK	Rho-GTPases, WNK4, vinculin
	CAROM	

4. Cancer invasion, angiogenesis and metastasis

Metastasis, the spread of cancer cells to tissues and organs beyond where the tumor originated and the formation of new tumors (secondary and tertiary foci) is the single event that results in the death of most patients with cancer. At the time of diagnosis of cancer, at least half of the patients already present clinically detectable metastatic disease [36]. A higher number of patients will also have micrometastases that would be beyond conventional detection techniques.

Thus, metastasis is the most life threatening event in patients with cancer. The process is composed of a number of sequential events which must be completed in order for the tumor cell to successfully metastasize, the so called metastatic cascade. This process contributes to the complexity of cancer as a multiplex disease. The metastatic cascade can be broadly separated into three main processes: invasion, intravasation and extravasation (Fig. 1).

Malignant tumor cells must dissociate from the primary tumor mass by loss of cell–cell adhesion capacity and invade the surrounding stroma; the process of invasion. This involves the secretion of substances to degrade the basement membrane and extracellular matrix and also the expression/suppression of proteins involved in the control of motility and migration. The tumor must also initialize angiogenesis, without which the tumor would fail to develop, as local diffusion for transport of nutrients to and removal of waste products from the tumor site would suffice for tumors up to 2 mm in diameter [37]. An interesting model can be found in Mullin [38], an “epithelial wounding” model. For tumors to continue to grow, a connection must be made to the blood supply. The blood vessel within the tumor's vicinity can then provide a route for the detached cells to enter the circulatory system and metastasize to distant sites; the process of intravasation [39,40]. Interaction between the tumor cell and the surrounding stroma is extremely important in the development of tumor angiogenesis [41]. The detached tumor cells must enter the circulatory system and survive the forces involved and the immune system to arrive intact at a distant site. Once the tumor cell has arrived at a likely point of intravasation, it interacts with the endothelial cells by undergoing biochemical interactions (mediated by carbohydrate–carbohydrate locking reactions, which occur weakly but quickly) develop adhesion to the endothelial cells to form stronger bonds, and thus penetrate the endothelium and the basement membrane; the process of extravasation. The new tumor can then proliferate at this secondary focus.

Thus, as TJ exist between the cancer cells themselves, the cells of the stroma and the cells of the endothelium, the TJ is the first structure impeding the path to successful metastasis of the cancer cells. For the tumor cell to proceed effectively, the TJ structure must be disturbed and dismantled to enable penetration of the cancer cell.

5. Metastasis and TJ

It is evident that the interaction and penetration of endothelium by the metastasizing tumor cell is therefore a key step in the formation of metastasis [28,42,43]. As our knowledge and understanding of the molecular structure, mechanism of action and function of TJ is expanded it has become apparent that the TJ can be regarded as a potentially important target for anti-cancer research and possible area for future therapeutics.

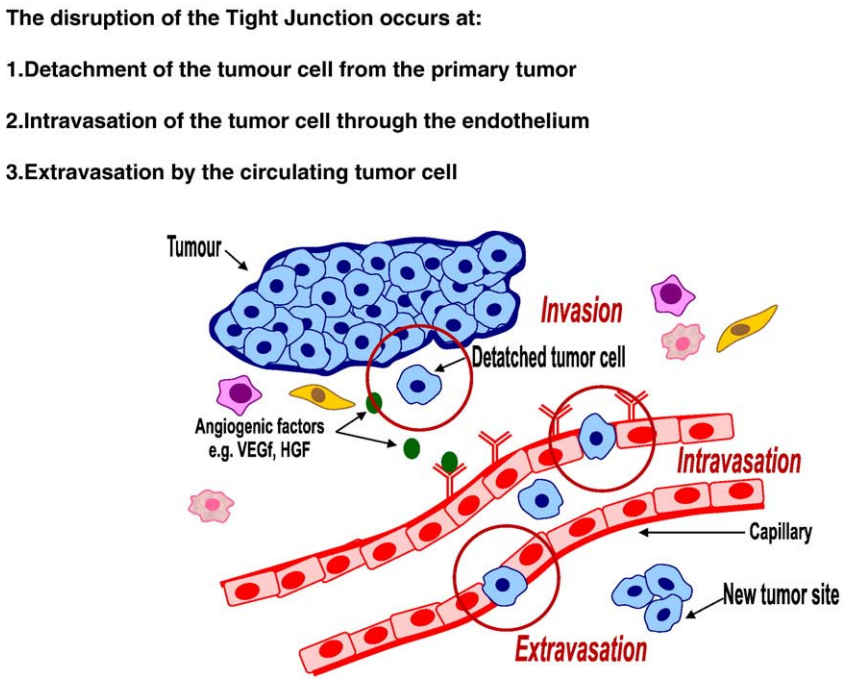

Fig. 1. Schematic illustrating events in the metastatic cascade.

5.1. Penetration of the mesothelium by tumor cells

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 neoplastic epithelium is necessary for invasion of surrounding stromal elements and subsequent metastatic events [18]. The association between TJ permeability of human epithelium has been investigated for some time. Tobioka et al. [44] have shown that the enhancement of TJ function reduced the penetration of tumor cells through mesothelial cells. Soler et al. [45] examined the permeability in normal human and rat colon epithelia and in colon tumors. TER and paracellular influx rate revealed the TJ of colon tumors – both natural and induced – were "leakier" than those of normal colon that was suggestive of an increased permeability of colon epithelium and that a decrease in epithelium barrier function precedes the development of colon tumors. Host cell signalling pathways such as phosphorylation of myosin light chain and the regulation of the TJ proteins claudin-4 and claudin-5 are the results of *Helicobacter pylori* induced chronic gastritis and may progress to gastric cancer [46]. Interestingly, it was noted that DMSO (Dimethyl sulphate up to 10%) produced no significant alteration in TJ permeability or in the cell–cell TJ complex in Caco2/TC7 colon cancer cells, an unexpected result as DMSO is usually used to solubilize poorly soluble drugs in permeation assays [47]. Mullin et al. [48] have opined that TJ leakiness is a late event in epithelial carcinogenesis but allows for growth factors in luminal fluid compartments to enter intercellular and interstitial fluid spaces for the first time, binding receptors located only on the baso-lateral cell surface, causing changes in epithelial cell kinetics, concluding that TJ leakiness is a promotional event unique to epithelial tumors. This conclusion was the result of data showing that adenocarcinomas in rat and human colon have uniformly leaky TJ, whereas most human colon hyperplasic and adenomatous polyps contain non-leaky TJ, though adenomatous polyps with dysplastic changes did posses leaky TJ. Moreover, protein kinase C activation and translocation results in increased permeability of LLC-PK1 cells and failure to regulate this activation is correlated with multilayered cell growth and persistent leakiness. Clarke et al. [49] further this by stating that TJ leakiness associated with protein kinase C activation (and its downstream effectors) suggests a potentially useful role for TJ leakiness as a marker for early cancer diagnosis. These protein kinase C activators are all tumor promoters, which supports the concept of TJ being integral to the progression of cancer. HGF/SF (hepatocyte growth factor or scatter factor), a cytokine secreted by stromal cells and key to the development and progression of cancer, particularly during metastasis has been shown to is capable of modulating expression and function of TJ molecules in human breast cancer cell lines [23]. HGF decreased trans-epithelial resistance and increases paracellular permeability of human breast cancer cell lines, MDA-MB-231 and MCF-7. Q-PCR showed that HGF modulated the levels of several TJ molecule (occludin, claudin-1 and -5, JAM-1 and -2) mRNA transcripts in MDA MB 231 and MCF-7 cells. Western blotting and immunohistochemistry also showed modulation of expression of the TJ molecule, occludin. It is suggested that HGF disrupts TJ function in human breast cancer cells by effecting changes in the expression of TJ molecules at both the mRNA and protein levels. The conclusion was that regulation of TJ could be of fundamental importance in the prevention of metastasis of breast cancer cells (Fig. 2).

Oncogene mutations have also been shown to result in increased "leakiness" of TJ in cancer. Ras mutations can modulate the expression of a number of TJ molecules [50]. The abundance of claudin-2 declined to undetectable levels in ras-overexpressing cells compared with vector controls whereas levels of occludin and claudins 1, 4, and 7 increased and the abundance of claudins-3 and -5 remained unchanged. An increase in extracellular signal-regulated kinase-2 phosphorylation suggests that the downstream effects on the tight junction may be due to changes in the mitogen-activated protein kinase signalling pathway and such selective changes in permeability may influence tumorigenesis by the types of solutes now able to cross the epithelial barrier. During epithelial morphogenesis the apically-localized proteins of the Par (Par3-Par6-aPKC-Cdc42) and Crumbs groups (Crb3-PALS1-PATJ) and the basolaterally localized proteins of the Dlg group (Dlg1-Scribble-Lgl) participate in a complex network of interdependent interactions that define the position and functional organization of adherens junctions and tight junctions. Stucke et al. [51] found an interaction between endogenous hDlg1 and MPP7, a

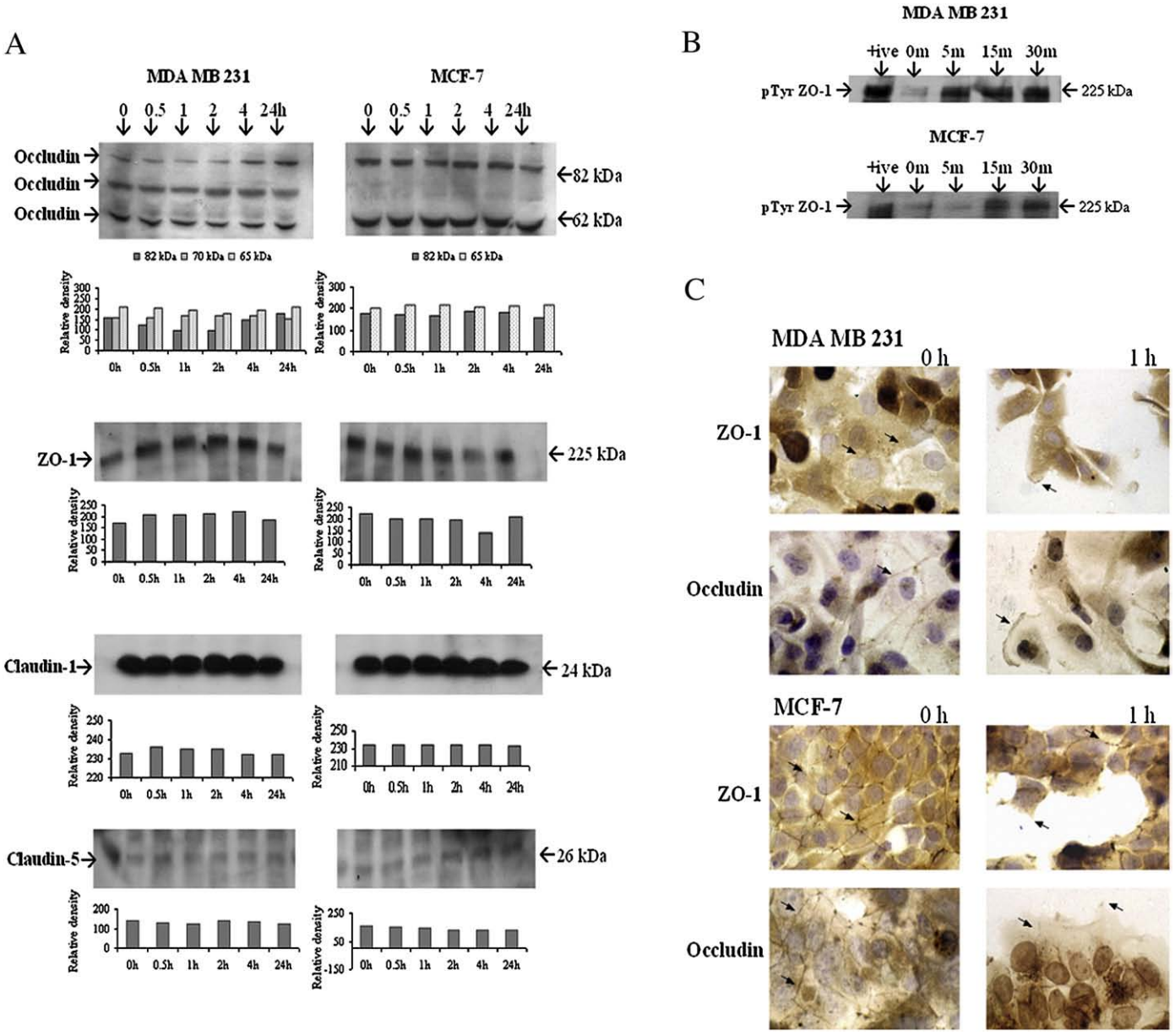

Fig. 2. Effect of HGF on expression of TJ molecules in human breast cancer cells. (A) Western blots of TJ proteins after HGF treatment. (B) HGF and the increased phosphorylation status of ZO-1 in human breast cancer cell lines. This suggests deactivation of ZO-1 by HGF. (C) Immuno-staining of human breast cancer cell lines treated with HGF for 1 h. Cells were stained with ZO-1 or occludin. MCF-7 cells, but not MDA MB 231 cells showed typical TJ pattern staining for ZO-1 and occludin at 0 h. HGF reduced staining of both by 1 h. Both cell lines show increased cytosolic staining and re-location of occludin and ZO-1 to ruffled membrane areas.

previously uncharacterized MAGUK-p55 subfamily member. MPP7 targets to the lateral surface of epithelial cells via its L27N domain, through an interaction with hDlg1. Loss of either hDlg1 or MPP7 from epithelial Caco-2 cells results in a significant defect in the assembly and maintenance of functional tight junctions, concluding that the formation of a complex between hDlg1 and MPP7 promotes epithelial cell polarity and tight junction formation. It is obvious that all these factors in the tumor microenvironment predispose cells to TJ leakiness.

5.2. Penetration of the endothelium by tumor cells

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 [52]. Tumor blood vessels are more permeable on macro-molecular diffusion than normal tissue vessels. However, the cause and mechanism of hyperpermeability of human vessels had not been clear [52]. Tumor cells release a number of factors that can assist their transmigration through the endothelium after treating endothelial cells with conditioned media from a highly invasive and metastatic melanoma cell line [52], with TJ being irreversibly damaged (as assessed using TER-trans-epithelial resistance). The invasion and metastasis of rat oral carcinoma cells can be inhibited by malotilate (MT) through modification of the host endothelial cells [53]. MT did not however affect the growth of human squamous cell lines (SAS, Ca9-22, HSC-2, 3, 4) through a rat lung endothelial invasion model. MT treatment of the endothelial cells did inhibit invasion in SAS, Ca9-22 and HSC-4 cells. ZO-1 protein levels were elevated dose dependently, with enhanced TJ function. HGF has been shown to decrease TER and increase PCP (paracellular permeability) in human endothelial cells [2]. NK4, an antagonistic variant of HGF was shown to inhibit this reduction in TJ function and to inhibit HGF-stimulated invasion of endothelium by human breast cancer cells (MDA-MB-231) [6]. HGF decreased the protein expression of ZO-1 and increased tyrosine

phosphorylation, with no associated changes in expression of occludin, claudin-1 or claudin-5. NK4 successfully prevented HGF-derived ZO-1 expression changes.

6. TJ molecules in cancer progression

Most cancers originate from epithelial tissues and are characterized by aberrant growth control and loss of differentiation and tissue architecture. It is a fundamental property of cancer cells that their mutual adhesiveness is significantly weaker than that of normal cells. Reduced cell–cell interaction allows cancer cells to disobey the social order, resulting in destruction of overall tissue architecture, the morphological hallmark of malignancy. Loss of contact inhibition, which reflects disorder in the signal transduction pathways that connect cell–cell interactions are typical of both early (loss of cell polarity and growth control) and late (invasion and metastasis) stages of tumor progression. An increasing number of studies have shown that numerous TJ components are directly or indirectly involved in cancer progression including ZO-1, ZO-2, claudin-7, claudin-1 and occludin. Highly differentiated adenocarcinomas with well developed TJ provide an important insight into the usefulness of TJ molecules are possible prognostic indicators and future targets for therapy. In breast cancer, ZO-1 has been demonstrated to be decreased in poorly differentiated tumors and correlated with increasing Grade and TNM (tumor-nodal) status [24]. Such observations indicate that TJ molecules could be used to identify poorly differentiated tumors and hence patients with poor prognosis.

There are a respectable number of reports describing the dysregulation of transmembrane proteins in human cancers and in cell lines. This dysregulation can be the result of both up-regulation and down-regulation of expression, epigenetic changes and changes in activation and location of the proteins. The following section will discuss such dysregulation by tumor type.

6.1. Breast cancer

6.1.1. Transmembrane proteins in breast cancer

Claudin-1 (first described by [54]) is normally expressed in mammary gland-derived epithelial cells, but is absent in most human breast cancer cell lines. Claudin-1 expression was not detectable in subconfluent MDA-MB-435 and MDA-MB-361 breast cancer cells [9]. Neither of these cell lines expresses occludin protein, and MDA-MB-435 does not express ZO-1 protein. Claudin-1 retroviral transduced breast cancer cells showed expression of Claudin-1 at the usual cell–cell contact sites, suggesting that other proteins may be able to target claudin-1 to the TJ in the absence of occludin and ZO-1. Moreover, paracellular permeability was reduced in these transduced cells. The authors suggest that *Claudin-1* gene transfer may be in itself enough to exert TJ mediated gate function in metastatic breast cancer cells even in the absence of other TJ associated proteins such as occludin. This indicates a possible tumor suppressor function. In sporadic and hereditary breast cancer, there were no genetic changes, implying that regulatory or epigenetic factors may be involved in the downregulation of the *claudin-1* gene during breast cancer development [19].

Claudin-1 cDNA isolated from human mammary epithelial cells (HMECs) was highly expressed in comparison to low or undetectable levels of expression in a number of breast tumors and breast cancer cell lines [19]. This indicated a possible tumor-suppressor effect for claudin-1. In sporadic tumors and hereditary breast cancer patients, there was no evidence to support the involvement of aberrant claudin-1 in breast tumorigenesis. Likewise, in breast cancer cell lines, no genetic alterations in the promoter or coding sequences were identified to explain the loss of claudin-1 expression. It was suggested that other regulatory or epigenetic factors may be involved in the downregulation of this gene during breast cancer development.

Loss of claudin-7 has been found to correlate with histological grade in both ductal carcinoma in situ and invasive ductal carcinoma of the breast [18]. The expression of claudin-7 is lost in both preneoplastic and invasive ductal carcinoma of the breast occurring predominately in high grade lesions. Expression is also frequently lost in LCIS correlating with the increased cellular discohesion observed in LCIS. Additionally, the majority of IDC cases displaying a low claudin-7 expression have a positive lymph node status. Such findings suggest that the loss of claudin-7 may aid in tumor cell dissemination and augment metastatic potential. Moreover, silencing of claudin-7 expression correlated with promoter hypermethylation in 3/3 breast cancer cell lines but not in invasive ductal carcinomas (0/5). In addition, HGF treatment results in disassociation of MCF-7 and T47D cells in culture, and a loss of claudin-7 expression within 24 h. Sauer et al. [55] more recently showed that primary and recurrent/metastatic breast lesions expressed Claudin-7. 46% of cases had full expression and reduced expression was found in 54%. In cases with reduced expression, the percentage of stained cells was usually high, and no smear showed <50% stained tumor cells. The staining pattern was heterogeneous and always mixed membrane/cytoplasmic. Claudin-7 expression was significantly correlated with tumor grading local recurrences and metastatic diseases, nodal involvement and cellular cohesion in invasive carcinomas, but not with tumor size or subtype.

Tokes et al. [56] compared levels of protein and mRNA expression of three members of the claudin family in malignant breast tumors and benign lesions. Altogether, 56 sections from 52 surgically resected breast specimens were analyzed for claudin-1, -3 and -4 expression by immunohistochemistry and real-time PCR. Claudins were rarely observed exclusively at TJ structures. Claudin-1 was present in the membrane of normal duct cells and in some of the cell membranes from ductal carcinoma in situ, and was frequently observed in eight out of nine areas of apocrine metaplasia, whereas invasive tumors were negative for claudin-1 or it was present in a scattered distribution among such tumor cells (in 36/39 malignant tumors). Claudin-3 was present in 49 of the 56 sections and claudin-4 was present in all 56 tissue sections. However, claudin-4 was highly positive in normal epithelial cells and was decreased or absent in 17 out of 21 ductal carcinoma grade 1, in special types of breast carcinoma (mucinous, papillary, tubular) and in areas of apocrine metaplasia. Claudin-1 mRNA was downregulated by 12-fold in the tumor group. Claudins-3 and -4 mRNA exhibited no difference in expression between invasive tumors and surrounding tissue. The significant loss of claudin-1 protein in breast cancer cells suggests that this protein may play a role in invasion and metastasis. The loss of claudin-4 expression in areas of apocrine metaplasia and in the majority of grade 1 invasive carcinomas also suggests a particular role for this protein in mammary glandular cell differentiation and carcinogenesis.

Soini [57] also evaluated the expression of claudins-2, -3, -4, and -5 in 20 cases of Paget's disease (13 mammary and 7 extramammary cases), and compared the results with those of other neoplastic skin lesions, including actinic keratoses, basal cell carcinomas, and malignant melanomas. Membrane-bound claudins-3 and -4 expression was seen in all cases of Paget's disease, whereas claudin-5 was seen in 50% of cases and claudin-2 was seen in 32% of cases. However, claudins-3, -4, and -5 were not seen in the other skin lesions, and claudin 2 was seen in most of them, suggesting an inverse expression of these claudins between Paget's disease and epidermal and nevocytic lesions. Claudin expression in breast carcinomas was claudin-2 in 52%, claudin-3 in 93%, claudin-4 in 92%, and claudin-5 in 47%. Claudins-2 and -5 were found more often in ductal carcinomas than in lobular carcinomas. Expression of the claudins was frequently associated with each other. They were not associated with estrogen or progesterone receptor status or with tumor grade. No significant differences were found between claudin expression in Paget's disease and breast carcinomas. The results demonstrate that claudins could be useful in diagnosing Paget's disease and in differentiating these

lesions from other epidermal lesions, such as actinic keratoses, basal cell carcinomas, and nevocytic lesions. The lack of difference in claudin expression between Paget's disease and breast tumors suggests that changes in the phenotype of claudins-2, -3, -4, and -5 are not necessary for epidermal invasion.

Claudin-16 (paracellin-1), ponsin, ZO-2, AF6, vinculin and nectin are reduced with poor prognosis of patients with breast cancer however JAM-2 does not show differences in expression [58]. The levels of transcripts of claudin-16 and vinculin were significantly lower in patients that had poor prognosis (with metastasis, recurrence or mortality), compared with those that remained healthy after a median follow-up of 72.2 months. Immunohistochemistry confirmed these results, as there was a decreased level in staining for claudin-16 and AF6. In normal tissue, staining was confined to the intercellular regions whereas in the tumor tissues the staining was diffuse and cytosolic. The conclusion was that low levels of TJ molecules claudin-16 and vinculin in breast cancer are associated with poor prognosis in patients, underscoring the idea that regulation of TJ could be of fundamental importance in the prevention of metastasis of breast cancer cells.

Interestingly, the nectin family has been little studied as regards TJ in cancer, being originally described as molecules involved in adherens junctions only. Recently however, it has become apparent that nectins are also involved in recruitment and maintenance of proteins within the TJ [59]. Nectin-4 was not detected in normal breast epithelium. By contrast, nectin-4 was expressed in 61% of ductal breast carcinoma and in 6% in lobular type. Expression of nectin-4 strongly correlated with the basal-like markers EGFR, P53, and P-cadherin, and negatively correlated with the luminal-like markers ER, PR and GATA3. All but one ER/PR-negative tumors expressed nectin-4. The detection of nectin-4 in serum improves the follow-up of patients with MBC: the association CEA/CA15.3/nectin-4 allowed to monitor 74% of these patients compared to 67% with the association CEA/CA15.3. Serum nectin-4 was a marker of disease progression, and levels correlate with the number of metastases. Serum nectin-4 was also a marker of therapeutic efficiency and correlated, in 90% of cases, with clinical evolution. Nectin-4 appears to be a new tumor-associated antigen for breast carcinoma and possibly new bio-marker whose use could help refine breast cancer taxonomy and improve patient follow-up. Nectin-4 emerges as a potential target for breast cancer immunotherapy [59].

The claudins-1, -3, and -4 have been found to be differentially expressed in the mammary gland during pregnancy, lactation, and involution, suggesting different roles for these proteins at different stages of mammary gland function [60]. In addition, claudins-1 and -3 are detected in mammary tumors and the wide distribution of claudin-3 in particular, appears to suggest specific roles for these proteins in mammary tumorigenesis.

Osanani et al. [61] had previously demonstrated that epigenetic silencing of occludin resulted in the acquisition of apoptotic resistance to various apoptogenic stimuli, causally contributing to the enhanced tumorigenicity of cancer cells. In a recent study, the authors demonstrated that forced expression of occludin induced anoikis and promoted oxidative stress-induced premature senescence in breast carcinoma cells, accompanied by upregulation of negative cell cycle regulators such as p16INK4A, p21Waf1/Cip1 and p27Kip1 but not p53. Endogenous re-expression of occludin mediated by a synergistic effect with a demethylator and histone deacetylase inhibitor or retinoids that stimulate retinoic acid receptor was also sufficient for provoking the senescent phenotype. In addition, tumors developed from occludin-expressing cells in mice showed a feature of cellular senescence that has not been described as a consequence of occludin signalling. These findings suggested that the loss of occludin expression could be partially involved in the senescence-escape program during mammary tumorigenesis.

The Coxsackie-adenovirus receptor (CAR) is the primary site for adenovirus attachment during infection and has been used as a delivery mechanism for gene therapies. Martin et al. [62] evaluated the expression of CAR in human breast cancers. Staining intensity of CAR was increased within tumor sections compared to background tissue. Q-PCR revealed significantly elevated levels of CAR transcript in breast tumors. CAR expression also increased with grade of tumor. Patients who had tumor metastases also showed elevated levels of CAR expression, however those with local recurrences had reduced levels of CAR. Ductal carcinomas expressed lower levels of CAR compared to tumors of other types. Tumors with nodal involvement were also associated with higher levels of CAR. Levels of CAR were significantly correlated with long-term survival over a period of 6 years. It appears that CAR expression is elevated in primary breast cancers. This raises pertinent questions in two areas: could this provide a key to treatment using viral vectors, or, does this elevated expression result in dysregulation of barrier function? Further research is required to uncover these questions.

6.1.2. Peripheral plaque proteins in breast cancer

MAGUKs may play a vital role in cellular functions preventing tumorigenesis as indicated by neoplastic phenotypes in *Drosophila*; Normal breast tissues have shown the expected intense staining at cell–cell junctions; however, ZO-1 staining is found to be reduced or lost in 69% of breast cancers analyzed using immunohistochemistry [20]. Normal tissue showed intense staining for ZO-1 at the position of the epithelial TJ, but this was lost or reduced in 69% of breast cancers analyzed. In infiltrating ductal carcinomas there was a reduction in staining in 42% of well differentiated, in 83% of moderately differentiated and in 93% of poorly differentiated tumors. ZO-1 was positively correlated with tumor differentiation, and more specifically with the glandular differentiation of tumors. The ZO-1 gene *tjp-1* was mapped relative to other markers flanking the gene. There was a loss of heterozygosity in 23% of informative tumors. 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.

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 responsible [21]. Analysis of the ZO-2 promoters (PA and PC) showed that lack of expression of ZO-A in neoplastic pancreatic cells is caused by inactivation of the downstream promoter PA, probably due to structural or functional alterations in the regulatory elements localized outside the analyzed promoter region as hypermethylation was not a convincing reason in early cancers. However, methylation of PA is responsible for the inactivation of the suppressed promoter at the late stages of tumor development [33]. ZO-2 was found to be de-regulated in breast adenocarcinoma, but not in colon or prostate adenocarcinoma, both of which are considered to be of acinar rather than ductal type. Also, in 18 breast cancer cell lines, the most poorly-differentiated, fibroblastic cell lines were ZO-1 negative, and were highly invasive [63].

Martin et al. [24] investigated the expression of ZO-1, ZO-2 and ZO-3, and MUPP-1 in patients with primary breast cancer (Fig. 3). Standardised transcript levels of ZO-1 and MUPP-1 were significantly lower in patients with metastatic disease compared with those remaining disease-free (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 tumor 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. It was concluded that low levels of TJ plaque molecules, such as ZO-1 and MUPP-1 in breast cancer are associated with poor patient prognosis.

ZO-1 is able to upregulate HER-2/neu expression in vitro by sequestering a repressor of the *Her-2/neu* gene promoter [22]. ZO-1 expression was examined in a series of breast cancers: one group

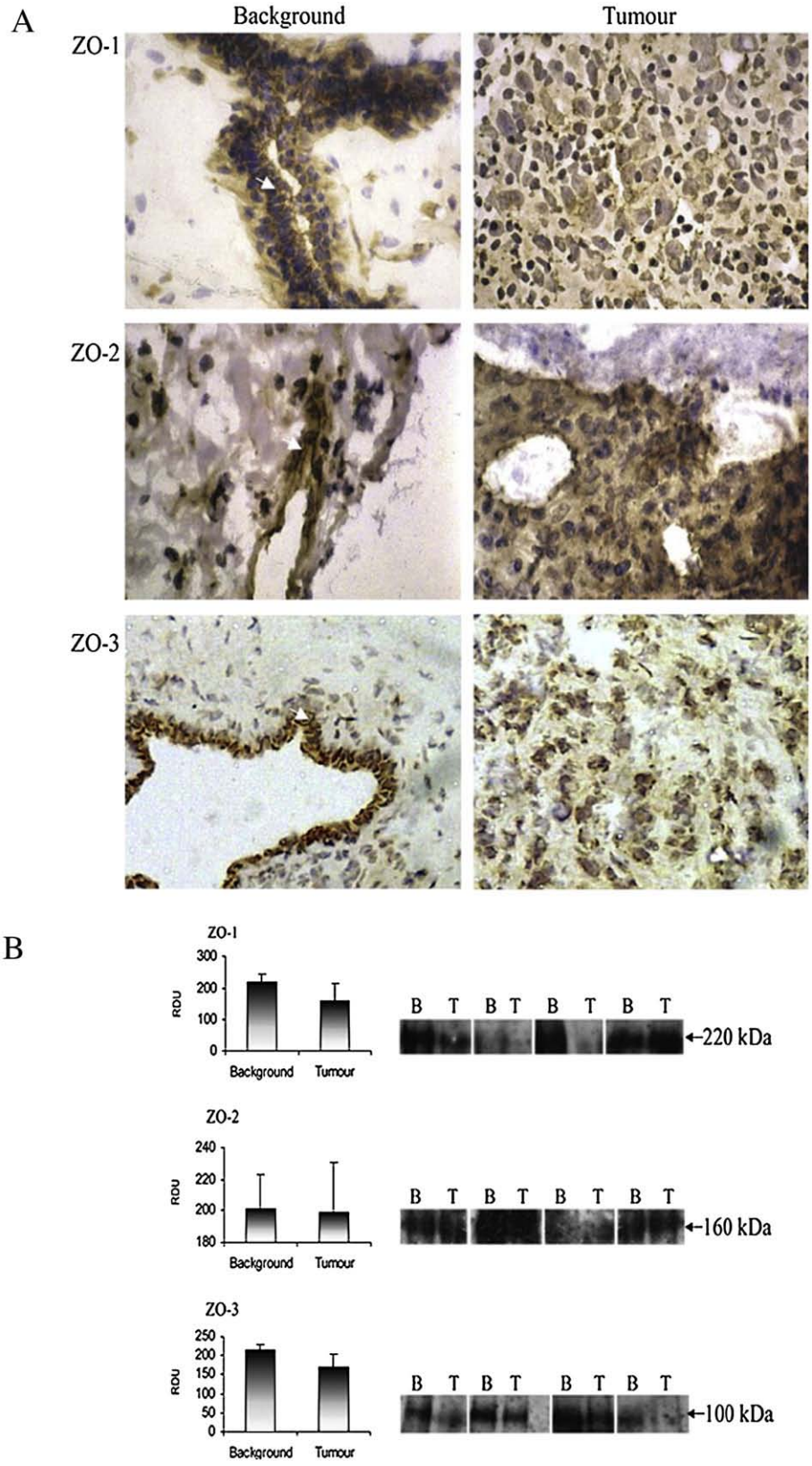

Fig. 3. Panel shows the differential expression of peripheral/plaque proteins in representative sections from patients with breast cancer. (A) Immunohistochemical staining (X100) of ZO-1, -2 and -3 in human breast cancer tissues. Clear staining is shown in normal tissue (left), reduced staining for ZO-1 and -3 shown in the right. (B) Western blotting of paired normal and tumor tissues and densimetric analysis.

contained those invasive cancers scoring for HER-2/neu status and was analyzed by IHC: 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 IHC stained DCIS were positive for ZO-1 in 18/20 cases, with 4/18 negative for ZO-1 in the invasive tumor.

The acquisition of a migratory/invasive phenotype by tumor cells is characterized by the loss of cell–cell adhesion contacts and the expression of degradative properties. Polette et al. [64] examined the effect of the disorganization of occludin/ZO-1 complexes on the expression of membrane-type 1 matrix metalloproteinase (MT1-MMP). The expression of MT1-MMP in invasive breast tumor cell lines correlated with the absence of occludin and with a cytoplasmic localization of ZO-1. In contrast, non-invasive cell lines displayed a membrane staining for both ZO-1 and occludin and did not express MT1-MMP. Cytoplasmic ZO-1 and MT1-MMP could be detected in invasive tumor clusters of human breast carcinomas. ZO-1 small interfering RNA transfection down-regulated MT1-MMP mRNAs and proteins and subsequently decreased the ability of tumor cells to invade. The authors conclude that ZO-1 can intervene in signalling events promoting tumor cell invasion. It is apparent that not only is down-regulation of TJ proteins important in effecting an invasive phenotype, but that intracellular mislocalization can be just as important: if the TJ protein in question has not been targeted correctly, the structure and function of the TJ will be impaired.

6.2. Bladder cancer

6.2.1. Transmembrane proteins in bladder cancer

Recently, interest in the role of TJ in bladder carcinoma has increased. A timely review explored the current understanding on the role and regulation of TJ function in the normal and diseased bladder [65]; however, few studies have materialised to further interest in this area. Boireau et al. [66] analyzed the expression and localization of claudins-1, -4, and -7 in human bladder carcinoma. Claudin-4 expression was significantly altered in 26/39 tumors, contrasting with the rare modifications detected in the expression of claudins 1 and 7. Overexpression of claudin-4 in differentiated carcinomas was followed by a strong downregulation in invasive/high-grade tumors, and this expression pattern was associated to the 1-year survival of bladder tumor patients. A CpG island was identified within the coding sequence of the *claudin-4* gene, and treatment with a methyl-transferase inhibitor restored expression of the protein in primary cultures prepared from high-grade human bladder tumors. Claudin-4 expression also correlated with its gene methylation profile in healthy and tumoral bladders from 20 patients. Delocalization of claudins-1 and -4 from TJ was observed in most human bladder tumors and in the bladder tumor cell line HT-1376. Although the *claudin-4* gene was unmethylated in these cells, inhibition of methyl transferases re-addressed the two proteins to TJ, resulting in an increase of cell polarization and transepithelial resistance. These biological effects were prevented by expression of claudin-4-specific siRNAs, demonstrating the important role played by claudin-4 in maintaining a functional regulation of homeostasis in urothelial cells. The authors concluded that the TJ barrier is disrupted from early stages of urothelial tumorigenesis and that hypermethylation was the leading to the alteration of claudin-4 expression and localization in bladder carcinoma.

6.3. Colorectal cancer

6.3.1. Transmembrane proteins in colorectal cancer

Since Soler et al. [45] revealed the TJ of colon tumors – both natural and induced – were "leakier" than those of normal colon there have been a number of studies looking at colorectal carrier function. It has been demonstrated that there is an inverse relationship between the expression of claudin-1 and Smad4, a tumor suppressor protein, in colon cancer cell lines and in human colon cancer tissue samples [67]. Smad4 expression in Smad4-deficient colon cancer cells inhibited claudin-1 expression through transcriptional regulation. Further analysis suggested the important role of h-catenin/T-cell factor (TCF)/lymphocyte enhancer factor (Lef) activity in the Smad4-dependent regulation of claudin-1 expression. In addition, the inhibition of claudin-1 expression contributes to the ability of Smad4 to inhibit invasion in colon cancer cells. Smad4-dependent inhibition of claudin-1 expression was a direct effect of Smad4 expression and not due to modulation of TGF-h signalling. Resnick et al. [14] investigated the pattern of expression and prognostic value of claudin-1, claudin-4, occludin and ZO-1 in a cohort of TNM stage II colon cancer using tissue microarray technology and retrospectively analyzed samples form 129 patients with TNM stage II colonic carcinomas for claudin-1, claudin-4, occludin and ZO-1 protein expression by immunohistochemistry. Seventy-five, 58, 56 and 44% of the tumors exhibited normal to elevated expression levels of claudin-1, claudin-4, occludin and ZO-1 respectively. Low expression levels of claudin-1 and ZO-1 were directly associated with higher tumor grade. Multivariate analysis indicated that lymphovascular invasion and low levels of claudin-1 expression were independent predictors of recurrence and that reduced claudin-1 expression was associated with poor survival. This was the first study to comprehensively examine the expression of several TJ associated proteins in colonic neoplasms and to correlate their expression with disease progression. Loss of claudin-1 expression proved to be a strong predictor of disease recurrence and poor patient survival in stage II colon cancer.

A more recent study looked at the expression analysis of genes encoding TJ proteins to display differential gene expression on RNA and protein level and to identify and validate potential targets for colorectal cancer therapy [68]. Claudins-1 and -12 are frequently over-expressed in colorectal cancer, whereas claudin-8 showed down-regulation in tumor tissue at the RNA level. Quantification of proteins confirmed the overexpression of claudin-1 in tumor tissues, whereas changes of claudins-8 and -12 were not significantly detectable on protein level. IHC confirmed the markedly elevated expression level of claudin-1 in the majority of colorectal cancer, showing membranous and intracellular vesicular staining. The authors concluded that differential expression of genes encoding claudins in colorectal cancer suggests that these TJ proteins may be associated to and involved in tumorigenesis. As claudin-1 is frequently up-regulated in large proportion of colorectal cancers and may represent potential target molecule. Added to their intrinsic properties in a correctly functioning TJ, it is becoming apparent that the over-expression of absence of expression of these proteins might provide evidence of prognostic value. In adenocarcinoma tissues the expression of claudins-1, -3 and -4 has been found to be upregulated [69]. Tokunaga et al. [70] investigated whether or not occludin is expressed in rosette or gland-like structures in human rectal carcinoid tumors. The expression profiles of occludin in 40 carcinoid tumors were examined immunohistochemically, using an anti-occludin monoclonal antibody. In eight (20%) samples of typical carcinoid tumors, a small number of rosette-like tubular structures outlined by occludin were detected. Thus occludin might be considered to be one of the most characteristic structural markers of polarized glandular structures. The results of this study provided supportive evidence that carcinoid tumor cells are capable of glandular differentiation. The significance of claudin-4 expression in colorectal cancer and its association with clinicopathological factors was examined [71]. The levels of claudin-4 expression in a total of 129 colorectal cancers and 44 metastatic tumors were examined by immunohistochemistry. A small interfering RNA (siRNA)-mediated claudin-4 knockdown examination was also conducted to assess the biological role(s) of claudin-4 in cultured cells. Expression of claudin-4 at the intercellular membrane was well preserved at the surface of tumors, with decreased claudin-4 expression detected in colorectal cancers, particularly at the invasive front. Interestingly, decreased claudin-4 expression was detected in metastatic lesions of colorectal cancer. siRNA-mediated claudin-4 knockdown in SW480 claudin-4-positive colorectal cancer cells upregulated cell motility, whereas no significant change was detected

in cell proliferation. Disruption of claudin-4-mediated TJ construction enhances cancer cell invasion and metastasis in human colorectal cancer and claudin-4 might be a good biomarker for diagnosing the risk of distant metastasis. It is interesting to note that the *claudin-1* gene is regulated by β-catenin [72]. Expression of claudin-1 decreases significantly in response to reduction of intracellular β-catenin by adenovirus-mediated transfer of wild-type APC into the APC-deficient colon cancer cells, with two putative Tcf4 binding elements in the 5′ flanking region of claudin-1 being responsible for activating its transcription. The authors again demonstrate increased expression of claudin-1 in primary colorectal cancers. Furthermore, immunohistochemical staining demonstrated that claudin-1 was weakly stained at apical boarder of lateral membrane of noncancerous epithelial cells and that it was strongly stained at all cell–cell boundaries and in the cytoplasms of cancer cells. Such results imply that claudin-1 is involved in the beta-catenin-Tcf/LEF signalling pathway. Moreover, it has been reported that there is an increased expression of claudin-1 in human primary colon carcinoma and metastasis and in cell lines derived from primary and metastatic tumors [73]. There was frequent nuclear localization of claudin-1 in these samples. Genetic manipulations of claudin-1 expression in colon cancer cell lines induced changes in cellular phenotype, with structural and functional changes in markers of epithelial–mesenchymal transition. It was also demonstrated that changes in claudin-1 expression had significant effects on growth of xenografted tumors and metastasis in athymic mice. Data suggests that the regulation of E-cadherin expression and β-catenin/Tcf signalling is a possible mechanism underlying claudin-1-dependent changes. Kinugasa et al. [74] also state that claudin-1 and claudin-2 were found to be over-expressed in colorectal cancer tissues. They may be useful as tumor markers and targets for the treatment of colorectal cancer.

6.3.2. Peripheral plaque proteins in colorectal cancers

Tubular gland structures of colorectal cancer have been demonstrated to undergo dedifferentiation at the primary site, with the gland structures re-formed in liver metastases. Kaihara et al. [75] examined the degree of differentiation of the gland structure of 48 cases of colorectal cancers (24 cases with synchronous liver metastasis, 24 cases without metastasis) by the modified Gleason grading system. The role of ZO-1, in the morphological changes (dedifferentiation and redifferentiation) at the primary site and liver metastases was also looked at. Liver-metastasized colorectal cancers showed a lower score in the modified Gleason grading system than the corresponding primary tumors. The tumor cells had undergone redifferentiation at liver metastases. ZO-1 was expressed at the apical cell borders of normal colorectal epithelium, the luminal side of which has tubular gland structures. In comparison with this normal epithelium, the ZO-1 expression level was frequently reduced in primary colorectal cancer with liver metastasis (20.8%) and ZO-1 was re-expressed in liver metastasized cancers (79.2%). Immunoprecipitation of colorectal cancers with liver metastasis showed that ZO-1 bound to epidermal growth factor receptor (EGFR) irrespective of the phosphorylation status of EGFR, and that EGFR associated ZO-1 was highly tyrosine-phosphorylated only in the primary colorectal cancers, but was dephosphorylated in the liver-metastasized cancers. The authors suggest that tyrosine phosphorylation of ZO-1 leads to down-regulation of the function of ZO-1 and dedifferentiation of the glands in colorectal cancers, and these phenomena contribute to liver metastases, and redifferentiation of the glands occurs in the liver metastases.

6.4. Eesophageal cancer

6.4.1. Transmembrane proteins in esophageal cancer

The majority of studies on TJ in esophageal cancer have concentrated on transmembrane proteins in the claudin family. This is also reflected by the growing number of studies indicating the importance of TJ function in the precancerous predecessor to esophageal cancer, Barrett's esophagus. Recently, Miyamoto et al. [76] examined 54 esophageal cancer cases to assess immunohistochemical expression patterns of claudin-1 with decreased expression of claudin-1 being statistically correlated with recurrence status. Decreased expression of claudin-1 was also correlated with short disease-free and overall. The results suggest that claudin-1 expression is correlated with the recurrence status and poor prognosis in esophageal cancer and claudin-1 expression may be a good indicator of recurrence in esophageal cancer.

Upregulation of claudins 3, 4, and 7 was identified in gastric adenocarcinoma Montgomery et al. [77]. While normal gastric mucosa lacked claudin 3, 4, and 7 expression, intestinal metaplasia and dysplasia showed these proteins. The authors hypothesized that claudins would be similarly overexpressed in Barrett's esophagus (BE) adenocarcinoma. The findings suggest that alterations in claudin proteins are an early event in tumorigenesis and may provide targets for diagnosis and directed therapy for esophageal adenocarcinoma and its precursors. Earlier studies [78] showed that reduced expression of claudin-7 at the invasive front of the esophageal cancer was significantly associated with the depth of invasion, lymphatic vessel invasion and lymph node metastasis. In contrast, significant association was not detected between claudin-1 expression and clinicopathologic factors except for histologic differentiation of the tumor. Claudin-7 expression at the invasive front of the primary tumor and its corresponding metastatic lymph nodes revealed significant reduction in claudin-7 expression in the metastatic lymph nodes suggesting that the reduced expression of claudin-7 at the invasive front of esophageal squamous cell carcinoma may lead to tumor progression and subsequent metastatic events. Moreover, Lioni et al. [79] examine the expression of claudin-7 in squamous cell carcinoma (SCC) of the esophagus and its possible role in tumor progression. In this context, the claudin-7-overexpressing cells became more adhesive and less invasive associated with increased E-cadherin expression. Claudin-7 was mislocalized during the malignant transformation of esophageal keratinocytes. This demonstrated that there might be a critical role for claudin-7 expression in the regulation of E-cadherin in these cells, suggesting that this may be one mechanism for the loss of epithelial architecture and invasion observed in esophageal SCC.

Patients with Barrett's [80] were observed to exhibit a transepithelial leak to sucrose whose mean value was threefold greater than that seen in healthy control subjects or patients with reflux but without any mucosal defect. A parallel study of claudin tight junction proteins in endoscopy biopsy samples showed that whereas Barrett's metaplasia contains dramatically more claudin-2 and claudin-3 than is found in normal esophageal mucosa, it is markedly lower in claudins 1 and 5, indicating very different tight junction barriers. A later study of 21 claudins in Barrett's oesophagus [81] (BE) and specialized columnar epithelium (SCE) that develops as replacement for damaged squamous epithelium (SqE) in subjects with reflux disease, demonstrated that in SCE, claudin-18 was the most highly expressed at the mRNA level and this finding is paralleled by marked elevation in protein expression on immunoblots. In contrast in SqE, claudin-18 was minimally expressed at the mRNA level and undetectable at the protein level. Immunofluorescence studies showed membrane localization of claudin-18 and colocalization with ZO-1. This prompted the authors to conclude that claudin-18 is the dominant claudin in the TJ of SCE and propose that the change from a claudin-18-deficient TJ in SqE to a claudin-18-rich TJ in SCE contributes to the greater acid resistance of BE. An early study by Rendon-Huerta et al. [82] observed that occludin is in fact found in normal esophageal tissue, in contrast to an earlier publication reporting its absence in esophagus [82]. They also observed that the amounts of occludin on a per-mg-total-protein basis are not different for biopsies from Barrett's metaplasia compared with adjacent normal

esophageal epithelium. However, the situation is very different for the claudins with claudin-1 being fairly abundant in normal esophagus but is absent in some Barrett's metaplasia biopsies and sharply reduced in most others. Claudin-2 presented a somewhat opposite picture, that is, consistently nondetectable in normal esophageal epithelium but detectable at low-to-moderate levels in two of eight Barrett's biopsies.

6.4.2. Peripheral plaque proteins in esophageal cancer

Kimura et al. [83] investigated occludin expression in conjunction with ZO-1 in normal epithelia and cancers of human digestive tract. ZO-1 was expressed as a single line at the apical cell border. In the esophagus ZO-1 was expressed in the spinous layer. As for tumors, ZO-1 showed the same expression in differentiated adenocarcinoma cells as in normal epithelium, but in poorly differentiated adenocarcinomas, the expression was reduced. There was a significant correlation between tumor differentiation and expression of these proteins. It was posited that ZO-1 could be involved in the formation of gland-like structures.

6.5. Gastric cancer

6.5.1. Transmembrane proteins in gastric cancer

Disruption of the TJ observed in the study by Fedwick et al. [46] implicates host cell signalling pathways, including the phosphorylation of myosin light chain and the regulation of tight-junctional proteins claudin-4 and claudin-5, in the pathogenesis of *Helicobacter pylori* infection. As gastric carcinoma remains one of most serious malignant tumors worldwide with *Helicobacter pylori* being the definite carcinogen this is an interesting area in determining the role of TJ barrier function [84]. The *Helicobacter pylori* components, cytotoxin-associated gene A (*CagA*), vacuolating toxin A (VacA) and blood-group antigen-binding adhesin gene (*BabA*), can mimic and bind to specific receptors or surface molecules both on gastric epithelial cells and platelets, in which CagA and VacA may also be directly involved in loosening of TJ in monolayers of polarized gastric epithelial cells. It has been shown that a history of *Helicobacter pylori* infection is found in the majority of patients with GC, and that anti-CagA, anti-VacA and anti-BabA antibodies targeting both *Helicobacter pylori* components and host mimic molecules can be detected in them with increased levels. Patients with GC who are positive for *Helicobacter pylori* prospectively have a better outlook than those negative. The stimulation of mentioned autoantibodies in antigen processing and presentation and subsequent T-cell activation and proliferation improves host immune status. On the other hand, in an autoimmune response, autoantibodies can induce the cross-reaction against those localized or circulating GC cells, which are characterized by mimic or absorbed *Helicobacter pylori* antigens, and lead to the killing and even suppressing of metastasis of cancer cells [84].

In the poorly differentiated gastric cancer cell line TMK-1, ZO-1 and occludin have been shown to be predominantly localized to the cytoplasm, although there is some weak expression at the cell–cell contact [85]. Epidermal growth factor (EGF), a growth factor that is often overexpressed in gastric cancer causes ZO-1 and occludin to be rapidly translocated from the cytosol to the cell–cell contact. These effects induced by EGF were attenuated in the presence of protein kinase C (PKC) inhibitors calphostin C and bisindolylmaleimide I, but not another PKC inhibitor Gö6976, PD98059 (MAPK inhibitor), LY294002 (PI3 kinase inhibitor) or KT5720 (protein kinase A inhibitor). Yoshida et al. [85] suggest that EGF can rapidly alter the localization of ZO-1 and occludin via a protein kinase C signalling pathway in TMK-1 gastric cancer cells. In situ hybridization was used to evaluate the expression of occludin mRNA in 42 gastric carcinoma specimens obtained by surgery and 23 relatively normal gastric mucosa obtained by gastric endoscopy [86]. Occludin mRNA was found positive in the cytoplasm of gastric glandulous epithelia as blue particles with intensive stain in 14 of 42 gastric carcinomas (33.3%), 23 of 42 paracancerous gastric tissues (54.8%), 14 of 23 relatively normal gastric tissues (60.9%), 9 of 16 well differentiated carcinomas (56.3%), 4 of 14 moderately differentiated carcinomas (28.6%), 1 of 10 poorly differentiated carcinomas (10.0%) and none of 2 mucosal carcinomas. There were significant differences in occludin mRNA positive rate between relatively normal gastric tissue and gastric cancer as well as between paracancerous gastric tissue and gastric cancer. The expression of occludin mRNA in moderately and poorly differentiated groups was gradually reduced when compared with well differentiated group, which suggests that there be a significant correlation between tumor differentiation and the expression of occludin mRNA. Furthermore, the positive signals of occludin mRNA distributed extensively in the cytoplasm of SGC7901/VCR cell, being vincristine resistant, derived from parental gastric cell line SGC7901. The positive signals of SGC7901/VCR were stronger than those of SGC7901 cells. The authors suggest that occludin mRNA, being mainly located in epithelial cells and its expression correlated with tumor differentiation, may be involved in the development of multi-drug resistance in gastric cancer.

A new claudin-based gastric cancer classification system for gastric cancer has been proposed [87]. The authors examined the expression of gastric (claudin-18) and intestinal (claudin-3 and claudin-4) claudins in non-neoplastic gastric mucosa (with intestinal metaplasia [IM], 78 cases; without IM, 88 cases) and 94 gastric cancers was analyzed immunohistochemically, as was the expression of gastric (MUC5A and MUC6) and intestinal (CD10 and MUC2) mucins. Heterogeneous expression of claudin-3, claudin-4 and claudin-18 was detected in advanced gastric cancer; however, there was no significant association between the claudins and the clinicopathological parameters. These gastric cancer tissues were also sub classified into claudin-based phenotypes: gastric claudin, 28 cases (30%); intestinal claudin, 41 cases (44%); and unclassified claudin, 25 cases (26%). Interestingly, the gastric cancers with unclassified claudin had worse malignancy grades, not only in size and invasiveness but also in potential metastatic ability and patient outcome. Although the mucin-based gastric cancer classification was also assessed, no significant correlation was found between mucin production and clinicopathological parameters. These observations suggest that loss of claudin expression may enhance the grade of malignancy of gastric cancer in vivo. Classification of gastric cancers using gastric and intestinal claudins is a good biomarker for assessing the risk of poor prognosis. Quantitative real-time reverse transcriptase-polymerase chain reaction and immunohistochemistry have shown that claudin-7 is overexpressed in 10 Tff1−/− gastric dysplasia samples [88]. Comparison with a serial analysis of gene expression database of human gastric cancer revealed similar deregulation in human gastric cancers. Quantitative real-time reverse transcriptase-polymerase chain reaction of human gastric adenocarcinoma samples indicated that, of these three genes, *claudin-7* was the most frequently up-regulated gene. Using immunohistochemistry, both mouse and human gastric glands overexpressed claudin-7 in dysplastic but not surrounding normal glands. Claudin-7 expression was observed in 30% of metaplasia, 80% of dysplasia, and 70% of gastric adenocarcinomas. Interestingly, 82% of human intestinal-type gastric adenocarcinomas expressed claudin-7 whereas diffuse-type gastric adenocarcinomas did not. These results suggest that claudin-7 expression is an early event in gastric tumorigenesis that is maintained throughout tumor progression [88]. Kuo et al. [89] continued to explore the roles of claudin-4 in the two histologically distinct types of gastric cancer; we selected 45 IGC (intestinal-type gastric cancer) and 48 DGC (diffuse-type gastric cancer) cases and then analyzed the expression of the protein using immunohistochemistry. The authors discovered that the overexpression of claudin-4 was greater in IGC than in DGC. A trend was observed between the overexpression of claudin-4 and lymph node metastasis, however, this association was not statistically

significant. The results showed that the expression of claudin-4 was lower in DGC. Possibly it played a role in determining the diffuse phenotype and loose cohesion of cells in DGC in a similar manner as E-cadherin.

Resnick et al. [90] determined the expression pattern of claudins-1, -3, and -4 as well as ZO-1 in large series patients with gastric cancer and to correlate expression with clinicopathologic and prognostic variables. Tissue microarrays were created from paraffinized samples from 146 patients with distal gastric adenocarcinomas (61 intestinal and 85 diffuse or mixed subtypes). In addition, cores of normal mucosa and intestinal metaplasia were taken from most cases. The microarrays were stained for claudins 1, 3, and 4 and ZO-1, and the intensity of staining was determined using a 3-point scale. Moderate claudin 1 and ZO-1 membranous staining were present, whereas only focal weak claudins 3 and 4 membranous staining was present in normal gastric epithelium. Moderate to strong staining of claudins 1, 3, 4, and ZO-1 was disparate in intensity. Cox multivariate analysis revealed that tumor stage, diffuse subtype, and moderate to strong claudin 4 staining were associated with decreased survival. The authors state that these TJ proteins were strongly expressed in most gastric intestinal-type adenocarcinomas but less frequently in diffuse gastric cancers. The up-regulation of claudin expression during gastric carcinogenesis suggests their potential utility as diagnostic biomarkers and possible targets for therapeutic intervention.

Park et al. [91] demonstrated that claudin-7 was up-regulated in gastric carcinoma. Claudin-7 was significantly more often expressed in intestinal metaplasia, adenoma and cancer than in normal gastric epithelium. Claudin-7 was more often unexpressed in diffuse type gastric cancer than in intestinal type. Compared to normal gastric epithelium, intestinal type gastric cancer significantly more often expressed claudin-7, but diffuse type did not. The expression pattern of claudin-7 did not change as cancer progressed. In this study we show that claudin-7 expression changed with the gastric carcinogenic process and that this is implicated in cancer characteristics.

6.5.2. Peripheral plaque proteins in gastric cancer

Similar patterns of expression have been noted for E-cadherin and claudin-4, but ZO-1 expression differed in gastric cancer tissues [92]. According to the Lauren classification, the reduced expression of E-cadherin and claudin-4 was more frequent in diffuse than intestinal type tumors with the reduced expression of E-cadherin and claudin-4 correlated with poor differentiation. Western blot analysis and RT-PCR also showed decreased claudin-4 expression in diffuse type tumors and poorly-differentiated adenocarcinoma. The reduced expression of claudin-4 and E-cadherin correlates with disruption of glandular structure and loss of differentiation, which suggests that the dysfunction of claudin-4 may play a role in the disruption of cell-to-cell adhesion in diffuse type gastric cancer and in a loss of differentiation.

6.6. Gynaecological cancers

6.6.1. Transmembrane proteins in gynaecological cancers

Cell–cell and cell–extracellular matrix interaction is crucial in tumor progression [93]. The expression of occludin and claudins (and syndecan-1) in early stage cervical carcinogenesis showed that occludin and claudin-2 were found colocalized in the basal layer, while syndecan-1 and claudins-1, -4 and -7 were coexpressed in the parabasal and intermediary layers in normal epithelia. Intensity of occludin staining decreased in CIN/CIS lesions, although it was more extended towards the upper epithelial layers with inverse relation with grades, as seen in the case of claudin-2 expression. Claudins-1, -2, -4 and -7 were detected in the entire epithelium in CIN, showing decrease in CIS. The progression of CIN was associated with reduced syndecan-1 expression, in contrast to claudins-1, -4 and -7 which increased toward CIS. It is thought that significant changes occur in the composition of cell adhesion complexes even in early stages of cervical carcinogenesis [92]. In ovarian cancer, laboratory generated human ovarian surface epithelial (HOSE) cells constitutively expressing wild-type claudin-3 and claudin-4 [94]. Expression of these claudins in HOSE cells increased cell invasion and motility. Conversely, knockdown of claudin-3 and claudin-4 expression in ovarian cancer cell lines reduced invasion. Claudin expression also increased cell survival in HOSE cells but did not significantly affect cell proliferation. Moreover, the claudin-expressing ovarian epithelial cells were found to have increased matrix metalloproteinase-2 (MMP-2) activity indicating that claudin-mediated increased invasion might be mediated through the activation of MMP proteins. siRNA inactivation of claudins in ovarian cancer cell lines did not have a significant effect on the high endogenous MMP-2 activity present in these cells. This could be construed as malignant cells having alternative or additional pathways to fully activate MMP-2 and suggests that claudin overexpression may promote ovarian tumorigenesis and metastasis due to increased invasion and survival of tumor cells.

Claudin-7 has been found to have highly differential expression in ovarian carcinoma [95]. 110 patients with various histologic types of epithelial ovarian carcinomas were studies, with claudin-7 transcript found significantly overexpressed in both primary and metastatic tumors compared to normal human ovarian surface epithelium cell lines. At the protein level, claudin-7 expression was found significantly higher in tumors of primary and metastatic origin when compared to normal ovaries, regardless of the histologic type, the grade of differentiation, and the pathologic stage of the disease. Claudin-7 was thus found to be significantly overexpressed in all main histologic types of epithelial ovarian cancer and in single neoplastic cells disseminated in peritoneal cavity and pleural effusions, suggesting its potential role as novel diagnostic marker in ovarian cancer. Claudin-4 is also overexpressed in epithelial ovarian cancer [96]. Claudin-4 overexpression in did not correlate with survival or other clinical endpoints and is associated with hypomethylation. Claudin-4 overexpression did correlate changes in barrier function by treatment with the *Clostridium perfringens* enterotoxin in a dose- and claudin-4-dependent noncytotoxic manner. There is a weak or absence of expression of claudin-3 and claudin-4 in surface human ovarian surface epithelium changed to typical cell-border localization with of inclusion cysts in the normal ovarian stroma [97]. Semiquantitative estimations of immunoblots showed that claudin-3 was significantly increased in ovarian adenocarcinomas compared to benign and borderline-type tumors. Claudin-4 was significantly increased in both borderline-type and ovarian adenocarcinomas compared to benign tumors, whereas no changes were found for claudin-1 or -5. Claudin-3, but not claudin-4, was significantly increased in moderately, poorly and undifferentiated adenocarcinomas compared to well differentiated and borderline-type tumors (FIGO grade). The authors concluded that both claudins-3 and -4, even though they differ in expression during ovarian malignant transformation, might be used as novel markers for ovarian tumors. A small region of the claudin-4 promoter is critical for its expression [98]. This region contains two Sp1 sites required for promoter activity. However, because of the ubiquitous expression of Sp1, these sites, although necessary, are not sufficient to explain the patterns of gene expression of claudin-4 in various ovarian tissues. The claudin-4 promoter was found to be further controlled by epigenetic modifications of the Sp1-containing critical promoter region. Cells overexpressing claudin-4 exhibit low DNA methylation. The authors conclude that as claudin-4 is elevated in a large fraction of ovarian cancer, the mechanism leading to deregulation may represent a general pathway in ovarian tumorigenesis and may lead to novel strategies for therapy and an overall better understanding of the biology of this disease [98]. D'Souza et al. [99] showed that claudins-3 and -4 can be phosphorylated in ovarian cancer cells, suggesting that claudin-3 phosphorylation by PKA, a kinase frequently activated in ovarian cancer, may provide a

mechanism for the disruption of TJ in this cancer. These results have general implications for the regulation of TJ in normal epithelial cells.

In ovarian cell lines, ET-1 (ETAR)/endothelin-1 axis (ET-1) induces loss of adherens and tight-junction protein expression, E-cadherin, b-catenin, and ZO-1, and gain of N-cadherin and vimentin expression [100].

Normal endometrial glands and samples of endometrial hyperplasia and endometrioid carcinoma grade 1 fully expressed occludin at the apical cell border [101]. In endometrioid carcinomas grades 2 and 3, however, occludin disappeared in solid areas of the carcinomatous tissues. Occludin was also found at the apical borders of the cancer cells that formed glandular structures. Occludin expression decreased progressively in parallel with the increase in carcinoma grade, and the decreased occludin expression correlated with myometrial invasion and lymph node metastasis. These results suggest that the loss of TJ has a close relationship with structural atypia in the progression of human endometrial carcinomas and their malignant potential [102].

6.7. Prostate cancer

6.7.1. Transmembrane proteins in prostate cancer

Long et al. [102] investigated the expression of claudin-3 and claudin-4 in human prostate tissue as potential targets for CPE toxin-mediated therapy for prostate cancer. On human multiple-tissue Northern blot analysis, mRNAs for both claudin-3 and claudin-4 were expressed at high levels in prostate tissue. In normal prostate tissue, expression of claudin-3 was localized exclusively within acinar epithelial cells by in situ mRNA hybridization. Compared with expression within prostate epithelial cells in surrounding normal glandular tissue, expression of claudin-3 mRNA remained high in the epithelium of prostate adenocarcinoma (10 of 10) and prostatic intraepithelial neoplasia (5 of 5). Prostate adenocarcinoma cells metastatic to bone were obtained from a patient with disease progression during anti-androgen therapy. These metastatic cells were prostate-specific antigen-positive by immunohistochemical staining and also expressed functional CPE receptors. The persistent high level of claudin-3 expression in prostate adenocarcinoma and functional cytotoxicity of CPE in metastatic androgen-independent prostate adenocarcinoma suggests a new potential therapeutic strategy for prostate cancer.

Zheng et al. [103] described two forms of claudin-7, a full length form of with 211 amino-acid residues and a C-terminal truncated form with 158 amino-acid residues. These two forms of are able to regulate the expression of a tissue-specific protein, the prostate-specific antigen (PSA), in the LNCaP prostate cancer cell line. The authors also found that the expression of claudin-7 was responsive to androgen stimulation in the LNCaP cell line, suggesting that this protein is involved in the regulatory mechanism of androgen. Both forms of claudin-7 were expressed in human prostate, kidney and lung samples, and in most samples, the full-length form of claudin-7 was predominant. However, in some prostate samples from healthy individuals, the truncated form of claudin-7 is predominantly expressed. It appeared that unlike other claudins, claudin-7 has both structural and regulatory functions, and the two forms of claudin-7 may be related to cell differentiation in organ development.

There is only one reported study documenting the pattern of claudin expression in prostatic adenocarcinomas [104]. Decreased expression of claudin-1 correlated with high tumor grade and biochemical disease recurrence, whereas decreased claudin-7 correlated with high tumor grade. In contrast, expression of claudin-3 correlated with advanced stage tumors and recurrence and expression of claudin-4 correlated with advanced stage. On multivariate analysis, advanced stage and decreased claudin-1 protein expression independently predicted disease recurrence. The authors conclude that immunohistochemical expression and prognostic significance of claudins are variable in prostatic adenocarcinomas, with decreased expression of claudin-1 emerging as an independent prognostic variable warranting further research. An earlier study found that occludin was also lost in polygonal (unpolarized) cells of Gleason grades 4 and 5, but remained expressed in all cells facing a lumen in all grades of cancer [105]. Downregulation of occludin in prostate cancer was thus seen to be associated with loss of cell polarity and coincides with the formation of the complex glandular architecture of Gleason grade 4 pattern or complete loss thereof in Gleason grade 5 patterns.

6.8. Lung cancer

6.8.1. Transmembrane proteins in lung cancer

Paschoud et al. [106] found a statistically significant correlation between diagnosis and positivity of tumors with either claudin-1 or claudin-5. Squamous cell carcinomas and basal cells of bronchial epithelium were positive for claudin-1 and negative for claudin-5, whereas adenocarcinomas, normal cylindrical cells and pneumocytes were positive for claudin-5 and negative for claudin-1, suggesting different pathways in tumor development and progression. Claudin-4 and ZO-1 staining were detected in both types of tumors, whereas cingulin was not detected in squamous cell carcinomas. In squamous cell carcinomas, there were statistically significant decreases in the mRNA levels of JAM-1, occludin, claudin-3, claudin-4, claudin-7, cingulin, ZO-2 and ZO-3, and an increase in claudin-1 mRNA. In adenocarcinomas, when transcript levels were compared with bronchial cells, there were also statistically significant decreases in the mRNA levels of claudin-1, claudin-3, claudin-4, claudin-7, ZO-2 and ZO-3. These results indicate that characterization of TJ protein expression in human lung tumors can be an additional diagnostic tool and provide new insights on their histogenesis. In small cell lung carcinomas, differential expression was confirmed for claudin-1 in 82.1% of lung tumor tissues, by quantitative real-time reverse transcription-PCR analysis [107].

An earlier study of 68 lung carcinomas and surrounding normal lung tissues found that in normal lung tissues occludin strongly stained the apicoluminal borders of the bronchial/bronchiolar epithelia and bronchial glands as a dot or short line [108]. Occludin also stained the intercellular borders of alveolar epithelia. In cancer cells that faced lumina of all adenocarcinomas, regardless of grade, including bronchioloalveolar carcinomas, occludin showed an expression pattern identical to that of the normal bronchial and alveolar epithelia. Occludin reactivity was not noted in any cases of squamous cell carcinoma, large cell carcinoma, small cell carcinoma, or large cell neuroendocrine carcinoma [108]. It was suggested that occludin could serve as an immunohistochemical indicator of the “true” glandular differentiation that forms tubulo-papillary structures in human lung carcinoma tissues.

6.9. Melanoma

6.9.1. Transmembrane proteins in melanoma cancer

Leotela et al. [109] used tissue microarray technology to reveal that claudin-1 was overexpressed in melanoma, and aberrantly expressed in the cytoplasm of malignant cells, suggesting a role other than transport. Indeed, melanoma cells in culture demonstrate no TJ function. It has been shown that protein kinase C (PKC) can affect expression of claudin-1 in rat choroid plexus cells, and there was a correlation between levels of activated PKC and claudin expression. It was subsequently found that PKC activation by PMA caused an increase in claudin-1 transcription and protein. Inhibition of PKC signalling in cells with high claudin-1 expression resulted in decreased claudin-1 expression. Transient transfection of melanoma cells with claudin-1 increased MMP-2 secretion and activation, and subsequently, motility of melanoma cells. Conversely, knockdown of claudin-1 resulted in the inhibition of motility, as well as decreases in

MMP-2 secretion and activation, implicating claudin-1 in melanoma progression.

6.10. Pancreatic cancer

6.10.1. Transmembrane proteins in pancreatic cancer

There has been found to be a correlation between TJ and cancer cell dissociation, as well as the involvement of MEK2 in regulation of TJ in cell dissociation of pancreatic cancer [110]. After incubation with conditioned medium of PC-10 cells, plasma membrane distribution of claudin-1 was obviously disrupted, and expressions of MEK2 and p-MEK1/2, as well as dissociation of cell colonies, were significantly induced in PC-1 and CAPAN-2 cells. However, U0126 (a MEK1/2 inhibitor) treatment apparently induced the plasma membrane distribution of claudin-1 and aggregation of single cells in PC-1 and AsPC-1 cells, synchronously seriously suppressed MEK2 and p-MEK1/2 expression. Arrangement of expression and distribution of claudin-1 is closely related to cell dissociation status in pancreatic cancer cells through MEK2 activation.

Borka et al. [111] analyzed protein and mRNA expressions of different claudins in human pancreatic endocrine tumors and ductal adenocarcinomas. Normal acini and ducts showed strong claudins-1, -3, -4, and -7 and scattered claudin-2 protein expressions, while Langerhans islands revealed only claudin-3 and -7 expressions. Claudin-2 expression was found in the half of ductal adenocarcinomas, while the vast majority of endocrine tumors were negative. Claudins-1, -4, and -7 immunohistochemistry were positive in all adenocarcinomas, whereas endocrine tumors were completely negative for claudins-1 and -4. Claudins-3 and -7 proteins were detected in all endocrine tumors, while claudin-13 in ductal adenocarcinomas was negative. The mRNA expression of claudins showed differences between endocrine tumors and ductal adenocarcinomas, with high expressions of claudin-3 in endocrine tumors and claudin-4 in ductal carcinomas which make them attractive targets for adjuvant therapy.

6.10.2. Peripheral plaque proteins in pancreatic cancer

An early study investigating ZO-1 in pancreatic cancer [112] showed that expression of ZO-1 mRNA was increased sixfold in PDAC samples in comparison with normal samples. Confocal microscopy revealed the presence of ZO-1 in the apical and apicolateral areas of ductular cells in the normal pancreas. Similarly, in CP, ZO-1 was localized at apical and apicolateral areas of small proliferating ductular cells and large metaplastic ducts. In PDAC, however, ZO-1 expression was observed irrespective of whether the cancer cells formed duct-like structures or exhibited a diffuse infiltrating pattern. Metastatic pancreatic cancer cells within lymph nodes display variable staining patterns for ZO-1, ranging from apical and apicolateral to a diffuse membranous staining suggesting that ZO-1 is overexpressed in PDAC and raise the possibility that this overexpression may confer a metastatic advantage to pancreatic cancer cells.

A later study [113] investigated the translocation of ZO-1 and the activation of epidermal growth factor receptor (EGFR) to demonstrate the involvement and correlation of TJ protein translocation and EGFR activation in the cell dissociation and subsequent invasion of pancreatic cancer. The obvious translocation of cell–cell junction localized ZO-1 protein to the cytoplasm and nucleus, simultaneous activation of EGFR, as well as the dissociation of cell colonies of non-dissociated pancreatic cancer cells were induced by dissociation factor treatment. However, EGFR inhibitor, AG1478, treatment significantly induced the redistribution of ZO-1 protein to the sites of cell–cell junction and the cell aggregation, as well as simultaneous suppression of EGFR activation in both the dissociated and the non-dissociated pancreatic cancer cells. In addition, AG1478 treatment markedly enhanced the in vitro invasion of non-dissociated pancreatic cancer cells. Translocation of TJ protein ZO-1 as thus found to be closely involved in the induction of invasion through EGFR activation in pancreatic cancer cells.

An early study [114] identified a fragment present in normal pancreatic duct cells that is not expressed in pancreatic duct carcinoma cells. Sequence analysis showed an 88% and 82% identity, respectively, to the cDNA of the canine and human TJ ZO-2 gene. Semi-quantitative RT-PCR analysis of human ZO-2 revealed a striking difference in the expression of various regions of the ZO-2 transcript in normal and neoplastic cells and the presence of an abnormality at the 5′-end of mRNA. RACE analysis identified 2 human ZO-2 mRNAs that encode proteins of different lengths, designated as ZO-2A and ZO-2C. The difference between the 2 forms of ZO-2 is the absence of 23 amino acid residues at the N terminus of ZO-2C compared with ZO-2A. Although ZO-2C was expressed in normal pancreatic cells and a majority of neoplastic tissues analyzed, ZO-2A was undetectable except in one case in all of the pancreatic adenocarcinomas analyzed.

6.11. Other cancers

6.11.1. Multi-cancer studies

As the coxsackie and adenovirus receptor (CAR) is involved in epithelial cell TJ [115], the authors examined CAR's role in tumor metastasis using a B16 melanoma and CT26 colon adenocarcinoma model of experimental metastasis. In lung metastasis, the colony number of B16 cells stably expressing CAR (B16CAR) was significantly lower than that of the control CAR-negative B16 cells. B16 and CT26 cells transiently expressing CAR, which were transduced with adenovirus (Ad) vector expressing CAR, also reduced lung metastasis, suggesting that CAR plays a role in the early stage of metastasis. CAR expression significantly decreased the accumulation of B16 cells in the lung after i.v. injection and the migration in vitro. CAR expression reduced expression of alpha (v), alpha (4), beta (3) and beta (1) integrin, which play important roles in attachment to cells or basement membrane. CAR expression likely acts as a metastatic suppressor in these cancer types.

6.11.2. Malignant brain tumors and the blood–brain-barrier (BBB)

Malignant brain tumors cause cerebral edema because they have leaky endothelial TJ, which allow plasma fluid to enter the brain from the microvessel lumen [116]. In order to identify molecular abnormalities in tumor endothelial TJ, occludin expression in microvessels from adult human non-neoplastic brain tissue was investigated. The proportions of microvessels immunolabelling for occludin were >2/3 in 5/5 non-neoplastic brain tissue samples, >1/3 in 5/5 low grade (Daumas-Duport I or II) astrocytomas and <1/3 in 5/5 high grade (III or IV) astrocytomas and 6/6 metastatic adenocarcinomas. Six non-neoplastic brain tissue immunoblots gave a 55-kDa occludin band, three low-grade astrocytomas gave 55-kDa and 60-kDa bands, 13 high-grade astrocytomas gave 60-kDa or no band and four adenocarcinomas did not give an occludin band. Expression of 55-kDa occludin inversely correlated with the presence of contrast enhancement on computed tomograms. Electron microscopy showed open endothelial TJ in 0/2 non-neoplastic human brain specimens and 2/2 high-grade astrocytomas. It is thought that loss of 55-kDa occludin expression in human brain tumors may contribute to endothelial TJ opening.

Septic encephalopathy is associated with breakdown of the blood–brain barrier and cerebral edema [117]. These features are also common properties of brain tumors. Perimicrovessel edema, disruption of associated astrocyte end feet and neuronal injury occur in a porcine model of acute septic encephalopathy. The adrenergic system has been implicated in the inflammatory response to sepsis and may play a role in controlling blood–brain barrier permeability, since the β2-adrenoceptor agonist dopexamine inhibits perimicrovessel edema formation whereas the α1-adrenoceptor agonist methoxamine provokes it. Electron microscopy revealed TJ opening in high-grade astrocytoma microvessels. Expression of the TJ protein occludin is

reduced in these microvessels and this reduction is inversely correlated with the degree of cerebral edema [117]. Normal astrocytes secrete factors that induce barrier properties in endothelial cells, whereas high-grade astrocytomas secrete vascular endothelial growth factor, which stimulates angiogenesis, down regulates occludin and increases endothelial cell permeability [117]. The water channel protein aquaporin-4 is normally expressed in astrocyte foot processes around cerebral microvessels. Its expression is massively up-regulated in high-grade astrocytoma and around metastatic adenocarcinoma. There is a significant correlation between aquaporin-4 expression and the degree of cerebral edema, but it is not clear whether increased aquaporin-4 expression enhances edema formation or clearance. These results suggest that the pathophysiology of brain edema is multifactorial, but that there may be common processes operating regardless of the aetiology [117].

The quality of the blood–brain barrier (BBB), represented mainly by endothelial TJ is now believed to be dependent on the brain microenvironment and influenced by the basal lamina of the microvessels [118]. In the highly vascularized glioblastoma multiforme (GBM), a dramatic increase in the permeability of blood vessels is observed but the nature of basal lamina involvement remains to be determined. Agrin, a heparan sulphate proteoglycan, is a component of the basal lamina of BBB microvessels, and growing evidence suggests that it may be important for the maintenance of the BBB [118]. This study provided the first evidence that agrin is absent from basal lamina of tumor vessels if the TJ molecules occludin, claudin-5 and claudin-1 were lacking in the endothelial cells [118]. If agrin was expressed, occludin was always localized at the TJ, claudin-5 was frequently detected, whereas claudin-1 was absent from almost all vessels. Furthermore, despite a high variability of vascular phenotypes, the loss of agrin strongly correlated with the expression of tenascin, an extracellular matrix molecule which has been described previously to be absent in mature non-pathological brain tissue and to accumulate in the basal lamina of tumor vessels. These results support the view that in human GBM, BBB breakdown is reflected by the changes of the molecular compositions of both the endothelial TJ and the basal lamina [118].

The development of peritumoral edema is thought to be due to extravasation of plasma water and macromolecules through a defective blood–brain barrier (BBB), but the exact mechanism by which occurs is poorly understood [119]. Biopsies of 25 patients with pathological diagnosis of astrocytic tumors were examined. Both open and close TJ were observed in the micro-blood vessels, inclusive in a same tumor. Cytoskeletal disorganization associated with disintegrated perijunctional actin filaments was seen. The paracellular space showed enlargement and commonly occupied by fluid proteinaceous, endothelial cells display oncotic and ischemic changes; basal lamina reveals enlargement, edema, vacuolization and collagen fibers disposed in irregular array. Pericytes exhibited edema and phagocytozed material, astrocytic perivascular-feet showed signs of oncosis and necrosis, cooption vessels totally surrounding by neoplastic cells also were seen [119]. The ultrastructural abnormalities observed in both junctional complexes and vascular microenvironment suggest a multi-factorial pathobiology process, probably hypoxia intratumoral, calcium overload in endothelial cells, and degradative effects of metalloproteinases over the basal membrane appear as determinant factors that leading to structural modifications of junctional complexes, therefore, treatment with both HIF-1a and metalloproteinases inhibitors possibly can contribute with the pharmacological handling of the peritumoral edema associated with astrocytic tumors [119].

6.11.3. Other cancers and associated endothelium

Hepatocyte growth factor (HGF) is a multi-function cytokine that has been shown to regulate the expression of cell adhesion molecules in human endothelial cells. It is also a key cytokine in the development and progression of cancer, particularly during metastasis. NK4 is a variant of HGF that has already been shown to be antagonistic to HGF. The study by Martin et al. [6] shows that HGF decreased transendothelial resistance and increased paracellular permeability in human vascular endothelial cells that such effects can be inhibited by addition of the NK4 variant. In addition, HGF-stimulated invasion of endothelium by breast cancer cells was inhibited by the addition of NK4. Western blotting revealed that HGF/SF decreased the protein level, and increased tyrosine phosphorylation of ZO-1, but did not cause a change in level of occludin or claudin-1, both molecules involved in TJ function. RT-PCR revealed that addition of HGF/SF caused no change in signal for claudin-5 or junctional adhesion molecule (JAM), but there was a decrease in the signal for claudin-1. NK4 was also able to prevent the decrease in levels of ZO-1 protein by HGF/SF indicating that TJ permeability can be modulated therapeutically.

Disruption of TJ can lead to leaky vascular bed and potentially to edema and swelling of tissues, the [120] aetiology of mastalgia. These changes may also cause vascular spread of cancer cells. In human endothelial cells GLA (gamma-linolenic acid), I (iodine), and Se (selenium) individually increased transendothelial resistance in the presence of 17beta-estradiol (17β-estradiol), which causes leakage of endothelial cells by disruption of TJ. The combination of all three agents also had a significant effect on TER (Fig. 4). Addition of GLA/Se/I reduced PCP of the endothelial cells. Treatment with GLA/Se/I reversed the effect of 17b-estradiol in reducing TER and increasing PCP. Immunofluorescence revealed that after treatment with Se/I/GLA over 24 h there was increasing relocation to endothelial cell–cell junctions of the TJ proteins claudin-5, occludin, and ZO-1 [120]. Interestingly, this relocation was particularly evident with treatments containing I when probing with claudin-5 and those containing Se for occludin. There was a small increase in overall protein levels when examined by Western blotting after treatment with GLA/Se/I when probed with claudin-5 and occludin. It was observable that GLA, I, and Se alone, or in combination are able to strengthen the function of TJ in human endothelial cells, by way of regulating the distribution of claudin-5, occludin, and ZO-1. Interestingly, this combination was also able to completely reverse the effect of 17β-estradiol in these cells [120].

6.11.4. Oral cancer

Oku et al. [121] investigated whether claudin-1 regulated invasion activity in oral squamous cell carcinoma (OSC) cells. Compared with OSC-7, both OSC-4 and NOS-2 more strongly expressed claudin-1 and possessed high activities of MMP-2 and MMP-9. Tumors formed in the tongues of SCID mice xenografted with OSC-4, NOS-2, and OSC-7 immunohistochemically revealed strong, moderate, and weak expression of laminin-5; 2 chains, respectively, and laminin-5; 2 chains were secreted in the conditioned medium of the cancer cells in parallel with the in vivo results. Claudin-1 siRNA largely suppressed the invasion of OSC-4 and decreased the activation of MMP-2, the expression of membrane-type MMP-1 (MT1-MMP), and the cleavage of laminin-5;2. In addition, not only antibodies against MT1-MMP and epidermal growth factor receptor (EGFR) but also MMP-2 and EGFR inhibitors strongly suppressed the invasion activity of OSC-4. These results suggest that claudin-1 upregulates cancer cell invasion activity through activation of MT1-MMP and MMP-2, which results in enhanced cleavage of laminin-5;2 chains.

6.11.5. Liver and hepatocellular cancer

VEGF induces a marked loss of pseudocanaliculi and disruption of occludin-delineated tight junctions in HepG2 cells. This effect of VEGF was mimicked by phorbol-12-myristate-13-acetate (PMA) and was sensitive to protein kinase C (PKC) inhibition by Gö6850. VEGF also induced the translocation of the PKCa-isoform to the plasma-membrane, but had no effect on the activity of Erks and p38MAPK. Sections from surgically removed human HCC showed expression of VEGF in the tumor and occludin disassembly in normal liver

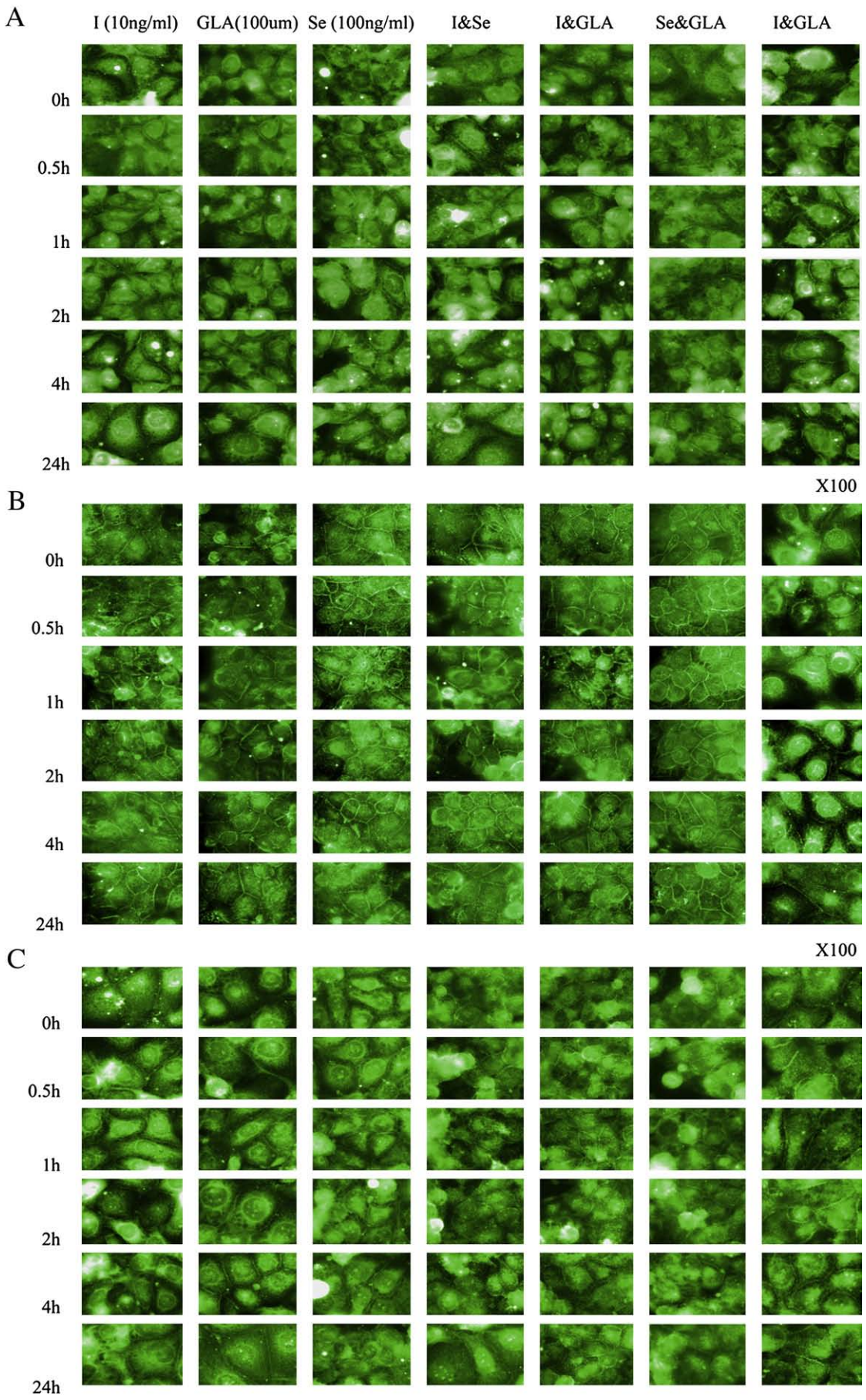

Fig. 4. Effect of iodine, selenium and GLA on the location of claudin-5 (A), occludin (B) and ZO-1 (C) in human endothelial cells.

parenchyma next to the tumor [122]. In conclusion, VEGF induced disruption of TJ in a PKC-a dependent manner. In addition to its known angioneogenic properties, the authors suggest that VEGF may promote HCC spreading into normal liver parenchyma. The data may provide another rationale for the use of VEGF antagonists for tumor therapy.

Ip et al. [123] investigated claudin-10 function in two different hepatocellular carcinoma cell lines observing that overexpression of claudin-10 conferred malignant phenotypes to hepatocellular carcinoma cells, Hep3B, which lack claudin-10 expression, by promoting cancer cell survival, motility, and invasiveness. More importantly, MMP2 was up-regulated. Increase in mRNA transcription and protein expression of membrane type 1-MMP (MT1-MMP) was also observed in the claudin-10 transfectants, and in addition, claudin-1, claudin-2, and claudin-4 were up-regulated in claudin-10 overexpression transfectants, indicating that the expression of claudin-10 in cancer cells might affect the expression levels of its family members. On the contrary, small interfering RNA-based knockdown of claudin-10 in HLE, an invasive cell line with high level of claudin-10 expression, abolished invasion and strongly decreased activation of MMPs and claudin member's expression.

6.11.6. Synovial cancer

Synovial sarcoma, a soft tissue sarcoma that develops in adults, is pathologically subclassified into monophasic spindle synovial sarcoma and biphasic synovial sarcoma with epithelial components [124]. Expression profiles of 21 claudins in 17 synovial sarcoma tumor samples, including 9 biphasic tumors, identified claudin-4, claudin-7, and claudin-10 as biphasic tumor-related claudins, and immunohistochemical analyses demonstrated the localization of these claudins in the epithelial component in biphasic tumors, with claudin-7 the most closely associated with the epithelial component. The mRNA expression and protein localization of claudin-7 coincided with those of the ELF3, an epithelia-specific member of the Ets family of transcription factors. Inhibition of ELF3 expression by small interfering RNA simultaneously down-regulated the mRNA expression of the *claudin-7* gene and the introduction of ELF3 expression in claudin-7-negative cell lines induced mRNA expression of the *claudin-7* gene. Therefore, the induction of claudin-7 expression by ELF3 appears critical to the formation of the epithelial structures in biphasic synovial sarcoma [124].

6.11.7. Thyroid cancer

In ninety-one thyroid neoplasms (15 follicular adenomas, 15 follicular carcinomas, 26 papillary carcinomas, 16 papillary microcarcinomas, 8 medullary carcinomas, 3 poorly differentiated carcinomas, and 8 undifferentiated carcinomas) occludin was mainly expressed in the form of intracytoplasmic vesicles, whereas all claudins tested exhibited membranous immunostaining [125]. Thirteen out of 15 follicular adenomas, 10/15 follicular carcinomas, 24/26 papillary carcinomas, 15/16 papillary microcarcinomas, 1/8 medullary carcinomas, 2/3 poorly differentiated carcinomas and 2/8 undifferentiated carcinomas exhibited claudin-1 expression, whereas claudin-4 was expressed in 13/15, 12/15, 23/26, 13/16, 7/8, 2/3 and 2/8 of the tumors, respectively, and claudin-7 expression was found in 67, 33, 73, 69, 25, 0 and 13% of the cases, respectively [125]. Occludin was expressed in 100% follicular adenomas, 80% follicular carcinomas, 96% papillary carcinomas, 50% papillary microcarcinomas, 50% medullary carcinomas, 33% poorly differentiated carcinomas and 88% undifferentiated carcinomas. Occludin expression was reduced in papillary microcarcinomas, medullary carcinomas and poorly differentiated carcinomas. All claudins exhibited reduced expression in undifferentiated carcinomas [125]. Claudin-1 was additionally reduced in medullary carcinomas and claudin-7 in follicular, medullary and poorly differentiated carcinomas. A correlation between loss of claudin-1 expression and worse disease-free survival was noted on univariate analysis. The authors suggest that dedifferentiation of the thyroid carcinomas is accompanied by reduction in claudin-1, -4 and -7 expression. A differential expression of TJ proteins in the different histologic types of thyroid gland is noted. Additionally, claudin-1 expression may be an important prognostic indicator of recurrence in thyroid carcinomas [125].

6.11.8. Neurofibroma

In a study of 16 neurofibromas from 12 patients with neurofibromatosis type 1 (NF1) cell–cell contacts with typical ultrastructural morphology of TJ were seen between adjacent perineurial cells surrounding the small nerves and between contacting perineurial cell processes embedded in tumor stroma [126]. Immunohistochemistry showed expression of claudin-1, claudin-3, and ZO-1 in the intercellular junctions of a subpopulation of tumor cells. Occludin was present mainly in perineurium and claudin-5 localized to the blood vessels. Claudin-1 positive cells were also positive for type IV collagen and epithelial membrane antigen but not for S-100 protein a labelling pattern consistent with a perineurial cell phenotype. Using claudin-1 as a marker, the authors showed that clusters of perineurial cells are distributed around the rudimentary nerves within cutaneous neurofibromas and at the periphery of some neurofibromas [126].

6.11.9. Testicular cancer

In normal seminiferous epithelium, specialized TJ between Sertoli cells constitute the major component of the blood–testis barrier [127]. Sertoli cells associated with CIS exhibit impaired maturation status, but their functional significance remains unknown. In normal tubules, ZO-1 and ZO-2 immunostaining was observed at the blood–testis barrier region of adjacent Sertoli cells. Within CIS tubules, ZO-1 and ZO-2 immunoreactivity was reduced at the blood–testis barrier region, but spread to stain the Sertoli cell cytoplasm. Western blot analysis confirmed ZO-1 and ZO-2, and their respective mRNA were shown by RT-PCR. In conclusion, Sertoli cells associated with CIS show an altered distribution of ZO-1 and ZO-2 and lose their blood–testis barrier function [127].

7. Prevention of metastasis: promising new targets for cancer diagnosis and therapy

It is apparent from this overview of the studies carried out on TJ function and aberrant expression in cancer, that individual components within the TJ complex offer intriguing and novel targets for the prognosis, detection and, indeed, possible modes of treatment for patients with cancer. Until now, most of the work has concentrated on the use of claudins, and the reader is directed to a number of reviews showcasing this [128–130]. Of interest is the work carried out by Skrovanek et al. [131] who found that restriction of sulfur-containing amino acids (SCAA) in LLC-PK (1) renal epithelial cells resulted in reduction of methionine by 90%. Cell growth and differentiation were maintained, and both confluent cell density and transepithelial short circuit current were unaffected. Occludin and claudins-1 and -2 did not have altered expression, however, claudins-3 and -7 were significantly decreased and claudins-4 and -5 were markedly increased. The functional result of these structural changes was improved barrier function. In contrast to normal cells, tumor cells have absolute requirement for methionine. In animal models, methionine restriction limits tumor growth and reduces tumor volume. However, interruption of methionine restriction induces the regrowth of tumors. Moreover methionine restriction induces cell modifications suggesting it had a use in association with conventional chemotherapy [132]. That there is a link between methionine restriction and TJ protein expression leads to interesting possibilities for future therapies. It might be anticipated that as further work sheds light on the seemingly diverse yet vital functions of the other TJ molecules, there will be increasing possibilities in utilising the TJ components as targets for therapy to prevent cancer metastasis.

8. Summary

TJ were first described as having altered form and function in tumor cells and tissues over thirty years ago [133,134]. It is increasingly evident from the rapidly growing research in this area, that the TJ has a vital role to play during cancer metastasis. The claudin family group has been most explored; however, it is vital that the other transmembrane and peripheral components attract as much attention. The TJ and changes in barrier function appear to be part of an essential mechanism that is awry during cancer metastasis; whether up-regulation promotes cell dissemination, or whether down-regulation, causing dismantling of the TJ structure leading to loss of polarity, loss of contact inhibition, uncontrolled growth, detachment and invasion of cancer cells and hence successful penetration of the endothelium (intravasation and extravasation) by aberrant cell surface expression (direct interaction) or via secretion of regulatory/degradative substances by the cancer cells. Moreover, dysregulation of the TJ can also lead to potentially exciting markers for the prognosis of a number of tumor types. Although TJ proteins have yet to be proven true suppressor proteins, all these data suggest that the TJ is vital to the prevention of successful cancer cell metastasis and further research should provide answers to using TJ as an essential point for intervention during the metastatic cascade.

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