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ORIGINAL PAPER

Different Expression of Occludin and ZO-1 in Primary and Metastatic Liver Tumors

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Abstract Tight junction (TJ) components were found to be correlated with carcinogenesis and tumor development. TJs are composed of three main integral membrane proteins; occludin, claudins and JAMs. Alteration of the TJ protein expression may play an important role in the process of cell dissociation, which is among the first steps of tumor invasion and metastasis. Reduced expression of ZO-1 has been reported to be associated with invasion of several tumors. The aim of the present study was to detect differences between occludin and ZO-1 expression in normal liver samples, HCCs and colorectal liver metastases. Expression of occludin and ZO-1 was analysed in 25 surgically removed human hepatocellular carcinomas (HCC) and 25 human colorectal liver metastases. Gene expression levels were measured by real-time RT PCR, protein expression was determined by immunohistochemistry, comparing tumors with the surrounding nontumorous parenchyma and with seven normal liver samples. Occludin and ZO-1 mRNAs showed significant downregulation in HCCs in comparison with normal liver and were also downregulated in the metastases when compared with normal liver. Occludin and ZO-1 proteins were weakly expressed on hepatocytes in normal liver, while strong expression was found on bile canaliculi. In HCCs occludin and ZO-1 did not show immunopositivity on tumor cells, while colorectal metastatic tumors revealed high levels of these molecules. HCCs and metastases are characterized by markedly different protein expression pattern of occludin and ZO-1, which phenomenon might be attributed to the different histogenesis of these tumors.

Keywords Hepatocellular carcinoma · Colorectal liver metastasis · Occludin · ZO-1

Abbreviations

ZO-1	Zonula Occludens-1
TJ	Tight Junction
HCC	Hepatocellular Carcinoma
CRC	Colorectal Cancer
ECM	Extracellular Matrix
JAM	Junctional Adhesion Molecule
TER	Transepithelial Electric Resistance
MAGUK	Membrane Associated Guanylate Kinase
PCR	Polymerase Chain Reaction

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Introduction

Hepatocellular carcinoma (HCC) is the most common malignant primary hepatic tumor. Metastatic liver tumors are significantly more common than HCCs. This can be explained by several factors, one of them being that the liver has no typical basement membrane and thus lacks one of the most important barriers limiting metastasis [1]. Tubular gland structures of colorectal cancer (CRC) have been demonstrated to undergo dedifferentiation at the

primary site, i.e., the gland structures are broken and the cancer cells, either a single or a few cells at a time, diffusely invade the submucosal layers of the intestine. Subsequently, when the cancer cells reach the liver via the portal vein, the gland structures are re-formed in liver metastases [2].

Cell contacts are highly important in this process. The cell adhesion molecules responsible for epithelial cell–cell connections form typical structures including tight junction (TJ), gap junction, adherent junction and desmosome. Besides, the different adhesion molecules play important role in cell–extracellular matrix (ECM) connections. Further, regulation of ion and solute movement and even influencing signal transduction pathways seem to be dominant functions [1].

Cell–cell junctions might have specific functions in different organs. TJs of biliary epithelial cells and hepatocytes prevent bile regurgitation from the biliary tract and serve as the only intercellular barrier between the biliary luminal space and the portal area [3]. On the other hand, gap junctions of hepatocytes are considered to enable ordered contraction of bile canaculi from centrizonal to periportal hepatocytes via their function of intercellular communication. Gap and tight junctions may thus play a crucial role in bile secretion, one of the most differentiated functions of the liver. As the result of impaired intercellular communication and leaky TJs, downregulation of gap and tight junctional functions was observed in intrahepatic cholestasis, which is a common pathological condition of the liver [4].

Loss of TJ function is critical for the development of a number of cancers, the breakdown of normal cell–cell adhesion between tumor cells is a key step in the acquisition of invasive phenotype [5].

TJs reveal very complex structure, are intimately associated with other structures such as the cytoskeleton, the nucleus, and also participate in proliferation and differentiation as part of the cellular signalling pathways [6].

TJs are composed of three main integral membrane proteins: occludin, claudins and junctional adhesion molecules (JAMs), from which claudins are the most important regulators of paracellular flux and transepithelial electric resistance (TER). The claudins form ion selective pores within TJ strands, whereas occludin and JAMs may have an adhesive and/or signal transducing function as they interact with various cytosolic complexes [7].

Occludin is described as exclusively localised at the TJs of epithelial and endothelial cells. First it was thought to be a dispensable component of TJ formation and function [8]. However, changes in occludin expression resulted in altered function of the TJ [9]. Occludin is capable of lateral oligomerisation and creates a paracellular barrier by forming a continuous line of adhesion between cells [7]. Occludin is a protein with four transmembrane domains, two extracellular loops of similar size, and three cytoplasmic domains. Both extracellular loops are enriched in tyrosine residues [9]. Overexpression of the mutant form of occludin in epithelial cells leads to changes in the gate and fence functions of TJs. Extracellular loops of occludin can connect each other in neighbouring cells. This connection regulates the permeability of TJ [9].

ZO-1 is a 210–225 kDa protein found at the submembranous domain of TJs in epithelia and endothelia. It is a PDZ-containing protein. ZOs are members of the MAGUK protein family (Membrane Associated Guanylate Kinase). Proteins in this family are recognised for having structurally conserved PDZ, SH3 and GK domains. Cells that do not form TJs, such as fibroblasts, show ZO-1 dispersely in the cytoplasm [10].

At the TJ ZO-1 is associated through its first PDZ domain to the carboxyl terminal end of claudins [11], by the second and third PDZs to JAM [12] and by its GK module to occludin [13]. ZO-1 serves as a link between occludin and the actin cytoskeleton [7], although occludin can also bind directly to actin [14]. ZO-1 has a scaffolding function, playing an important role in signal transduction by clustering critical membrane proteins [15]. A schematic illustration of the connection of occludin and ZO-1 with the membrane as well as with each other is shown in Fig. 1.

Previously we examined the claudin-4 expression in liver tumors and the difference between biliary tract cancers and hepatocellular carcinomas was determined [16]. The present study aimed to investigate the expression of other members of TJ proteins, especially occludin as well as the cytoplasmic scaffolding protein, the ZO-1, in normal liver, hepatocellular carcinoma and colorectal liver metastasis.

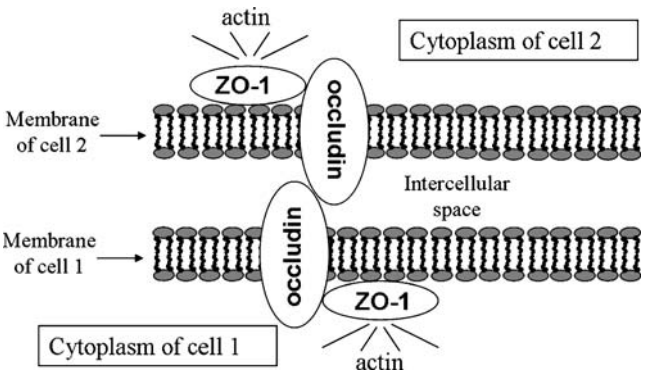

Fig. 1 Occludin is a transmembrane protein, while ZO-1 is a peripheral membrane protein. At the tight junction ZO-1 is associated through its guanylate kinase domain to occludin. It is also connected with actin cytoskeleton. Extracellular loops of occludin can connect each other in neighbouring cells. This connection regulates the permeability of TJ

Materials and Methods

Patients' Samples

Tissue blocks of 57 cases were taken from surgically removed liver tumors including hepatocellular carcinomas (HCCs) and surrounding nontumorous liver tissues (25 cases), colorectal liver metastases and surrounding non-tumorous liver tissues (25 cases). Seven normal liver samples served as control. Cases were analyzed with the permission of the Regional Ethical Committee of the Semmelweis University (#172/2003). (Mean age: 59.3 years in HCC patients, 62.8 years in metastatic cases. There was a male predominancy, showing 15/25 and 14/25 ratios in the HCC and metastatic groups, respectively.)

RNA Extraction and Real-time RT-PCR

Isolation of RNA was carried out from tissue samples stored in RNA-later (Sigma, St. Louis, MO, USA, R0901). RNA was isolated with Trizol (Sigma, St. Louis, MO, USA, T 9424, according to manufacturer's instructions) and stored at −80°C. One μg aliquot of total RNA was reverse transcribed using random hexamers and Mmulv reverse transcriptase (Applied Biosystems N8080127 and N8080018, Foster City, CA, USA), for 10 min at 25°C, 50 min at 42°C, and 5 min at 95°C. Each PCR was carried out in a 25 μl volume of 1× Sybr Green PCR buffer (BIORAD, Hercules, CA, USA) with 500 nM primers (Table 1) for 2 min at 95°C for initial denaturation. This was followed by 40 cycles at 95°C for 20 s, at 63°C for 30 s and at 72°C for 60 s after which melting analysis was performed from 55–95°C in AB 7000 Real Time PCR System (Applied Biosystems) with occludin, ZO-1, GAPDH, RNA–polymerase II and β-actin housekeeping gene primers.

Statistical Analysis

Statistical differences were evaluated by the program (REST) described by Pfaffl and co-workers, using Pairwise Fixed Reallocation Randomisation Test [17]. Relative quantification was performed using average of GAPDH, β-actin and RNA-polymerase II as internal controls. Real-time PCR results were carried out by three replicate measurements of each sample.

Immunohistochemistry

Samples were fixed in 10% buffered formalin, and embedded in paraffin. Three to five micrometer thick sections were stained with hematoxylin (Sigma H 3136) for 10 min and with eosin (Sigma E 4382) for 1 min to establish the diagnosis and select areas for immunohistochemistry. Necrotic and haemorrhagic foci were excluded from further analysis. Occludin and ZO-1 were detected by immunohistochemistry using rabbit polyclonal antibody against occludin (Zymed, San Francisco, CA, USA, 71–1500) and rabbit polyclonal antibody against ZO-1 (Zymed 61–7300). Deparaffinized sections were blocked for endogenous peroxidase activity with 3% H_2O_2 for 20 min. Antigen retrieval was performed with proteinase K (Dako, Glostrup, Denmark, S3020) for 15 min. Primary antibodies were used in a dilution of 1:100 at 4°C overnight. To exclude nonspecific binding, negative controls incubated with secondary antibody only (Envision anti-rabbit, Dako K 4003) were processed and revealed no signal. Immunostaining was examined in normal, HCC and metastatic liver tissues using light microscopy. Ten non-overlapping fields were photographed per slide. Slides were evaluated with Leica QWin Pro 3.1 software (Leica Microsystems Imaging Solutions Ltd., Cambridge, UK). Thresholds were set for red, green and blue colour components of the image, respectively. The system detects all pixels in the image equivalent or nearly equivalent to the colour levels of the immunopositive areas. Finally, the positive area as percentage of the total field was defined as the ratio of pixels set above the threshold compared to the total number of pixels within a defined area of interest.

For statistical analysis, the Mann–Whitney *U* test was used to compare protein expression in the different study groups. Probability values of $p<0.05$ were accepted as being significant.

Table 1 Primers used for real-time RT-PCR

Gene	Forward primer	Reverse primer
Occludin	CGG TCT AGG ACG CAG CAG AT	AAG AGG CCT GGA TGA CAT GG
ZO-1	CGA GTT GCAA TGG TTA ACG GA	TCA GGA TCA GGA CGA CTT ACT GG
β-actin	CCT GGC ACC CAG CAC AAT	GGG CGG GAC TCG TCA TAC
GAPDH	GAA GGT GAA GGT CGG AGT	GAA GAT GGT GAT GGG ATT TC
RNA polymerase II	GCA CCA CGT CCA ATG ACA T	GTG CGG CTG CTT CCA TAA

Table 2 Fold change differences in the expression of occludin and ZO-1 based on real-time RT-PCR reactions

	Occludin	ZO-1
NORM-HCC	D 8.193*	D 4.576*
	p=0.001	p=0.001
NORM-sHCC	D 3.673*	D 3.453*
	p=0.0095	p=0.022
NORM-sMET	D 9.832*	D 7.09*
	p=0.002	p=0.0075
NORM-MET	D 12.109*	D 6.415*
	p=0.001	p=0.001
sHCC-HCC	D 2.23*	D 1.325
	p=0.0435	NS
sMET-MET	D 1.232	U 1.105
	NS	NS
HCC-MET	D 1.478	D 1.402
	NS	NS

Relative quantification was carried out using the mean of GAPDH, β-actin and RNA-polymerase II as reference genes. The underlined group is compared with the other group

U Upregulated, *D* downregulated, *NS* non significant, *NORM* normal liver, *HCC* hepatocellular carcinoma, *MET* colorectal liver metastasis, *sHCC* surrounding tissue of HCC, *sMET* surrounding tissue of colorectal liver cancer

*p<0.05

Results

Real-time RT-PCR Analysis of Occludin and ZO-1 mRNA Expression

Table 2 and Fig. 2 show data on the mRNA expression data.

A significant downregulation of mRNA expression for occludin and ZO-1 was seen in all groups compared with the normal liver. In the HCC sample group occludin showed an 8.193 fold downregulation, as compared with the normal liver group and a 2.23 fold downregulation as compared with the surrounding tissue of HCC. In the metastasis sample group occludin was considerably downregulated by 12.109 folds, as compared with the normal liver group. Occludin showed 1.478 fold downregulation in the metastasis group when compared with HCC (not significant). In surrounding tissues of HCC and metastasis the mRNA level of occludin was significantly lower as compared with normal liver samples (3.673 and 9.832 fold, respectively). The difference between the expression of occludin in metastasis and the surrounding liver tissue of metastasis was not significant.

ZO-1 in the HCC group showed a 4.576 fold downregulation when compared with the normal liver and a 1.325 fold downregulation in comparison with the surrounding tissue of HCC. In the metastasis sample group ZO-1 was significantly downregulated by 6.415 folds upon comparison with the normal liver group. ZO-1 showed a 1.402 fold downregulation in the metastasis group when compared with HCC (not significant). In surrounding tissues of HCC and metastasis the mRNA level of ZO-1 was found significantly lower when compared with normal liver samples (3.453 and 7.09 fold, respectively). The difference between the expression of ZO-1 in metastasis and in the surrounding tissue of metastasis was not significant.

Immunohistochemistry

Occludin and ZO-1 showed membrane positivity: they were expressed weakly on hepatocytes and strongly on bile canaliculi (surrounding the luminal space of the bile ducts) in normal liver (Fig. 3d,e). In most of the samples occludin and ZO-1 did not show positivity in HCCs (Fig. 3f,g), with

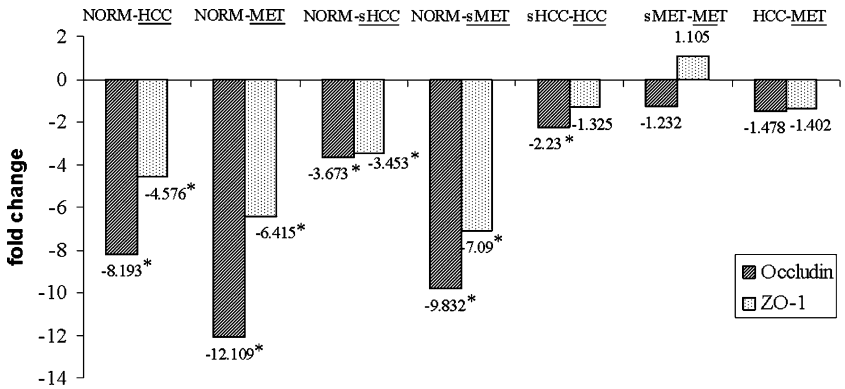

Fig. 2 mRNA expression analysis of normal liver, HCC, surrounding tissue of HCC, metastasis and surrounding tissue of metastasis (data shown in Table 2). Data indicate the fold change differences in the expression of occludin and ZO-1 in the underlined group as compared with the other group. *Asterisk* Significant difference (p<0.05), *NORM* normal liver, *HCC* hepatocellular carcinoma, MET colorectal liver metastasis, *sHCC* surrounding tissue of HCC, *sMET* surrounding tissue of colorectal liver cancer

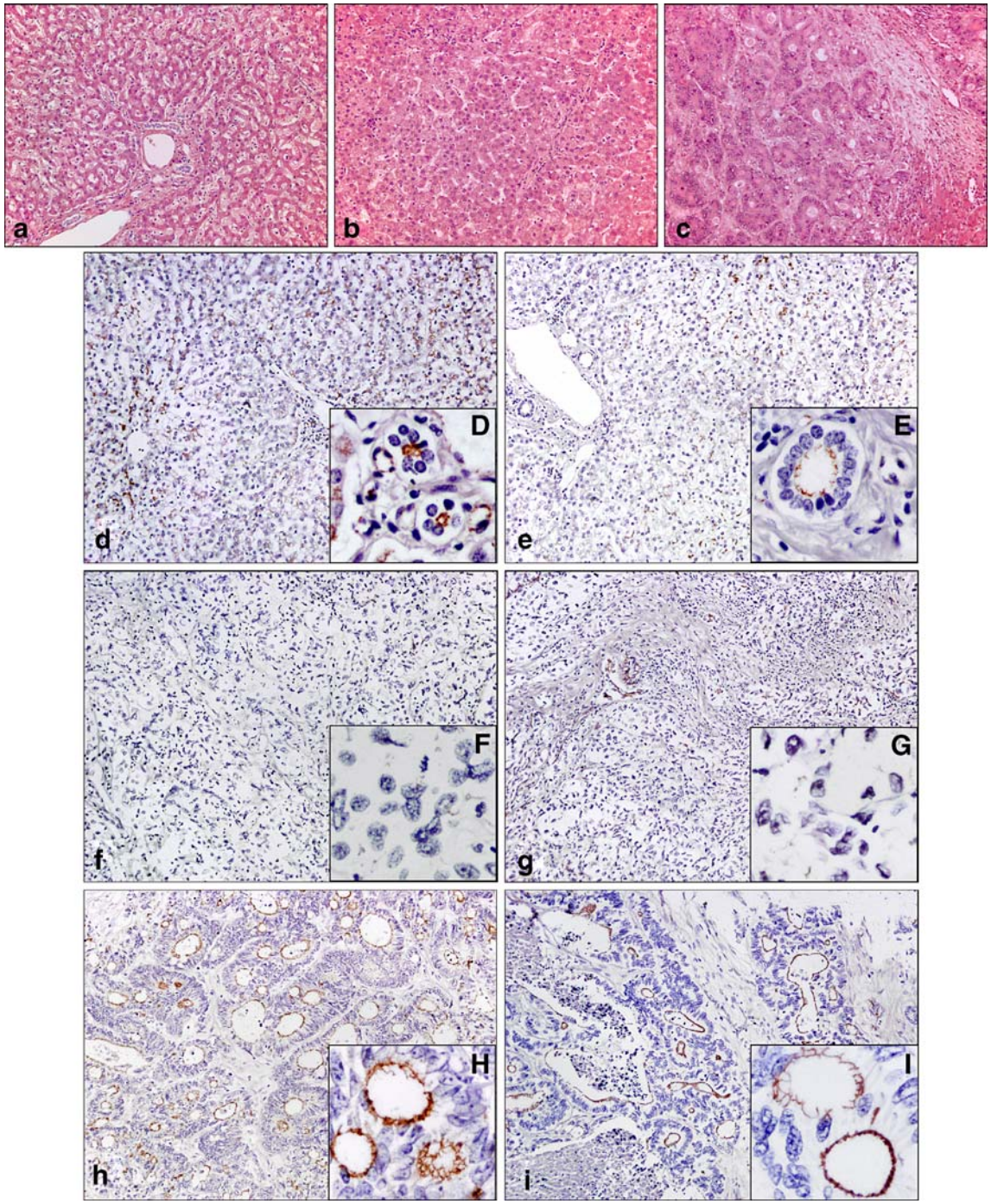

Fig. 3 Occludin and ZO-1 showed positivity on bile canaliculi in normal liver (occludin: **d**, *D*; ZO-1: **e**, *E*). Occludin and ZO-1 are not expressed in HCC (occludin: **f**, *F*; ZO-1: **g**, *G*). In colorectal liver metastasis occludin and ZO-1 are expressed in the luminar poles of the tumor (occludin: **h**, *H*; ZO-1: **i**, *I*). **a** Haematoxylin–eosin staining of normal liver, **b** haematoxylin–eosin staining of HCC, **c** haematoxylin–eosin staining of colorectal liver metastasis. (Magnification: **a**, **b**, **c**, **d**, **e**, **f**, **g**, **h**, **i**: ×200, *D*, *E*, *F*, *G*, *H*, *I*: ×600)

the exception of a few cases where small areas of tumor cells revealed membrane positivity. On the contrary, occludin and ZO-1 were strongly expressed in the cell membrane in metastatic tumor glands, especially at the luminar poles (Fig. 3h,i).

The percentage of immune-positive areas was the highest in metastasis (occludin: 1.594%, ZO-1: 1.255%). The expression of occludin and ZO-1 in metastasis showed significant differences in comparison with the expression seen in the other groups (Table 3 and Fig. 4).

Table 3 Results of immunostaining for occludin and ZO-1 in HCC, metastasis and normal liver samples

	Occludin	ZO-1
NORM-HCC	0.028–0.164	0.190–0.45
	NS	NS
NORM-MET	0.028–1.594	0.190–1.255
	$p=0.0004$	$p=0.0014$
sHCC-HCC	0.047–0.164	0.085–0.45
	NS	$p=0.0167$
sMET-MET	0.196–1.594	0.193–1.255
	$p<0.0001$	$p=0.0001$
HCC-MET	0.164–1.594	0.45–1.255
	$p<0.0001$	$p=0.0015$

Means and probability values by Mann–Whitney *U*-test for pairwise comparison of occludin and ZO-1
U Upregulated, *D* downregulated, *NS* non significant, *NORM* normal liver, *HCC* hepatocellular carcinoma, *MET* colorectal liver metastasis, *sHCC* surrounding tissue of HCC, *sMET* surrounding tissue of colorectal liver cancer

Discussion

Changes in the protein composition of TJ was found to be correlated with carcinogenesis and tumor development. As a pivotal transmembrane protein of TJ, occludin may play an important role in the process of cell dissociation, which is the first step of tumor invasion and metastasis [18].

In neoplastic transformation decreased intercellular adhesion and loss in cell polarity are observable, implying the downregulation and/or dysregulation of TJ protein expression [19]. However, occludin deficient animals present pleiotropic abnormalities, suggesting that occludin can have additional functions unrelated to the classic TJ function, or signalling events at the TJ might have more complex consequences than previously thought [8, 20].

The role played by ZO-1 in tumorigenesis is still unexplored. In most cancers, levels of ZO-1 were found typically downregulated, leading to increased motility and contributing to oncogenic behaviour, whereas in melanoma cells it was found to be upregulated localized at adherent junctions [21]. Tumor suppressor function has been raised since deletions or mutations in its gene have produced overgrowth, whereas downregulation of its expression has been found coupled to breast cancer progression [9]. The possible participation of ZO-1 in tumor suppression is rather complex, as many additional factors appear to be intervened. For example, in breast cancer cells, insulin-like growth factor I receptor (IGF-IR) induces E-cadherin mediated cell–cell adhesion by upregulating ZO-1. The expressions of IGF-IR and ZO-1 increase growth on the other hand, survival of the primary tumor may reduce the occurrence of metastasis [22]. Vitamin D3 promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and ZO-1, further by inhibition of β-catenin signalling and achieving translocation of ZO-1 from the nucleus to the plasma membrane [23].

Reduced expression of ZO-1 was reported to be associated with invasion of breast and gastrointestinal cancers. ZO-1 was found expressed at the apical cell borders of normal colorectal epithelium at the luminal side of tubular gland structures. In comparison with this normal epithelium, the ZO-1 protein expression level was frequently reduced in primary CRC. However, ZO-1 was reexpressed in liver metastases of CRC [2].

When the cancer cells reach the liver via the portal vein, the gland structures are re-formed in the liver metastases. The adhesion molecules in CRC lose their functions at the primary site, later recovering them in the metastatic phase [24]. In metastatic tumors, occludin and ZO-1 were both found surrounding the lumen of the glandular structures of colon metastases, therefore confirming the observation of Brabletz and coworkers [24].

Busch also showed a consistent occludin expression in prostate cancer cells facing the glandular lumen, regardless of grade. However, no occludin expression was seen in cells arranged without polarity [25]. These data reveal that tight junctions play a crucial role in the formation of organized tubular structures in glandular epithelia. Similarly, loss of tight junctions was related to loss of functionally organized tubular structures and neoplastic growth of the epithelia, and occludin expression was completely lost in poorly differentiated solid components of gastrointestinal tumors [26]. Overall, immunohistochemical studies of neoplastic and non-neoplastic glandular epithelia suggest that occludin is crucial for the formation of glandular

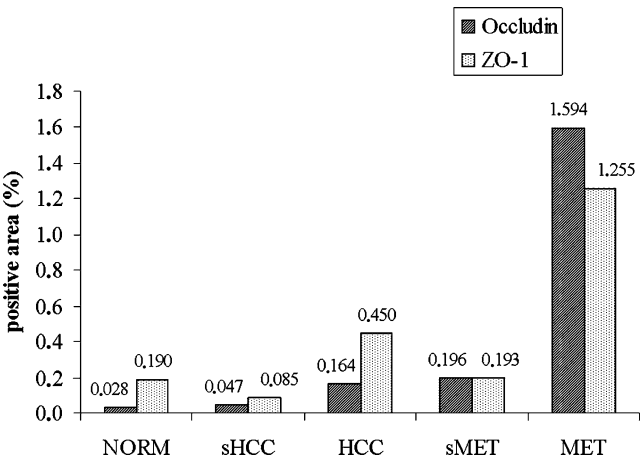

Fig. 4 Diagram illustrating the difference in occludin and ZO-1 expression detected by immunohistochemistry and evaluated by Leica QWin Pro software. *NORM* Normal liver, *HCC* hepatocellular carcinoma, *MET* colorectal liver metastasis, *sHCC* surrounding tissue of HCC, *sMET* surrounding tissue of colorectal liver cancer

structures [25, 26]. The expression of these proteins was reduced in poorly differentiated adenocarcinomas as compared with differentiated ones, where expression was found similar to normal epithelium. Kimura suggested that occludin could be a marker of differentiation for glandular epithelia [26].

Claudins are the other major constituents of tight junctions. Alterations in claudin expression pattern have been described in HCC and liver metastasis of colorectal carcinoma as well. Relatively high levels of claudin-1, -3, and -7 expression has been described in HCC contrary to the low expression of claudin-4 and claudin-5 [27]. Lodi et al. [16] reported that claudin-4 expression was prominent and stable in cholangiocellular carcinoma while no or weak expression was found in HCC. Claudin-10 expression has been reported to be associated with local recurrence and poor prognosis of HCC [28]. Kominsky et al. detected strong expression of claudin-3 and claudin-4 in the majority of brain metastases of colorectal carcinoma by immunohistochemical analysis, however, the expression of claudin-3 and claudin-4 was absent in adjacent normal brain tissue [29]. Interestingly, claudin-3 and claudin-4 expression in primary colorectal carcinoma was not associated with lymph node metastasis [30].

Occludin and ZO-1 mRNA content of HCC and metastasis was lower than in case of normal liver tissue, while occludin and ZO-1 protein expression of metastases was higher compared with the expression found in normal liver. Colorectal metastases revealed lower occludin and ZO-1 mRNA contents when compared with HCC, while the expression of these proteins was higher. One of the possible explanation for this discrepancy is lower protein turnover in these tumors: proteins (e.g. occludin and ZO-1) are not degraded for a long time, so mRNA level is lower, while higher protein level is detectable in tumors as compared with normal liver. Another possible reason might be posttranslational modification.

Cancer cells lose TJs with dedifferentiation, however, this is a secondary or late event in tumorigenesis, although TJs are considered to be deeply involved in the process [31].

Both primary and studied metastatic liver tumors show distinct features of TJ architecture, with the secondary tumors showing a conventional TJ organization, whereas HCC presents a TJ protein pattern difficult to interpret based on our current knowledge on tight junction function. HCCs and metastases are characterized by markedly different protein expression pattern of occludin and ZO-1, which phenomenon might be attributed to the different histogenesis of these tumors.

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