Examination of the expression pattern of different claudins in human biliary tract cancers, hepatocellular carcinomas and rat liver regeneration model

Doctoral (Ph.D.) theses

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1. Introduction

The connection of adjacent epithelial and endothelial cells occurs via different intercellular structures. Tight junctions (TJ), the most apical part of the intercellular junctional complex recognizable as a network of anastomosing strands, are crucial structures controlling paracellular permeability and maintaining cell polarity. TJs have been shown to recruit cytoskeletal and signaling molecules that participate in the regulation of cell proliferation, differentiation and gene expression.

The claudin superfamily proteins consisting of at least 24 members play a central role in modulating tight junction functions. The claudins have four transmembrane domains, two extracellular loops, and a cytoplasmic carboxyl-terminal tail domain. The expression pattern of claudins is tissue specific, which may explain the differences in paracellular transport between different epithelial cell types. TJs in single cells generally contain more than two species of the claudin superfamily. The exact combination of claudin proteins within a given tissue is thought to determine the selectivity and strength of the TJs.

Several human diseases are believed to be caused by the alteration of TJs. Down-regulation of TJ proteins have been associated with psoriasis, collagenous colitis, Crohn-disease and other chronic inflammatory diseases. Mutation of claudin-1 is implicated in neonatal sclerosing cholangitis associated with ichthyosis. Mutations in the gene encoding claudin-14 cause autosomal recessive deafness DFNB2. Claudin-16 plays a crucial role in paracellular Mg2+ transport and is defective in familial hypomagnesemia with hypercalciuria and nephrocalcinosis.

Claudin-3 and claudin-4 proteins have recently been shown to represent the natural receptors for Clostridium perfringens enterotoxin (CPE). Binding of CPE to claudins induces toxin-mediated cytolysis very rapidly.

Previous studies have shown that alterations of the claudin proteins are associated with various human tumor types. The exact role of these proteins in the process of carcinogenesis and tumor progression is unknown. In various tumors, claudins may be over-expressed or down-regulated. Down-regulation of claudins by decreasing intercellular adhesion may increase cell motility and promote metastasis formation. Over-expression of claudins can negatively interfere with proper tight junction formation and function, potentiating malignant transformation and metastasis.

Cell adhesion and cell polarity are features that play an important role in the acinar architecture and function of the liver, especially for hepatocytes forming sheets and trabecules

and cholangiocytes forming ducts. Stable adhesive interactions and dynamic adhesive events occur in developing tissues and in regeneration and are responsible for assembling cells together, determining the overall architecture of the tissue. In case of parenchymal cell necrosis or partial hepatectomy the missing liver mass is restored by replication of existing hepatocytes, while in case of massive liver damage or disturbed regeneration (carcinogenesis, necrosis, hepatotoxins) the potential stem cell compartment differentiates during the so-called oval cell (in rat) - or ductular-reaction. Oval cells or hepatic progenitor cells are bipotential liver stem cells, which are able to differentiate into hepatocytes and bile duct epithelia.

During the multistep process of hepatocarcinogenesis, the accumulation of the genetic alterations leads to aberrant cell growth. Previously the monoclonal origin of hepatocellular carcinomas has been proved. Based on our current knowledge, besides the hepatocyte origin, hepatocellular carcinomas may be derived from hepatic progenitor cells, too. The majority of hepatocellular carcinomas contain cells which phenotypically resemble hepatic progenitor cells expressing AFP, CK7, CK19, OV-6 and CK14 markers. It is debated, whether these markers appear related to dedifferentiation of mature hepatocytes or maturation arrest of immature hepatic progenitor cells.

The precise origin of cholangiocarcinomas is unknown, however recent studies suggest the remarkable role of hepatic progenitor cells in this process besides the dedifferentiation of the mature cholangiocytes.

2. Aims

1. To examine the claudin-4 (CLDN4) expression profile in normal human biliary system (gallbladder, choledochal duct, hepatic ducts, intrahepatic bile ducts).

2. To examine the CLDN4 protein and RNA expression in human biliary tract cancers and hepatocellular carcinomas. To compare CLDN4 expression in the same types of tumors of different grades, to characterize the correlation between CLDN4 protein expression and the state of tumor differentiation.

3. To investigate the CK7, CK19, CK20 and HSA (HepPar1) expression in human biliary and hepatocellular carcinomas. To compare our result with previously published data.

4. To study the specificity and sensitivity of diagnostic markers used in the differential diagnosis of human biliary and hepatocellular carcinomas.

5. To examine the claudin-1, -2, -3, -4 and -7 expressions of oval cells, hepatocytes and cholangiocytes in rat liver regeneration model.

3. Materials and methods

Samples from 53 cases of surgically removed biliary tract cancers (BCs) and 50 cases of hepatocellular carcinomas (HCCs) were obtained with the permission of the Regional Ethical Committee. The BCs arose in the gallbladder, common bile duct, hepatic ducts and intrahepatic bile ducts. Ten normal livers and 10 normal, non-tumorous extrahepatic biliary tissues removed for other reasons served as controls. The surgically removed tumors were immediately transferred to the pathology department (within 10 minutes), where blocks were macrodissected and cut out by an experienced liver pathologist for histopathology, frozen sections, and further molecular biological studies.

All HE-stained slides were reviewed, a tissue block with the representative tumor was selected from each case, and an area of the tumor on the corresponding slide was encircled. In addition, non-neoplastic liver as well as biliary tissues were also included on the tissue arrays. The area of interest in the donor block was cored twice with a 2.0 mm diameter needle and transferred to a recipient paraffin block (with 24 holes, arranged in four columns and six rows).

Whole sections and tissue microarray slides were treated with primary antibodies against claudin-4, CK7, CK19, CK20 and HSA (HepPar1) after treatment with appropriate antigen retrieval.

For the detection of claudin-4 protein on representative samples further immunofluorescence examination and Western blot analysis were also carried out.

Seven BCs, 10 HCCs and 4 normal liver specimens were selected for real-time RT-PCR analysis. REST (relative expression software tool) Pair Wise Fixed Reallocation Randomization Test was used to compare each sample group.

Male Fischer F-344 rats were used to examine liver regeneration. Oval cells were induced by the method of the 2-acetylaminofluorene (AAF)/partial hepatectomy (PH). AAF was daily administered to rats by gavage at 5 mg/kg body weight for 6 consecutive days. On day 7, a standard two-thirds PH was carried out, and then the daily administration of AAF at the same dosage continued for 6 days. Claudin-1, -2, -3, 4-, -7, OV-6, CK7 and laminin immunofluorescence examinations were carried out on livers 12 days after the PH, when the oval cell proliferation was at the maximum. Sections were examined by confocal laser-scanning microscope.

4. Results

4.1. CLDN4 expression in normal liver, BCs and HCCs

The normal biliary epithelium expressed CLDN4 at a low level as detected by immunohistochemistry. Membranous staining was seen on the extrahepatic bile duct epithelium and larger intrahepatic bile ducts. More apical membranous localization could be detected in the interacinar portal bile ductules. The hepatocytes did not react with the CLDN4 antibody.

All 53 biliary tract cancers reacted with anti-CLDN4, showing typical strong intense membrane-bound positivity. There was no difference in CLDN4 expression among the BCs of different origin, namely intrahepatic-, extrahepatic or gallbladder carcinomas. The grade of differentiation did not influence the CLDN4 immunoreaction, both well- and poorly differentiated tumor cells expressed intensive reaction with anti-CLDN4. In contrast, none of the HCCs showed positive immunostaining for CLDN4.

Using Western blot analysis a strong immunoreactive band of CLDN4 at M_r 22 000 was observed only in case of bile duct tumors. CLDN4 could not be demonstrated in HCCs and normal liver tissues.

The relative expression level of mRNA evaluated by real-time RT-PCR revealed that the CLDN4 was significantly higher in BC specimens than in HCC specimens (48-fold increase, $p < 0{,}001$) using β-actin as a reference gene (Fig. 6). CLDN4 was also up-regulated (95-fold, $p < 0{,}001$) in the BC sample group as compared with the normal liver sample group.

4.2. CK, HSA expression in BCs and HCCs

Biliary tract cancers showed different cytokeratin expression pattern according the site of the tumor origin. CK7 expression was observed in 92%, CK19 in 83%, CK20 in 26% of BC cases. HSA was detected in 86% of HCCs, however 8% of the BCs showed focal positivity as well.

In HCC cases CK19 reactivity was absent, 34% of the cases showed CK7 focal positivity and the expression of CK20 was observed in only 4%. HSA was detected in 86% of HCCs.

4.3. Specificity and sensitivity

Sensitivity and specificity of immunoreactions detected in BCs were calculated as 100% and 100% for CLDN4, 92% and 66% for CK7, 83% and 100% for CK19, 26% and 96% for CK20 and 8% and 14% for HSA, respectively.

4.4. Claudin-1, -2, -3, -4 and -7 expressions in rat liver regeneration model

Double immunofluorescence staining of the above-mentioned claudins and of oval cell markers (CK7, OV-6) clearly showed that proliferating claudin positive cells corresponded to oval cells in all cases (the same staining pattern was seen).

Large number of oval cells with strong claudin-1 immunostaining was observed in the regenerating liver cells at day 12. Weaker claudin-1 reactivity was noted in mature hepatocytes and bile duct cells.

Distinct claudin-2 expression was seen in oval cells, while only very few cholangiocytes were positive. Hepatocytes showed similar claudin-2 expression – increasing from periportal to pericentral hepatocytes - to that in normal liver.

Strong claudin-3 expression was also detected in oval cells, hepatocytes and bile duct cells expressed claudin-3 at lower level.

Distinct CLDN4 immunoreactivity was present in oval cells. Bile duct cells showed similar positivity, but CLDN4 reactivity was absent in all hepatocytes.

Claudin-7 expression was very strong in oval cells. The expression of claudin -7 in bile duct cells was similar to that found in oval cells, while the protein expression in hepatocytes was slightly visible.

5. New conclusions

- *The normal biliary epithelium expressed CLDN4 uniformly at a low level, regardless of the site of origin within the biliary tree.*

The normal human biliary epithelium expressed CLDN4 at a low level as detected by immunohistochemistry, regardless of the site of origin (gallbladder, choledochal duct, hepatic ducts, intrahepatic bile ducts). Weak membranous staining was seen on the extrahepatic bile duct epithelium and larger intrahepatic bile ducts. More apical membranous localization could be detected in the interacinar portal bile ductules. The intensity of staining of CLDN4-expressing cells was compared with that of normal colonic mucosa.

- *Biliary tract cancers showed strong CLDN4 positivity at both protein and RNA levels, regardless of the site of origin within the biliary tree.*

All the 53 human BCs reacted with anti-CLDN4, showing typical strong intense membrane-bound positivity. Western-blotting and Real Time RT-PCR results confirmed our immunohistochemical findings. The grade of differentiation did not influence the CLDN4 immunoreaction, both well- and poorly differentiated tumor cells expressed intensive reaction with anti-CLDN4. Based on our results (100% specificity and 100% sensitivity), CLDN4 can be used as a marker for extra- and intrahepatic BCs.

- *In HCCs, similarly as in normal hepatocytes, CLDN4 was absent at both protein and RNA levels.*

No expression of CLDN4 was detected in HCCs. Similarly, CLDN4 was also absent in normal human hepatocytes. The absence of CLDN4 in mature hepatocytes and HCCs and its increased expression in biliary epithelium and BCs suggest that the pattern and composition of TJs are quite stable and characteristic in epithelial cells of the liver and extrahepatic biliary system. Loss of CLDN4 expression might be considered a marker of hepatocytic differentiation, similarly to the loss of CK7.

- *Based on our results, CLDN4 differentiates BCs from HCCs at both protein and mRNA levels.*

CK7, 19 and 20 are well accepted markers of bile ducts and the tumors derived from them. Our results show that the majority of BCs express CK7 and CK19, which corresponds to previous studies. However, some HCCs also express CK7. CK20 was detected both in BCs and HCCs.

One of the most widely applied antibodies used in the differential diagnosis of hepatocellular tumors is HSA (HepPar1). In our study, the majority of HCCs expressed HSA, however some BCs showed focal positivity as well.

Based on our results, CLDN4 can better differentiate BCs from as compared with cytokeratin pattern or HSA expression.

It is important to note that although CLDN4 expression differentiates BCs from HCCs in the liver, there are no data on CLDN4 expression in liver metastasis originating from adenocarcinomas of other tissues. Therefore, the presence of increased CLDN4 expression should be interpreted with caution, and further examinations are needed to obtain the correct diagnosis.

- *All the examined claudins showed increased expression in proliferating oval cells as compared with normal hepatocytes and cholangiocytes.*

We report a distinct claudin protein expression on the membrane of oval cells in the AAF/PH model. It emphasizes the importance of the changes in the interaction of certain adhesion molecules and formation of cell polarity during differentiation in the liver. Cells adhere to each other through specialized structures that create interfaces between compartments, which serve to regulate concentration gradients driving transcellular transport of solutes, ions and macromolecules, influence cell motility and maintain cell polarity. Hepatic oval cells should participate in cell rearrangements involving cell migration in order to move toward the terminal veins of the liver acini, exchanging neighbors throughout the process. The exact function of individual claudins is still not properly understood, however high expression of claudins in hepatic oval cells, which is reduced significantly in the mature hepatocytes, suggests an important role of these molecules in tissue remodeling and cell rearrangement.

- *The inverse correlation between progenitor cells, showing high expression of claudin-7 in our study, and mature hepatocytes with reduced expression focuses on the dynamic nature of protein interaction in the architecture and function of TJs.*

Our data, as well as those of other studies suggest that the increased or decreased expression or loss of the TJ protein, claudin-7, is quite a dynamic process, which can occur during both differentiation and carcinogenesis. The inverse correlation between the progenitor cells showing high expression of claudin-7 in our study and the differentiated hepatocytes showing reduced expression might reflect upon the occurrence of a similar process during differentiation in the liver, and focuses on the dynamic nature of protein interaction in the architecture and function of TJs. Our data support the opinion that the complex interactions of several proteins act not only as selective barriers, or as a fence, but participate in the control of cell growth and differentiation.

List of publications

Publications related to the theses

Lódi, C., E. Szabó, A. Holczbauer, E. Batmunkh, A. Szíjártó, P. Kupcsulik, I. Kovalszky, S. Paku, G. Illyés, A. Kiss, and Z. Schaff. 2006. Claudin-4 differentiates biliary tract cancers from hepatocellular carcinomas. *Mod Pathol.* 19:460-9. IF (2006): 3.753

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Batmunkh, E., **C. Lódi**, Á. Holczbauer, E. Szabó, P. Tátrai, C. Páska, A. Kiss, P. Kupcsulik, I. Kovalszky, and Z. Schaff. 2005. High expression of agrin in hepatocellular and cholangiocellular carcinoma. 40th Annual Meeting of the European Association for the Study of the Liver, Paris, France, April 13-17, 2005. *J Hepatol.* Supplement No.2 Volume 42.

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