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RESEARCH

Increased Expression of Claudin-1 and Claudin-7 in Liver Cirrhosis and Hepatocellular Carcinoma

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Abstract Claudins have been reported to be differentially regulated in malignancies and implicated in the process of carcinogenesis and tumor progression. Claudin-1 has been described as key factor in the entry of hepatitis C virus (HCV) into hepatocytes and as promoter of epithelial-mesenchymal transition in liver cells. The objective of the current study was to characterize claudin expression in hepatocellular carcinoma (HCC) as well as HCC-surrounding and normal liver samples with respect to cirrhosis and HCV infection. Expression of claudin-1, -2, -3, -4, and −7 was measured by morphometric analysis of immunohistochemistry, and Western blotting in 30 HCCs with 30 corresponding non-tumorous tissues and 6 normal livers. Claudin-1 and −7 protein expression was found significantly elevated in cirrhosis when compared with non-cirrhotic liver. HCCs developed in cirrhotic livers showed even higher expression of claudin-1 contrary to decreased claudin-7 expression when compared with cirrhosis. With reference to HCV status, HCCs or surrounding livers of HCV-infected samples did not show significant alterations in claudin expression when compared with HCV-negative specimens. Cirrhotic transformation associates with elevated claudin-1 and -7 expressions in both non-tumorous liver and HCC. The fact that no significant differences in claudin expression were found regarding HCV-positivity in our sample set suggests that HCV infection alone does not induce a major increase in the total amount of its entry co-factor claudin-1. Increased expression of claudin-1 seems to be a consequence of cirrhotic transformation and might contribute to a more effective HCV entry and malignant transformation.

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Introduction

Hepatocellular carcinoma (HCC) is one of the leading cancers in the world, ranking fifth in malignant morbidity and representing the third most frequent cause of cancer-related death [1]. The incidence of HCC is rising, especially in the Western population [2], which is explained by the increasing prevalence of hepatitis C virus (HCV) infection. Apparently, cirrhotic rearrangement of the liver parenchyma has central role in both processes, since cirrhotic transformation may facilitate hepatocarcinogenesis and potentiate HCV infection as well. Altered cell-cell and cell-extracellular matrix connections are supposed to play significant roles in these processes.

The tight junction (TJ) complex is a dynamic structure, with claudins constituting its backbone [3–5]. The TJ sensitively reacts to pathological conditions such as HCV infection and structural rearrangement of the liver parenchyma in cirrhosis. It is not clearly known, however, to which extent these structures are involved in the maintenance of the chronicity of viral infection and further, in the disturbed cell-cell connection of hepatocytes in cirrhosis and in the development of HCC. The actual composition of claudin strands and possible interactions between them might deeply influence the morphology of the TJ [4]; thus, the different tissue expression of claudins under pathological conditions might be responsible for, or reflect the altered function of the TJ.

Effective viral entry of HCV into target cells seems to be a determining factor in the maintenance of chronic infection. Besides CD81 [6] and scavenger receptor class B type 1 (SR-B1) [7], two TJ proteins—claudin-1 [8] and occludin [9]—have emerged as key entry co-factors. Association of claudin-1 with CD-81 seems to define HCV entry into hepatocytes [10], further, targeting claudin-1 appears to be a promising approach to prevent HCV infection [11, 12]. Interestingly, both junctional (i.e. apical) and non-junctional (i.e. basolateral) forms of claudin-1 have been implicated in the viral entry process [13, 14]. Moreover, two studies have suggested that HCV infection per se could increase claudin-1 expression, although Reynolds et al. presented in vitro results and only cirrhotic cases, while Mensa et al. investigated the rapid time course of graft HCV reinfection after liver transplantation [13, 14].

The potential of altered claudin expression in the development of cirrhosis and in the process of hepatocarcinogenesis has not been so far clearly understood. Our recent study revealed that HCC and both colorectal liver metastases and pancreatic cancer liver metastases dispose distinct claudin expression profiles, and thus claudins might contribute to carcinogenesis and metastasis formation [15]. Moreover, claudin-1 has the potential to confer malignant phenotype on HCC and normal liver cell lines [16], and seems to be directly involved in signaling pathways resulting in the induction of epithelial-mesenchymal transition in human liver cells [17].

The question arises whether and how claudin expressions are altered in HCV-related and -unrelated cirrhosis and HCC, and whether these changes are attributable to the development of cirrhosis or rather to viral infection. The aim of our study was to characterize the protein expression of claudin-1, -2, -3, -4 and -7 in normal and cirrhotic livers as well as HCCs with respect to cirrhosis and HCV infection.

Patients and Methods

Patient Material

Surgical resection specimens of 30 human hepatocellular carcinoma (HCC) cases with 30 corresponding non-tumorous surrounding liver samples were investigated in our study, twenty of which were already characterized in our recent publication to compare claudin expression profiles of HCC and colorectal and pancreatic liver metastases. The mean age was 62 years in HCC patients. There was a male predominance: 20/30.

Seventeen HCC patients were serologically HCV positive. In 11/30 cases, HCC developed on the basis of cirrhosis (score 5–6 according to the modified fibrosis/cirrhosis index [18]) and 11/30 showed chronic hepatitis (CH) with fibrosis (score 3 or 4) but no cirrhosis. A total of 6/17 HCV positive cases coincided with cirrhosis while 11/17 with CH and fibrosis. Five out of the thirteen HCV-negative HCC patients developed cirrhosis of alcoholic etiology, whereas the other patients of this group did not show CH or fibrosis. Six age-matched HCV-negative normal liver samples from individuals who

Table 1 Tissue material. Formalin-fixed, paraffin-embedded samples were used for the immunohistochemical analysis. Tumor tissue and surrounding parenchyma were always analyzed separately

	HCV negative cases (*n*)	HCV positive cases (*n*)	Total (*n*)
Normal Liver	6	–	6
HCC with non-cirrhotic liver	8	11	19
HCC with cirrhotic liver	5	6	11
Total (*n*)	19	17	36

HCV hepatitis C virus, *HCC* hepatocellular carcinoma

died in accidents were similarly processed and analyzed (Table 1).

The studied material was obtained with permission from the Regional Ethical Committee of the Semmelweis University (#172/2003, #137/2008, #35/2011) and with written consent from the patients. The patients were not subjected to chemo- or radiotherapy prior to surgery.

Histology and Immunohistochemistry

Tissue blocks were fixed in 10 % neutral-buffered formalin and embedded in paraffin. Three-four μm thick sections were stained with hematoxylin-eosin, picrosirius red, PAS and Shikata's orcein.

Immunohistochemical detection of claudin-1, -2, -3, -4, and -7 proteins was performed with Ventana ES automatic immunostainer (Ventana Medical Systems; Tucson, AZ), using monoclonal mouse antibodies against claudin-2 and -4, and polyclonal rabbit antibodies against claudin-1, -3, and -7 (Zymed; San Francisco, CA, USA) diluted 1:100, as described previously [15].

Morphometry and Statistical Analysis

The immunohistochemical reactions detecting claudins were photodocumented using light microscopy (200× magnification, Olympus BX microscope). Ten randomly selected areas were assessed, and stained area percentages were quantified using Leica QWin morphometrical software (Leica Microsystems Imaging Solutions Ltd., Cambridge, UK) as described previously [15]. Portal areas were measured separately to calculate the contribution of bile ducts to overall positivity.

Statistical analysis was carried out by STATISTICA software v8.0 (StatSoft; Tulsa, OK). To compare the expression of individual claudins in the different groups, non-parametric Kruskal-Wallis and Mann–Whitney tests were used.

Western Blotting

Five representative tumors and surrounding non-tumorous livers were cut out separately, then samples were snap-frozen in liquid nitrogen and stored at −80 °C until further analysis. Snap frozen samples were homogenized in lysis buffer (100 mM NaCl, 1 % np-40, 2 mM EDTA, 50 mM TRIS, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM PMSF, pH 7.5). Equal amounts of proteins were mixed with 2X Laemmli sample buffer, and after boiling and centrifugation the samples were separated on 10 % SDS-polyacrylamide gel and transferred to nitrocellulose membranes. The blots were incubated with anti-claudin-1, -2, -3, -4 and -7 (diluted 1:200) as primary antibodies overnight. Biotinylated secondary antibodies (diluted 1:2000, DAKO) were applied on the blots for one hour at room temperature. The signals were visualized by chemiluminescent detection (ECL, Amersham Biosciences, Piscataway, NJ, USA) and evaluated on ChemiDoc (Bio-Rad Laboratories, Hercules, CA, USA) gel documentation system [19].

Results

Qualitative and Quantitative Immunohistochemical Evaluation of Claudin Expression

Mean values of immunopositive area proportions are listed for each group in Tables 2 and 3. Since bile duct proliferations in cirrhosis are strongly claudin positive, we demonstrate morphometric results including and excluding portal tracts in order to reveal hepatocytic contribution to overall area positivity (Tables 2 and 3, respectively).

Claudin-1: Elevated Expression in Cirrhosis and HCC

Claudin-1 reaction invariably resulted in plasma membrane staining. In normal and non-tumorous livers, strong apical staining appeared on the biliary epithelial cells and weak to moderate staining at the junction lines of hepatocytes (Fig. 1a). Hepatocytes in cirrhotic nodules showed moderate to strong expression, and unlike on normal hepatocytes, the membranous staining often appeared on the entire circumference of the cells, though with uneven intensity (Fig. 1b). This was especially seen on the periphery of cirrhotic noduli. HCCs showed variable degree of immunostaining, with the majority of tumor cells exhibiting moderate to strong membranous reaction (Fig. 1c).

In quantitative terms, claudin-1 expression was significantly elevated in cirrhotic surrounding livers (cSL) relative to non-cirrhotic surrounding livers (ncSL). In 7 cirrhotic cases intense membrane staining was detected in dysplastic hepatocytic nests as well. All HCCs, including those formed in cirrhotic and non-cirrhotic livers, showed increased claudin-1 expression relative to NL and ncSL, yet the difference was significant only in case of cirrhosis-based HCCs (cHCC). cHCC reacted even stronger than cSL, which became more evident when excluding ductular reaction from the evaluation. Claudin-1 immunoreaction in cHCC was also stronger than in non-cirrhosis-based HCC (ncHCC).

The cirrhosis- and HCC-related differences in claudin-1 expression are summarized in Fig. 2. Although ductular reaction in cirrhosis contributed to a notable proportion of immunostaining, the cirrhosis-related increase in claudin-1 expression was found to be significant even with the omission of bile

Table 2 Morphometry results and statistical analysis including bile duct positivity

	Group	Area %	SEM	K-W test *p*-value	vs ncHCC *p*-value	vs cSL *p*-value	vs ncSL *p*-value	vs NL *p*-value
Claudin-1	cHCC	2.54	0.50	<0.001*	0.018*	1.000	<0.001*	0.017*
	ncHCC	0.77	0.20			0.088	1.000	1.000
	cSL	1.48	0.24				0.001*	0.060
	ncSL	0.37	0.08					1.000
	NL	0.40	0.15					
Claudin-2	cHCC	1.80	0.33	<0.001*	1.000	0.017*	0.038*	0.946
	ncHCC	1.41	0.29			<0.001*	<0.001*	0.115
	cSL	3.77	0.17				1.000	1.000
	ncSL	3.43	0.24					1.000
	NL	3.10	0.55					
Claudin-3	cHCC	0.41	0.09	0.238	0.981	1.000	1.000	1.000
	ncHCC	0.27	0.07			0.674	1.000	1.000
	cSL	0.39	0.07				1.000	1.000
	ncSL	0.27	0.04					1.000
	NL	0.25	0.07					
Claudin-4	cHCC	0.00	0.00	<0.001*	1.000	0.019*	<0.001*	0.001*
	ncHCC	0.00	0.00			0.005*	<0.001*	<0.001*
	cSL	0.03	0.004				1.000	1.000
	ncSL	0.02	0.003					1.000
	NL	0.01	0.003					
Claudin-7	cHCC	0.54	0.15	<0.001*	0.315	1.000	0.144	0.054
	ncHCC	0.24	0.06			0.002*	1.000	1.000
	cSL	1.00	0.18				0.001*	0.001*
	ncSL	0.23	0.05					1.000
	NL	0.11	0.05					

Mean and standard error values of morphometric analysis are listed. Statistical comparison of horizontal versus vertical groups was performed using the Kruskal-Wallis test. Significant differences ($p<0.05$) are marked by asterisks. *SEM* standard error of mean, *K-W test* Kruskal-Wallis test, *cHCC* cirrhotic hepatocellular carcinoma, *ncHCC* non cirrhotic hepatocellular carcinoma, *cSL* cirrhotic surrounding liver, ncSL non cirrhotic surrounding liver, *NL* normal liver

ducts. Moreover, the enhancing effect of cirrhosis was also transferred to cHCC.

Claudin-7: Elevated Expression in Cirrhosis and HCC

Claudin-7 immunohistochemistry resulted in weak membrane staining on hepatocytes and very strong membrane positivity on bile duct cells in normal livers (Fig. 1d). In 7 cirrhotic cases intense membrane staining was detected in dysplastic hepatocytic nests. Hepatocytes in cirrhotic nodules showed moderate to strong claudin-7 expression (Fig. 1e). HCC cells were also moderately stained; a clearly apical pattern in tumor cell pseudoacini is shown in Fig. 1f.

By morphometry, hepatocytic claudin-7 immunostaining (i.e. stained area without bile ducts) was found significantly elevated in cSL when compared with NL and ncSL. cHCC, in its turn, was also significantly superior to NL and ncSL, but stained weaker than cSL.

Cirrhosis- and HCC-related alterations of claudin-7 expression are illustrated in Fig. 3. It is of note that in case of claudin-7, too, the ductular reaction contributes to a relevant portion of the staining in cirrhosis.

Claudin-2: Unaltered Expression in Cirrhosis, Decreased Expression in HCC

Claudin-2 reaction gave granular membranous and cytoplasmic staining in both non-tumorous and tumorous cells. No significant quantitative alteration was measured in cSL compared with either NL or ncSL. As a contrast, claudin-2 expression was found decreased in all HCCs, with no difference between cHCC and ncHCC. Claudin-2 immunohistochemical reaction of the normal liver versus HCC is shown in Fig. 4.

Table 3 Morphometry results and statistical analysis excluding bile duct positivity (i.e. hepatocytic staining)

	Group	Area %	SEM	K-W test *p*-value	vs ncHCC *p*-value	vs cSL *p*-value	vs ncSL *p*-value	vs NL *p*-value
Claudin-1	cHCC	2.54	0.50	<0.001*	0.028*	1.000	<0.001*	0.028*
	ncHCC	0.77	0.20			1.000	0.631	1.000
	cSL	1.06	0.19				0.014*	0.584
	ncSL	0.27	0.06					1.000
	NL	0.38	0.14					
Claudin-2	cHCC	1.80	0.33	<0.001*	1.000	0.017*	0.037*	0.980
	ncHCC	1.41	0.29			<0.001*	<0.001*	0.121
	cSL	3.76	0.17				1.000	1.000
	ncSL	3.42	0.24					1.000
	NL	3.09	0.55					
Claudin-3	cHCC	0.41	0.09	0.280	0.902	1.000	1.000	1.000
	ncHCC	0.27	0.07			0.914	1.000	1.000
	cSL	0.36	0.06				1.000	1.000
	ncSL	0.25	0.04					1.000
	NL	0.24	0.07					
Claudin-4	cHCC	0.00	0.00	–				
	ncHCC	0.00	0.00					
	cSL	0.00	0.00					
	ncSL	0.00	0.00					
	NL	0.00	0.00					
Claudin-7	cHCC	0.54	0.15	<0.001*	0.252	1.000	0.036*	0.016*
	ncHCC	0.24	0.06			0.236	1.000	1.000
	cSL	0.73	0.19				0.033*	0.015*
	ncSL	0.16	0.03					1.000
	NL	0.09	0.04					

Mean and standard error values of morphometric analysis are listed. Statistical comparison of horizontal versus vertical groups was performed using the Kruskal-Wallis test. Significant differences ($p<0.05$) are marked by asterisks. *SEM* standard error of mean, *K-W test* Kruskal-Wallis test, *cHCC* cirrhotic hepatocellular carcinoma, *ncHCC* non cirrhotic hepatocellular carcinoma, *cSL* cirrhotic surrounding liver, ncSL non cirrhotic surrounding liver, *NL* normal liver

Claudin-3: Weak Expression Unaffected in Both Cirrhosis and HCC

Claudin-3 resulted in very weak membrane staining on hepatocytes, while bile ductular cells gave stronger reaction. Weak membranous staining was detected on HCC cells. Morphometry failed to reveal any significant differences between the investigated groups.

Claudin-4: Expression Confined to Bile Ducts and HCC Pseudoglandules

Normal hepatocytes were not stained for claudin-4, while the majority of normal bile ducts revealed weak to moderate membranous expression. Claudin-4 expression decreased in HCCs in comparison to normal and surrounding liver; however, this was clearly attributable to the lack of bile ducts in HCC. The majority of HCCs did not show any staining, whereas in 4/30 cases tumor cells forming alveolar, glandule-like structures exhibited weak membrane staining mainly localized at the apical pole (Fig. 5).

The Effect of HCV Infection on Claudin-1 and -7

When comparing HCV-positive and HCV-negative subgroups within the ncSL and cSL groups as well as within the ncHCC and cHCC groups, only tendencies in immunopositive area percentages were revealed by morphometry (Supplementary Fig. 1. and 2.).

The only significant difference was seen in claudin-7 area percentage between HCV– and HCV+ non-cirrhotic surrounding livers ($p=0.013$, Mann–Whitney test), but it is of note that all HCV infected cases were associated with fibrosis in this group, while HCV negative cases showed no fibrosis.

In cirrhosis, the observed tendencies might suggest a possible upregulation of claudin-1 and -7 in relation to HCV

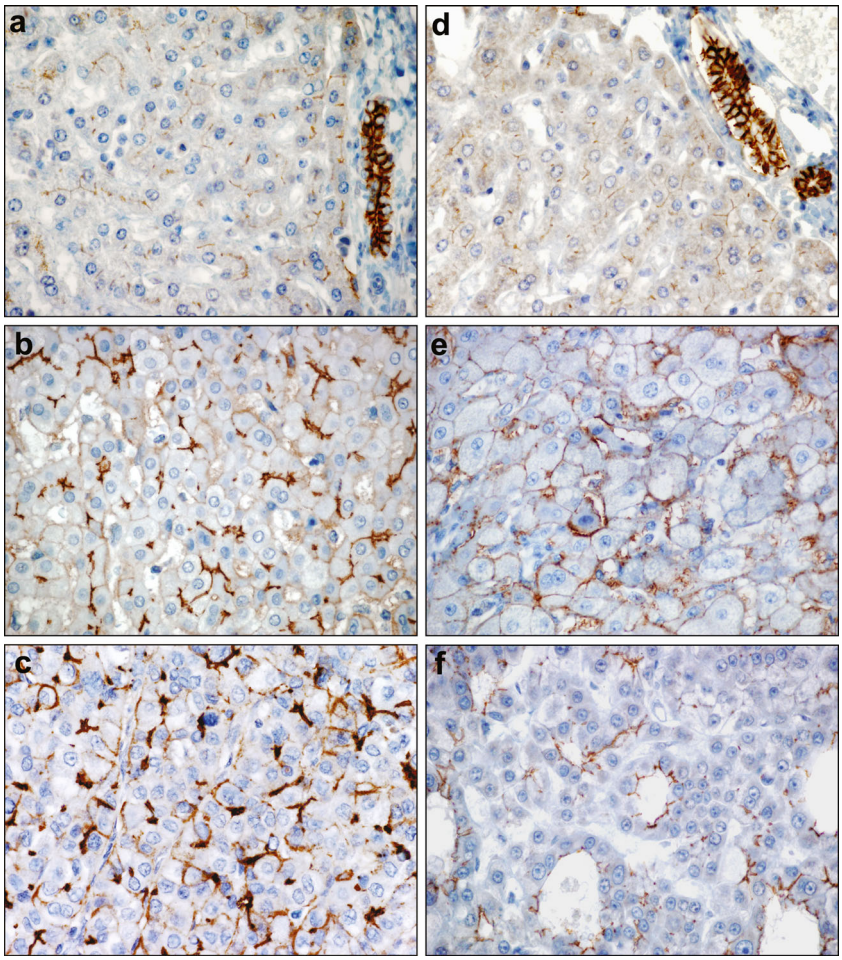

Fig. 1 Expression of claudin-1 and claudin-7 in normal liver, cirrhosis, and HCC. **a** Claudin-1 in normal liver; **d** Claudin-7 in normal liver. Strong expression was found in bile ducts (BD), while weak expression on hepatocytes (*arrow*). Original magnification: 600×. **b** Claudin-1 in cirrhosis; **e** Claudin-7 in cirrhosis. Strong expression on hepatocytes in cirrhotic nodules, occasionally extending to the basolateral surface of hepatocytes (*arrows*). Original magnification: 600×. **c** Claudin-1 in HCC that developed in cirrhotic liver. Even stronger expression of claudin-1 on tumor cells (*arrow*) as compared with cirrhosis. **f** claudin-7 in HCC that developed in cirrhotic liver. Claudin-7 shows weaker expression in HCC as compared with cirrhosis, mainly located at the apical surface of alveolar structures (*arrow*). Original magnification: 600×

infection, although the differences did not reach statistical significance. The fact that upregulation affects hepatocytes rather than bile ducts was supported by the fact that the differences became more accentuated with the exclusion of the portal tracts from the measurement.

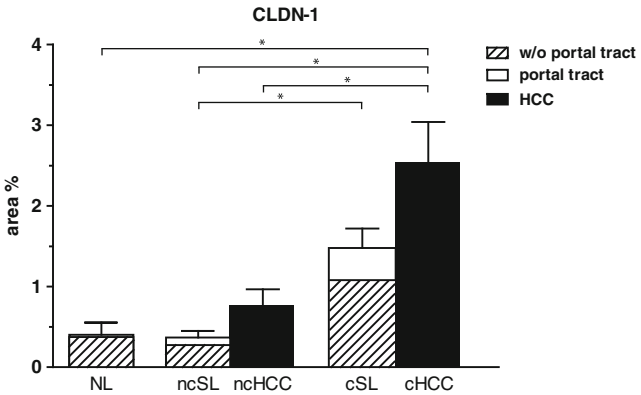

Fig. 2 Percentage of immunohistochemically stained area for claudin-1, including and excluding portal tracts. Significant differences between groups were the same with and without portal tract contribution. *Asterisk* indicates significant difference between two groups according to the Kruskal-Wallis test. (for exact *p* values see Tables 2 and 3). NL normal liver, ncSL non-cirrhotic surrounding liver, ncHCC hepatocellular carcinoma with non-cirrhotic surrounding liver, cSL cirrhotic surrounding liver, cHCC hepatocellular carcinoma with cirrhotic surrounding liver; w/o without

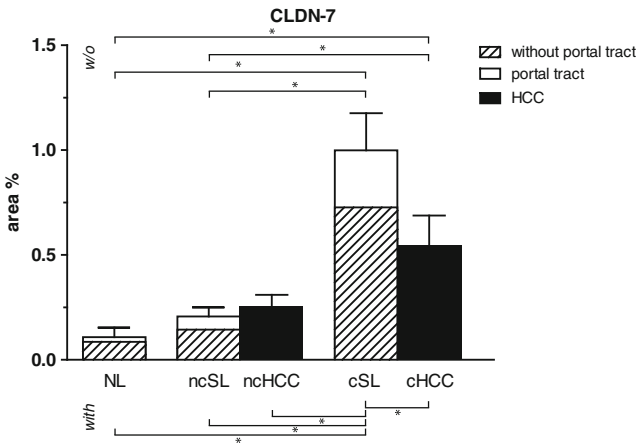

Fig. 3 Percentage of immunohistochemically stained area for claudin-7, including and excluding portal tracts. Significant differences between groups are presented including (below bars, *with*) and excluding (above bars, *w/o*) portal tract contribution. *Asterisk* indicates significant difference between two groups according to the Kruskal-Wallis test. (for exact *p* values see Tables 2 and 3). NL normal liver, ncSL non-cirrhotic surrounding liver, ncHCC hepatocellular carcinoma with non-cirrhotic surrounding liver, cSL cirrhotic surrounding liver, cHCC hepatocellular carcinoma with cirrhotic surrounding liver; w/o without

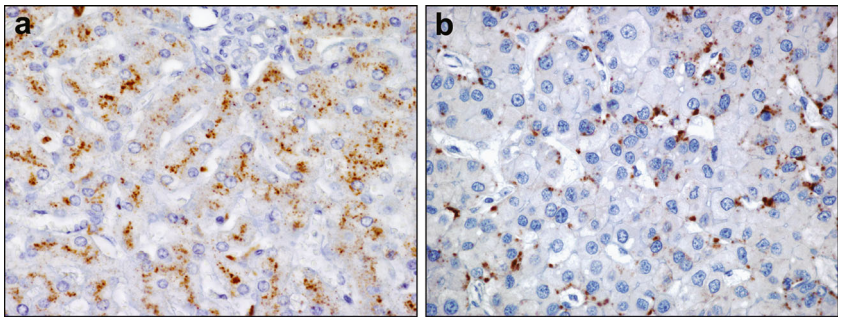

Fig. 4 Expression of claudin-2 in normal liver (**a**) and HCC (**b**). Decreased claudin-2 protein expression was found by immunohistochemistry in HCCs as compared with corresponding non-tumorous surrounding liver and normal liver

Western Blot Analysis of Claudin Expression

Western blot analysis (Fig. 6.) confirmed the data obtained by immunohistochemistry. The size of the detected claudins were accurate, 21, 23, 24, and 23 kDa for claudin-1, -2, -3, and -7, respectively, while claudin-4 was practically not detectable. Claudin-1 and claudin-7 expressions were elevated in cirrhosis compared with normal liver, and strong expression of claudin-1 was also detected in HCC. Claudin-2 signal was the weakest in the cHCC sample. Bands corresponding to claudin-3 were uniform across samples.

Discussion

Since chronic hepatitis plays an essential role in the development of cirrhosis and the profound regulatory alterations in cirrhosis may eventually precipitate in malignant transformation, HCV infection takes a major share in enhancing hepatocarcinogenesis. However, exactly how the development of cirrhosis promotes malignant transformation is largely unexplained. The impact of substantially altered liver architecture and rearranged intercellular connections between hepatocytes on TJ structure and composition has not been investigated in depth, although it was shown that claudins and other TJ components undergo dynamic changes in liver regeneration and in response to hepatotropic growth factors [20–22].

Several lines of research have focused on the role of adhesion molecules in tumor progression, especially in tumor invasion of breast [23, 24], pancreatic [25, 26], cervical [27] etc. cancers. Furthermore, several authors suggest that claudin-3 and -7 are specific markers of human hepatic stem cells which might be involved in regeneration-related neoplastic development in the liver [28–30]. The cell adhesion system is known to communicate with signal transduction pathways; thus, the complex junctional structures may be involved in the control of cell growth and differentiation [17, 31–33].

A recently revealed liver-specific function of claudins, equally relevant to hepatocarcinogenesis, is the co-receptor role of claudin-1 in a late step of HCV entry into hepatocytes [8]. Claudin-1 seems to exert its role in complex with other entry co-factors, which is supported by the fact that claudin-1 and CD81 do not only co-localize on the sinusoidal surface of the hepatocytes [14, 34], but define HCV entry as well [10]. Anti-claudin-1 antibodies were able to inhibit HCV infection by neutralizing this complex [11]. The prominent role of TJs in HCV internalization was further underlined with the recognition of occludin in the process [9]. However, occludin was not or just sparsely detected in HCC [35].

The chronic nature of HCV infection presumes the continuous effective viral entry into, and spread from hepatocytes. In our study, claudin-1 and -7 expressions were found increased in cirrhotic surrounding livers when compared with non-cirrhotic and normal livers. These observations are in line with Reynolds et al., who also found that claudin-1 expression was

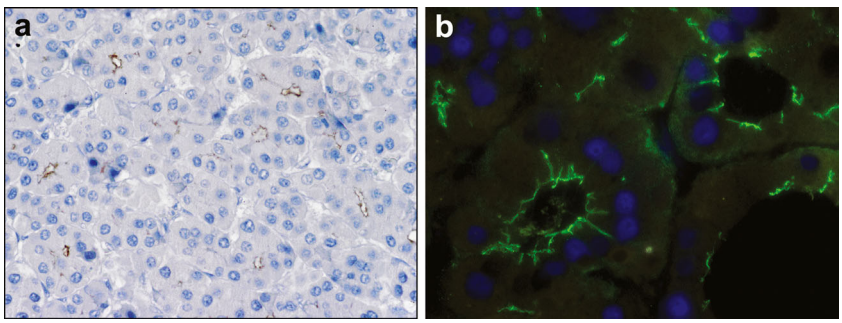

Fig. 5 Expression of claudin-4 in HCC. **a** Detection of CLDN-4 by immunohistochemistry. Original magnification: 600×. **b** Detection of CLDN-4 by immunofluorescence. Original magnification: 630×

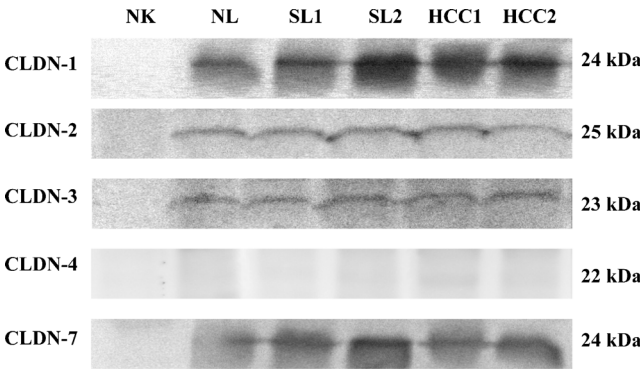

Fig. 6 Western blot analysis of HCC and surrounding liver for claudin-1, -2, -3, -4 and -7. CLDN: claudin; NK: negative control; NL: normal liver; SL1: non-cirrhotic surrounding liver, SL2: cirrhotic surrounding liver, HCC1: HCC with non-cirrhotic surrounding liver; HCC2: HCC with cirrhotic surrounding liver

increased in cirrhotic livers, with a more intense basolateral staining in HCV-positive cirrhotic cases [14]. Our immunohistochemical results also showed that claudin expression is not restricted to the apical pole of cells: both hepatocytes in cirrhotic nodules and HCC cells expressed claudins on their basolateral surfaces in our sample set. Similar findings regarding claudin-1 have been reported on squamous epithelium, where the molecule, besides TJs, is also located at the so-called "kissing points" of epithelial cells, a meshwork of serpiginously curved ridges reminiscent of, but not identical with, tight junctional ridges [36].

However, the in vitro data of Reynolds et al. on HCV infected Huh-7.5 cells, along with the results of Mensa et al., who found increased liver graft claudin-1 levels associated with HCV recurrence after liver transplantation[13], are suggestive of a direct HCV-induced upregulation of claudin-1 expression. In this regard, we did not find significant increase of claudin-1 staining in our HCV-infected sample set in comparison to HCV-negative samples neither in cirrhotic nor in non-cirrhotic or hepatocellular carcinoma groups. However, there was a tendency that in HCV-positive cirrhotic cases claudin-1 expression was higher in comparison to HCV-negative cirrhosis, as measured by morphometric analysis. Discordance with the above discussed literature might be attributable to different experimental designs and sample sets. Provided that claudin-1 facilitates HCV internalization, it may enhance the activity and severity of viral hepatitis. Hence, the elevated claudin-1 expression in HCV-related cirrhosis may at least in part account for a self-potentiating infection.

On the other hand, claudin-7 was significantly elevated in HCV infected non-cirrhotic livers compared to HCV-negative cases. Since there is no evidence of claudin-7 modulation by HCV infection so far, other factors, such as fibrosis—which was present in HCV-positive, but not in HCV-negative non-cirrhotic cases—might be associated with the observed elevation.

Alterations in claudin expression were also observed in HCCs. Tumors that developed in cirrhotic livers revealed increased expression of claudin-1 and -7 in comparison with the normal liver, but also with HCCs developed in non-cirrhotic livers. At the same time, claudin-2 expression in HCCs was found decreased in comparison to both corresponding surrounding and normal livers. The fact that HCCs developed in cirrhosis express both claudin-1 and claudin-7 stronger than non-cirrhosis-associated HCCs implies that these expressions might be 'inherited' from the originating cirrhotic liver. Thus, certain structural changes in TJs may begin well before malignant transformation takes place. Other changes, such as the downregulation of claudin-2 might foreshadow malignant transformation because this change was not anticipated in cirrhosis.

In our experience, claudin-1 expression at the protein level seemed to increase with malignant transformation, in contrast with claudin-7 protein expression that slightly dropped in HCCs compared to their surrounding livers. Interestingly, other authors found that poor differentiation and portal invasion of HCC correlated with the loss of claudin-1, while they also observed the preservation of claudin-1 in well-differentiated HCC [37]. Recently, Suh et al. suggested a direct involvement of claudin-1 in molecular pathways that contribute to tumor malignancy [17]. Overexpression of claudin-1 in HCC cells and even in normal liver cells induced epithelial-mesenchymal transition through activation of the c-Abl-ERK1/2 signaling pathway. Together with our findings on elevated claudin-1 expression in cirrhosis, this might be a novel mechanism by which cirrhotic transformation could potentiate malignant transition.

Previously, we reported that in contrast with the pronounced expression of claudin-4 in biliary tumors, this protein was not detected in HCCs by immunohistochemistry [19]. On a closer examination, however, a few HCCs in our new sample set showed weak and focal claudin-4 expression localized to acinary structures of HCC cells. That claudin-4 was markedly elevated in cirrhosis compared with non-cirrhotic and normal livers was clearly attributable to the increased number of bile ducts in cirrhosis. Conversely, the decreased expression of claudin-4 in HCCs as compared with normal livers and surrounding livers could be explained by the lack of bile ducts in HCC.

Taken together, our data indicate that cirrhotic transformation associates with elevated claudin-1 and -7 expression in both non-tumorous liver and HCC. Additionally, a marked yet statistically non-significant tendency of elevated claudin-1 and -7 levels in HCV-positive versus HCV-negative cirrhosis was detected. The lack of significant differences of claudin expression regarding HCV-positivity suggests that HCV infection alone does not induce a major increase in the total amount of claudin-1 in our samples. At the moment, there is no feasible explanation for the biological significance of

increased expression of claudin-7. Even if HCV infection alone fails to significantly alter claudin expression, the elevated levels of claudin-1 in cirrhosis may represent an indirect mechanism by which the effectivity of HCV entry is enhanced, thereby boosting HCV entry and chronicity of HCV infection, moreover claudin-1 might also play role in promoting carcinogenesis. This study adds further explanation to the expected effectivity of biological therapy with anti claudin-1 antibody.

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