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ORIGINAL PAPER

Expression of Matrilin-2 in Liver Cirrhosis and Hepatocellular Carcinoma

Erzsébet Szabó · Éva Korpos · Enkhjargal Batmunkh · Gábor Lotz · Ágnes Holczbauer · Ilona Kovalszky · Ferenc Deák · Ibolya Kiss · Zsuzsa Schaff · András Kiss

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Abstract The recently described matrilin protein family is part of the extracellular matrix, their pathophysiological role as well as distribution in liver diseases, however, have not yet been studied. Considering that matrilins have been found to play role in cell growth and tissue remodeling, their possible involvement in carcinogenesis has been raised. The main objective of this study was to investigate the changes in matrilin-2 expression which is one of the main components of basement membranes. Thirty-five cases of surgically resected hepatocellular carcinomas, 35 corresponding surrounding liver tissues and 10 normal liver samples were used for the study. In 15 of 35 cases the tumor developed on the basis of cirrhosis. Matrilin-2 protein expression was detected in normal liver around bile ducts, portal blood vessels, while sinusoids were negative by immunohistochemistry. Cirrhotic surrounding tissue showed intensive matrilin-2 staining along the sinusoids. Tumorous neovasculature was found strongly positive by immunohistochemistry. No differences, however, were detected by morphometry regarding the amount of protein expression based on the grade of hepatocellular carcinomas. Real-time RT-PCR did not show significant differences in matrilin-2 mRNA expression between normal, cirrhotic and tumor samples. This suggests posttranslational modification of matrilin-2 manifesting in altered distribution in liver fibrosis. Our data indicate that matrilin-2 is a novel basement membrane component in the liver, which is synthetised during sinusoidal "capillarization" in cirrhosis and in hepatocellular carcinoma. This is the first report to describe the expression and distribution of matrilin-2 in human normal and cirrhotic liver as well as in hepatocellular carcinoma.

Keywords Matrilin-2 · Basement membrane · Extracellular matrix · Hepatocellular carcinoma · Cirrhosis

Abbreviations
HCC hepatocellular carcinoma
ECM extracellular matrix

Introduction

Hepatocellular carcinoma (HCC) is one of the most malignant tumors and its incidence has increased in the Western world over the last decade [1, 2]. Malignant transformation of hepatocytes may occur in the context of chronic liver injury, regeneration and cirrhosis. More than 80% of HCCs are associated with cirrhosis, which is characterized by fibrous scarring and regenerating parenchyma during the process. HCC cells create a permissive soil by extracellular matrix (ECM) remodelling. Several studies have indicated that certain components of ECM have a double edged effect—promotion and suppression—on HCC, but the mechanism is indistinct [2].

E. Szabó · E. Batmunkh · G. Lotz · Á. Holczbauer · Z. Schaff (✉) · A. Kiss
2nd Department of Pathology,
Semmelweis University,
Üllői út 93,
H-1091 Budapest, Hungary
e-mail: schaff@korb2.sote.hu

É. Korpos · F. Deák · I. Kiss
Institute of Biochemistry, Biological Research Center of the Hungarian Academy of Sciences,
Szeged, Hungary

I. Kovalszky
1st Institute of Pathology and Experimental Cancer Research, Semmelweis University,
Budapest, Hungary

ECM is defined as a complex mixture of proteins, proteoglycans, and adhesive glycoproteins that provides structural and mechanical support to cells and tissues. The structural and regulatory proteins of the ECM cooperatively regulate a wide variety of cellular processes, both positively and negatively. These cellular events regulate physiological processes such as embryonic development, tissue morphogenesis, angiogenesis and probably also pathological processes such as transformation and metastasis [3–6]. The most important ECM proteins belong to the non-collagenous ECM. They play prominent role in cell-cell and cell-matrix connections. These molecules, e.g. fibronectin, vitronectin, laminin and tenascin show altered production during desmoplastic reaction [7].

The matrilins represent a recently discovered family of oligomeric non-collagenous ECM proteins [8, 9]. The matrilin family has four members that all share a structure made up of von Willebrand factor A domains, epidermal growth factor-like domains and a coiled-coil α-helical module [8, 10, 11]. Matrilin-1 (previously called cartilage matrix protein or CMP), and matrilin-3 are expressed mainly in cartilage [12, 13], while matrilin-2 and -4 occur in a wide variety of extracellular matrices. Matrilin-2 is the largest member of this family with a calculated molecular weight of 104,300 Da in mouse [14]. The mRNA of this protein has been detected in a variety of mouse organs including calvaria, uterus, heart and brain as well as fibroblast and osteoblast cell lines. Recent data indicate that matrilin-2 is an inherent component of all kinds of dense and loose connective tissue [8, 14], bone, cartilage, nervous system and in a variety of organs [11, 15]. However, there are only few studies regarding the expression and significance of matrilin-2 in human tissues [16].

The potential role of the matrilins in tumor development and progression has not been widely investigated. Matrilin-2 has been shown to appear in the early stage of cell differentiation during embryogenesis. It is the main component of the stroma and the basement membranes of many tissues [14, 15, 17]. We have recently shown that matrilin-2 is produced by hepatic oval cells in rats and deposited along the newly formed tubulo-trabecular structures in the periportal area [18]. According to our assumption, matrilin-2 has a possible putative role in the reorganization of the basement membrane during the "capillarization" of cirrhotic liver tissue together with other molecules mentioned above. Therefore, in the present study we examined the expression and localization of matrilin-2 in normal human liver tissues, liver cirrhosis and HCCs to evaluate its potential significance in these liver disorders.

Materials and Methods

Tissue Specimens and Tissue Preparation

A total of 35 surgically resected hepatocellular carcinomas (HCC) and 35 corresponding surrounding liver tissues were used for the study. The material was collected with the permission of the Regional Ethical Committee of the Semmelweis University (# 172/2003). Ten normal human liver samples were obtained from patients who died in accidents or from non liver related diseases. The absence of any pathological alterations was approved by pathologists (Zs. SCH; G. L., A. K.). The HCC cases included 19 well-, nine moderately and seven poorly differentiated tumors according to Edmonson–Steiner's classification corresponding to grade 1–3 (G1–G3). The male/female ratio was 27/8, the median age was 64.8 (44–82). In 15 of 35 cases HCC developed on the basis of cirrhosis corresponding to score 5 and 6 according to the modified staging described by Ishak et al. [19]. The other cases showed no or moderate fibrosis which were scored by 0–4. In 23 cases (13 HCCs with surrounding liver, 10 normal livers) the size of the specimen allowed detailed sampling for molecular biological analysis. Samples were snap frozen in liquid nitrogen or fixed in RNA later (Sigma, Saint Louis, Missouri, USA) and stored at −80°C until further analysis. For RNA isolation RNA later fixed material was used.

Histology

The surgically resected tumor samples with their surrounding nontumorous liver, as well as the samples from normal liver were fixed in 10% buffered (in PBS, pH 7,4) formalin for 24 h at room temperature, dehydrated in a series of ethanol and xylene and embedded in paraffin. The 3–4 μm thick sections were routinely stained with hematoxylin (Sigma H 3136) and eosin (Sigma E 4382; HE) and used to establish the diagnosis.

Immunohistochemistry

For immunohistochemistry the sections were washed in PBS buffer (pH 7.4) and treated with 3% hydrogen peroxide in water for 10 min to block endogenous peroxidase. For antigen retrieval the sections were digested by ready-to-use proteinase-K (DAKO, Carpenteria, CA, USA) for 10 minutes at room temperature. After incubation for 20 minutes with normal horse serum to block nonspecific binding of antibodies, the sections were incubated with rabbit polyclonal antiserum against matrilin-2 received from D. Piecha (University of Cologne, Germany) and diluted 1:150. The specificity of the anti-matrilin-2 serum was tested

in immunoblot and immunohistochemistry by Piecha and coworkers [14, 20]. After overnight incubation with primary antibodies at 4°C, the sections were incubated with the matching biotinylated secondary antibody (DAKO) for 30 min at room temperature. This step was followed by streptavidin-biotinylated peroxidase complex (ABC kit; Novocastra, Newcastle, UK). Signal amplification was enhanced by biotinylated-tyramine according to the protocol described by Merz and colleagues [21]. For all sections 3-amino-9-ethyl-carbazole (Biogenex HK 129-5K, San Ramon, USA) was used as chromogen and Mayer's hematoxylin as nuclear counterstain. Negative controls for nonspecific binding, incubated with secondary antibodies only, were processed and revealed no signal. Human skin tissue was used as positive control.

Morphometry

The immunohistochemical reactions detecting matrilin-2 were photodocumented using light microscopy (200× magnification, Olympus BX microscope). Ten randomly selected areas were assessed. Digital images showing immunoreactivity of matrilin-2 were quantified using Leica QWin Pro 3.1 software (Leica Microsystems Imaging Solutions Ltd., Cambridge, UK). Before making the measurements, the range of colors to be considered as positive was defined manually on the representative immunopositive areas of the image. Thereby, threshold levels were set for the red, green and blue components of the color of immunoreaction and these values were adjusted only to equalize for obvious intensity differences between the slides. Positive areas were defined as percentage of pixels above the threshold within the total area of the image.

The statistical analysis was carried out by GraphPad Prism software version 2.01 (GraphPad Software, Inc. San Diego, CA, USA). The Mann–Whitney *U* test was used to compare the expression of matrilin-2 in the different groups, p value <0.05 was accepted as being significant.

Western Blot Analysis

Snap frozen specimens of HCCs, surrounding cirrhotic, corresponding non-cirrhotic tissues and normal livers were used to obtain protein from tissues. Tissue samples of 0.3–1 g were homogenizated in lysis buffer consisting of 50 mM/l Tris-HCl (pH 7.6), 100 mM NaCl, 10 mM EDTA, 2 mM N-ethylmaleimide and 2 mM phenylmethylsulfonyl fluoride. After centrifugation at 1,000×*g* for 15 min, supernatants were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) without prior reduction. The protein concentration was measured and equal amounts of protein samples in equal volumes (30 µl) were loaded in SDS loading buffer [0.5 M/l Tris–HCl, (pH 6.8), 10% (*v*/*v*) glycerol, (0.5 *v*/*v*) bromophenol blue], then separated on 4–12% polyacrylamide gradient gels together with a prestained molecular weight marker (Rainbow-colored markers; Amersham Pharmacia Biotech, Little Chalfont, UK) and electrophoretically transferred to nitrocellulose membrane. Non specific binding was blocked with 5% (*w*/*v*) skim milk in TBS (pH 7.6). Blots were incubated with primary antibody against matrilin-2 overnight at 4°C. Specimens obtained from human kidney tissue samples were used as positive control. Blots were washed extensively and incubated with secondary horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma) diluted 1:1,000 in TBS (pH 7,6) containing 5%(*w*/*v*) skim milk. Bands were visualized by enhanced chemiluminescence (ECL, Amersham). The signals were finally evaluated on X-ray film.

Quantitative Real-Time RT-PCR

(a) RNA isolation from RNA later fixed samples

Thirteen HCCs (seven which developed in cirrhotic livers) with their surrounding liver parenchymas and 10 normal livers were analyzed. Liver samples were fixed in RNA later (Sigma) for 24 h then stored at −80°C. RNA was extracted with TRIZOL (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions [22]. Briefly, the 20 mg pieces of tissue were homogenized, and total RNA was extracted by chloroform and precipitated by isopropanol. The RNA pellet was washed once in 70% ethanol, dried and resuspended in 50 µl of RNase free water and kept at −80°C until further use. The integrity of total RNA was verified by gel electrophoresis.

(b) Reverse-transcription of RNA

An aliquot of total RNA, 1 µg (in 10 µl mix) was reverse transcribed with 2.5 units of M-MuLV reverse transcriptase (Applied Biosystems, Foster City, CA, USA) by using random hexamers for 10 min each at 25°C, 50°C, 42°C and 5 minutes at 95°C in the presence of RNase inhibitor (Applied Biosystem).

(c) Real-time RT-PCR

Real-time RT-PCR was performed by using matrilin mRNA specific primers. Specific real time PCR reactions to detect matrilin-2 and GAPDH were carried out with 2 µl cDNA template in a total volume of 25 µl, containing 1× Sybr Green PCR Master Mix (Applied Biosystems) with primers for MATN2 (GI: 62548859) forward 5′-GACGGA AGACGGTGCAAGAA-3′(20 bp), reverse 5′-CCAGTGA CAAACTGCTTCACGA-3′(22 bp), GAPDH (GI: 7669491)

forward 5′-CATGGGTGTGAACCATGAGAAGT-3′(23 bp) reverse 5′-TGGACTGTGGTCATGAGTCCTT-3′ 500 nM of each, using the ABI Prism 7000 sequence detection system (Applied Biosystems). After initial denaturation at 95°C for 10 s, 40 cycles were performed at 95°C for 20 s, at 63°C for 30 s and at 72°C for 30 s. Finally, melting analysis was performed from 55°C to 95°C to verify product homogeneity. In addition, the resulting real-time PCR products (10 μl) were run on a 2% agarose gel to ensure that a right size product was amplified in the reaction. Real-time RT-PCR reactions were carried out in duplicates for each sample.

Statistical Analysis of Real-time PCR Data

Evaluation of the data for relative quantification to reveal statistical differences between the groups to be compared was calculated with Relative Expression Software Tool (REST) by pairwise fixed reallocation randomisation test [23]. Relative quantification method was utilized for data analysis by using GAPDH as reference gene.

Results

Histopathology

All cases of well-differentiated HCCs (G1) possessed trabecular growth pattern with sinusoid-like spaces. The tumor cells showed variable degrees of pleiomorphism. The tumor cells in G2 HCC cases formed acinar structures. In poorly differentiated HCCs (G3), the tumor cells revealed a compact (solid) growth pattern with slit-like spaces for blood. Pleiomorphism of the tumor cells, including bizarre mononuclear and/or multinuclear giant cells, was prominent in many cases. No “rare” histological types of HCC as fibrolamellar HCC or hepatocholangiocarcinoma were included in the study.

In 15 of 35 HCC cases, cirrhosis was diagnosed in the surrounding liver. In the remaining 20 HCCs, mild to moderate fibrosis 19 was present in the surrounding liver, however, neither nodule formation nor significant bile ductular reaction were detected.

All normal liver specimens showed a regular architecture with normal-sized portal tracts, with no sign of fibrosis.

Immunohistochemistry

Normal and non-cirrhotic surrounding liver: Matrilin-2 was detected exclusively in the portal tracts (Fig. 1a). Basement membranes surrounding bile ducts and vessels reacted strongly for matrilin-2, however, no matrilin-2 expression was detected in the acini, along the sinusoids and terminal veins (Fig. 1a).

Cirrhotic surrounding liver: Intensive matrilin-2 protein expression was detected along the sinusoids in cirrhotic nodules and strong positivity was found around proliferating bile ducts and blood vessels in fibrous septa (Fig. 1b).

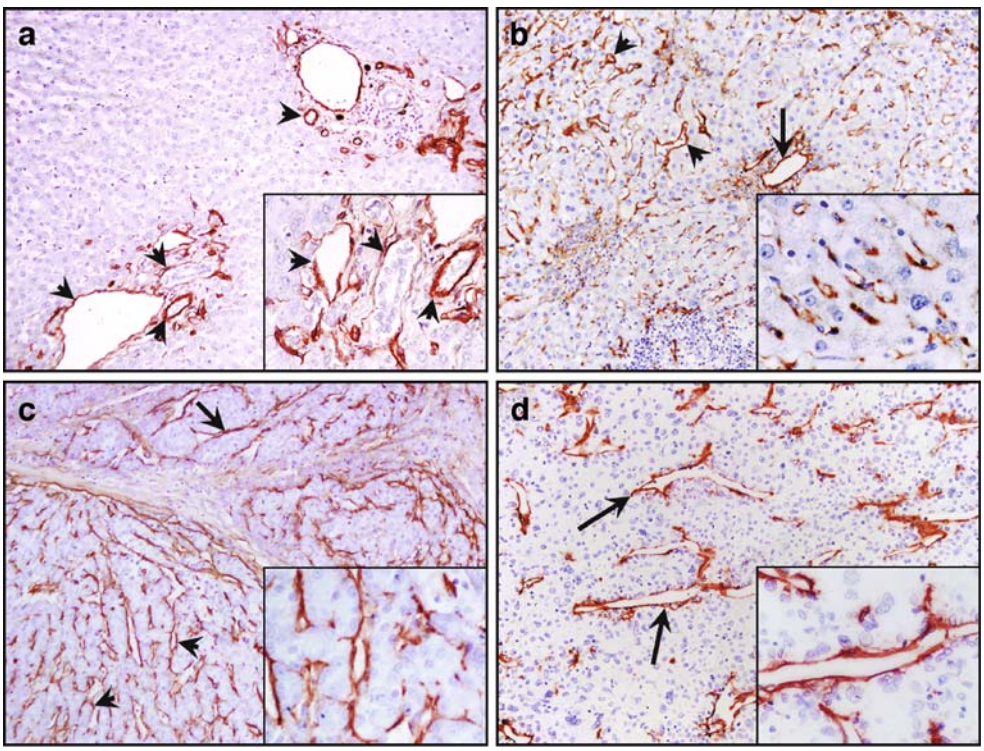

Fig. 1 Immunohistochemical detection of matrilin-2. **a** Normal liver. Matrilin-2 is expressed around the bile ducts (*arrowhead*) and blood vessels (*arrowhead*) in portal tracts. Sinusoids are mainly negative. **b** In cirrhotic nodules strong matrilin-2 reaction was detected in blood vessels (*arrow*) and along the sinusoids (*arrowhead*). **c**, **d** HCC shows intensive matrilin-2 expression along the neovascular formation in well- (*arrowhead*) and poorly (*arrow*) differentiated tumors. Heterogeneity can be detected within one tumor (**c**). Original magnification 200×, inset 600×, AEC

Hepatocellular carcinoma: There was no matrilin-2 present in intracellular localization, similarly to the observation in normal and surrounding liver. However, in all tumors, the staining pattern of matrilin-2 was different from that seen in normal liver tissue. Strong matrilin-2 expression was seen in HCC tissues, among the tumor cells mostly localized along the neovascular basement membrane (Fig. 1c,d). In well differentiated HCCs, the matrilin-2 followed the 1-3 cell- layer thick trabecules (Fig. 1c). Matrilin-2 surrounded the acinar formations of tumor cells of moderately differentiated cases. In poorly differentiated HCCs, larger groups of tumor cells were circumscribed by the positive reaction (Fig. 1c,d).

Quantitative analysis of the areas positive for matrilin-2 by immunohistochemistry resulted in the following mean values: 0.57% for normal livers, 0.59% for surrounding non-cirrhotic liver, 3.97% for surrounding cirrhotic livers, 4.26% for HCCs which developed on the basis of cirrhosis, 3.03% for HCCs without cirrhosis. These results show that significantly ($p<0.0001$) more matrilin-2 was detected in HCCs and cirrhotic livers as compared with the normal liver and non-cirrhotic surrounding liver (Fig. 2a). HCCs developing on the basis of cirrhosis did not show significantly higher amounts of matrilin-2 when compared with HCCs developing in non-cirrhotic livers. Further, in well- and moderately differentiated hepatocellular carcinomas the percentage of immunopositive areas revealed no significant differences when compared with poorly differentiated cases (Fig. 2b). Immunohistochemistry resulted in the following mean values: 3.78% for G1, 4.62% for G2 and 4.33% for G3 HCCs.

Western Blotting Analysis of Matrilin-2

Western blot analysis confirmed the presence of monomer matrilin-2 in normal livers, in HCCs and cirrhotic and non-cirrhotic tissues around HCCs. The immunoreactive band migrated faster than 97 kDa size marker band. This mobility value matches the size of matrilin-2 monomer, proteolytically cleaved close to the COOH end. The matrilin-2 oligomers represented a minor fraction and were slightly visible in the kidney (positive control) extracts and the liver samples. In each case the expression of matrilin-2 monomer was strong and there were no major differences in the intensity of the bands (Fig. 3).

Real-time RT-PCR Analysis of Matrilin-2 mRNA Expression

Matrilin-2 mRNA expression was analyzed by real-time reverse transcriptase based PCR. The melting analysis and gel electrophoresis of real-time RT-PCR products approved the specificity of the reaction. Matrilin-2 mRNA expression was slightly decreased in all nontumorous (cirrhotic and non-cirrhotic) surrounding liver specimens as well as in HCCs, as compared with the normal liver tissue (range, 1.21–1.91 fold). HCCs which developed on the basis of cirrhosis revealed higher matrilin-2 mRNA expression when compared with HCCs in non-cirrhotic livers (1.34-fold). Overall, these differences were not significant after statistical analysis.

Discussion

Determining the function of matrilins is a permanent challenge. Our knowledge on matrilins is still limited. Previously, matrilin-2—a recently discovered basement membrane component—was demonstrated in the ECM of different murine tissues, including kidney, heart, lung, esophagus [14]. Matrilin-2 has been shown to be present in the basement membrane of vessels, nerves, muscles, and

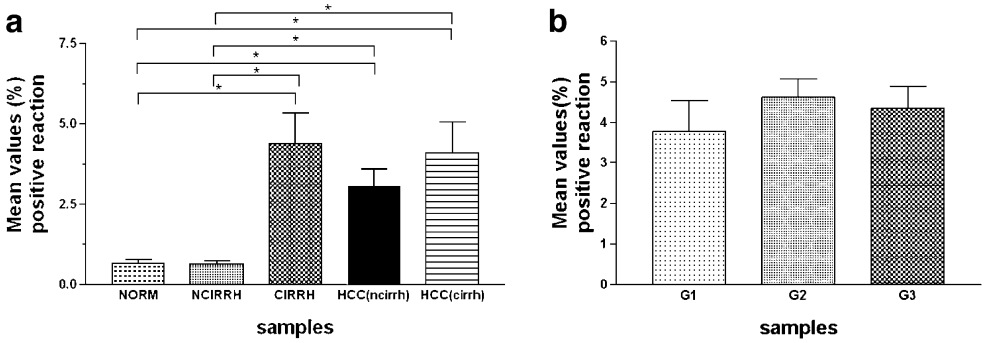

Fig. 2 Diagram illustrating the difference in matrilin-2 expression detected by immunohistochemistry and evaluated by Leica Qwin Pro 3.1 software. **a** Comparison of normal liver, cirrhotic liver groups and HCC. The mean values of cirrhotic and HCC groups were significantly higher as compared with the normal liver. The mean percentage of the area positive for matrilin-2 immunostaining in HCC samples was not significantly higher when compared with the cirrhotic surrounding liver. *NORM* normal liver, *NCIRRH* surrounding nontumorous, non-cirrhotic liver, *CIRRH* surrounding nontumorous cirrhotic liver, *HCC (ncirrh)* HCC developed in non-cirrhotic liver, *HCC (cirrh)* hepatocellullar carcinoma in cirrhotic liver, *asterisk* marks significant differences. **b** Comparison of HCC groups of different grades. There were no significant differences between the well- (G1), moderately (G2) and poorly differentiated (G3) HCC groups

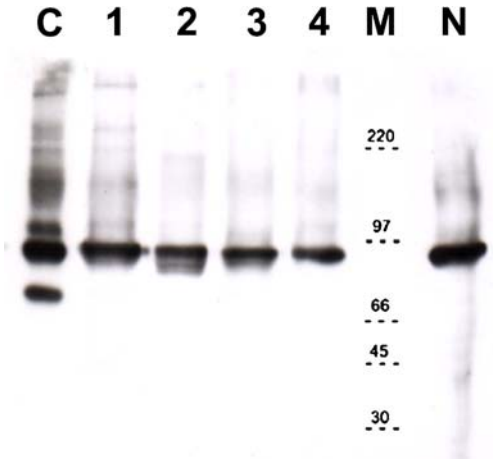

Fig. 3 Comparison of matrilin-2 protein content of liver samples by immunoblot analysis. Extracts from normal liver (*N*), HCC (*1*), corresponding surrounding liver without cirrhosis (*3*), HCC developed on the basis of cirrhosis (*2*), corresponding nontumorous liver (*4*), and human kidney tissue as positive control (*C*) were submitted to electrophoresis in SDS-polyacrylamide gels without prior reduction. Immunoblot analysis of parallel samples showed a specifically stained band at cca. 95 kDa. (*M*) molecular weight marker, size markers (in kDa) are indicated

sweat glands [14]. Matrilin-2 protein expression was elevated in the majority of human sporadic pilocytic astrocytoma (PA), therefore suggesting that matrilin-2 may be a specific and clinically useful biomarker for discriminating the indolent, clinically aggressive forms of this brain tumor [24].

Several basement membrane components and ECM proteins have already been described as having important roles in fibrogenesis and the pathogenesis of different tumor types, including HCC. For example, strong and homogeneous deposition of collagen type IV and laminin in sinusoids and overexpression of integrins by sinusoidal cells was described in cirrhosis and HCCs [25]. Until now, no data have been published on the expression of matrilin-2 in the human liver, including HCC. The present study therefore aimed at detecting the expression of matrilin-2 in normal liver, liver cirrhosis and HCC.

In our study, HCCs showed strong matrilin-2 immunoreaction along the neovasculature of tumors and the sinusoids in cirrhotic liver. In contrast, the positive reaction for matrilin-2 in normal liver was detected only in portal tracts: around portal veins, hepatic arteries, while terminal veins and sinusoids were negative. Our findings are in agreement with the observation that matrilin-2 is predominantly present in the basement membrane of other tissues [16]. Our immunohistochemical results indicate that matrilin-2 protein expression is significantly higher in HCCs and cirrhotic tissues as compared with normal and non-cirrhotic surrounding livers.

Western blot analysis confirmed the presence of matrilin-2 protein detected by immunohistochemistry as a strong immunoreactive band of about 95 kDa representing the monomeric form of the protein. The higher molecular weight matrilin-2 oligomers represented a minor fraction and were hardly visible or even missing. Our previous study on rat liver also showed that multiple electrophoretic bands resulting from proteolytic processing of the homo-oligomers are not detectable in the liver [18]. It is yet to be determined whether the absence of oligomers is a result of intensive proteolytic processing in the liver or another explanation is likely for this phenomenon. Western blot analysis, however, did not find major differences in matrilin-2 protein expression between normal liver, cirrhosis and HCC. The similar intensity of matrilin-2 bands in different samples can be explained by inappropriate solubilisation of the protein from cirrhotic and tumorous basement membranes in comparison with normal and non-cirrhotic liver samples. Another explanation for the discrepancy between immunohistochemistry and Western blot analysis, however, is that portal areas contain much higher amounts of matrilin-2 than the "increased" amount in the newly formed basement membranes along the sinusoids in cirrhosis and HCC. Immunohistochemistry reveals the differences in the distribution of matrilin-2 rather than providing exact data on the total amount of the protein. Both mRNA measurement and Western blot analysis use extracts from the whole tissue, thus higher amounts of portal tract components in the case of normal liver might "compensate" the lower amount of matrilin-2 expression in the finer (thinner) vascular elements in the cirrhotic and HCC cases.

Compared with normal liver HCC samples displayed lower, but not significantly lower, mRNA expression. This might reflect the higher rate at its utilisation in cirrhosis and HCC and might also suggest that posttranslational modification plays a significant role in matrix protein expression.

Earlier studies have shown that matrilin-2 has been implicated in the interaction of extracellular matrix components and cells by binding to collagens, proteoglycans, laminins, fibronectins and other members of ECM molecules and it also plays prominent role in the extracellular assemblies of ECM components as well as in the formation of collagen network [17]. This function of matrilin-2 may be relied upon the presence of vWFA-domain, which contributes to its macromolecular interaction with other matrix proteins [14]. Matrilin-2, together with interacting proteins, participate in the formation of collagen-associated filamentous networks in the reorganization of ECM during "capillarization" supporting macromolecular assembly of collagens. However, the role of this anchorage and the underlying molecular interactions are not exactly known [26].

In our previous study we detected the appearance of matrilin-2 in the basement membranes around the components of the portal triad in normal rat liver as well as in ductular formation during oval cell induced liver regeneration [18]. This finding also supports its possible function among the essential structural constituents in the high density basal membrane of the liver. This proposed function of matrilin-2 protein is similar and comparable to the earlier described function of a similar molecule, nidogen [27]. Nidogen is a probable key molecule during the formation and differentiation of bile ducts because of its co-localization with laminin, which suggests that they function as anchorage for hepatoblasts and hepatocytes. As matrilin-2 is supposed to be in complexes with these molecules it might maintain and strengthen the stability of this anchorage with nidogen. Similar to the described role of agrin [28, 29], matrilin might play a role in the vascular proliferation of cirrhosis and may promote tumor progression by supporting stromal cell growth.

Our findings confirm previous observations detecting matrilin-2 almost in all structures containing basement membranes [16] and emphasize the difference between the portal triad and sinusoids.

In conclusion, matrilin-2 expression is limited to the portal tract in normal liver, significantly increased in HCC and cirrhotic liver tissues, suggesting that matrilin-2 is involved in the reorganization of tissue architecture in cirrhosis and HCC. The increased expression of matrilin-2 in cirrhosis and HCC might reflect the altered function of ECM in the newly formed structural arrangement.

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