Laminin-5 Chains Are Expressed Differentially in Metastatic and Nonmetastatic Hepatocellular Carcinoma

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ABSTRACT

Purpose: The purpose of this work was to study the expression of the extracellular matrix protein laminin-5 (Ln-5) in hepatocellular carcinoma (HCC), which is the fifth most frequent cancer and the third most common cause of tumor-related death in the world. The occurrence of metastasis is the main problem in HCC patients. Ln-5 is an extracellular matrix component that promotes adhesion and migration; it is present at the basement membrane and has recently been associated with cancer metastasis. Although Ln-5 has been shown to promote motility and scatter of rat liver cells, it has never been found in the liver.

Experimental Design: We studied the expression and localization of the α3, β3, and γ2 chains of Ln-5 in 40 HCC patients. We analyzed tissue samples collected from the HCC primary nodule and from peritumoral and metastatic tissues. The presence of Ln-5 was investigated by immunohistochemistry, reverse transcription-PCR, and Northern blot analysis. The clinical outcome of the patients was evaluated over a 4-year follow-up period.

Results: This study provides the first report that Ln-5 is present in the HCC primary nodule, but not in normal or peritumoral cirrhotic tissues. In particular, the γ2 chain is strongly associated with the occurrence of metastasis (96%; P < 0.001) and with worse prognosis. In peritumoral tissues, Ln-5 has been detected along the advancing edge of the metastatic nodule.

Conclusions: Ln-5 is associated with a more metastatic phenotype of HCC, and its detection could be an important finding both as an unfavorable prognostic factor and as a diagnostic marker for detecting micrometastasis in peritumoral tissues.

INTRODUCTION

HCC is the most frequent epithelial cancer of the liver, the fifth most frequent cancer in the world, and the third most common cause of tumor-related death (1). Its frequency is estimated to be at risk of constant growth in upcoming years, particularly in Mediterranean countries, likely because of the spread of hepatitis C virus and hepatitis B virus infections (2). In these countries as well as in North America, the occurrence of HCC represents the last stage in the natural history of chronic virus-related liver disease.

Our group and others (1, 3, 4) have reported that in HCC, the most reliable and important prognostic factor is the occurrence of metastasis, which severely affects survival. In the liver, cancer cells do not lie on a BM structure, as other epithelial tumor cells do, but live in trabecular structures surrounded by a tissue enriched by ECM proteins secreted as a consequence of the underlying cirrhosis (5). Therefore, HCC cells need to penetrate through these ECM tissue boundaries to spread to surrounding or distant sites. However, the mechanisms underlying this cellular invasiveness are not fully understood. In various epithelial malignancies, the interaction of cancer cells with ECM components such as Ln-5 has been reported to play an important role (6, 7).

Ln-5 is a heterotrimeric glycosylated protein that belongs to the Ln family and is formed by α3β3γ2 chains assembled with disulfide bonds that are the product of three different genes named LAMA3, LAMB3, and LAMC2, respectively (8). It is a main component of the BM structure, where it promotes different functions such as adhesion or migration, depending on whether or not it has been proteolytically remodeled by MMPs such as MMP-2 and/or MT1-MMP (9, 10). In particular, the γ2 chain of Ln-5 has been detected at the advancing edge of several tumors, and a number of reports suggest its involvement in the spread and metastatic activities of cancer cells (11–14).

Ln-5 is widely expressed in the human body (15), but it has never been described in the liver, although one of the groups that first isolated the protein (referred to as ladsin) reported that a Buffalo rat liver cell line efficiently scatters in the presence of Ln-5 (16). However, no studies have thus far investigated the expression of Ln-5 in the liver under pathological conditions such as HCC or the expression of all of the chains in cancer tissues in the same patient.

In this study, we investigated the expression of Ln-5 in...
normal, peritumoral, and HCC liver tissues and correlate the presence/absence of Ln-5 with the occurrence of metastasis and with the patients’ clinical outcome.

MATERIALS AND METHODS

Patients. We studied tissues obtained by means of surgical resection or fine-needle biopsy from 40 patients with HCC. The patients were considered to have metastatic or nonmetastatic disease based on a number of criteria such as histological observation of micronodules at the periphery of the HCC, vascular invasion, or computed tomography scan findings (17). Following these criteria, we classified the disease as metastatic (28 patients) and nonmetastatic (12 patients); the clinical features have already been reported (17). As a control, we studied five subjects with normal liver who underwent surgery for trauma. The study was performed in accordance with the Helsinki Declaration, and informed written consent was obtained from all patients before surgery or biopsy.

The presence of Ln-5 in normal and peritumoral tissues was determined by investigating the presence of each Ln-5 chain in all of the tissue specimens using immunohistochemistry, whereas in the primary lesions of 20 HCC patients (14 patients with metastatic disease and 6 patients with nonmetastatic disease), the presence of Ln-5 was also confirmed by RT-PCR and Northern blot analysis.

Immediately after collection, the liver tissues of normal, HCC primary lesion, and peritumoral type, obtained by surgery and/or fine-needle biopsy, were cut into two parts: the main portion was processed for routine histology to determine the histological diagnosis and the presence of micrometastasis in the peritumoral tissues; whereas the remaining portion was immediately snap frozen in liquid nitrogen.

Immunohistochemistry. Frozen tissues were included in OCT 4583 embedding compound (Miles Laboratories, Inc., Naperville, IL), and indirect alkaline phosphatase was performed as described previously on serial 5-µm-thick sections (18). Sections were incubated with the following monoclonal primary antibodies: BM2 and 6F12 directed against α3 and β3 chains, respectively, were a gift of B. Burgeson (Charlestown, MA); and D4B5 and GB3 directed against the γ2 chain were purchased from Chemicon (Temecula, CA) and a gift of C. Meneguzzi (Nice, France), respectively. As negative controls, sections were processed under the same experimental conditions but with the secondary antibody only. Reactions were developed using red fuchsin as chromogen and abundantly washed for 20 min. Finally, randomly chosen microscopic fields were captured, and positive staining was defined according to an image analysis software assisted system (Lucia; Nikon Corp.), as reported previously (19).

RNA Extraction and RT-PCR Analysis. Total RNA was extracted from ground frozen tissues (60–80 mg) mechanically homogenized according to the manufacturer’s instructions for the RNeasy minikit (Qiagen, Valencia, CA), and 2 µg of total RNA were reverse-transcribed using the RETRO-script (Ambion, Austin, TX) according to the manufacturer’s instructions.

Table 1  Expression of the Ln-5 chains in HCC patients with and without metastasis

<table>
<thead>
<tr>
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<th>Metastatic HCC (28 patients)</th>
<th>Nonmetastatic HCC (12 patients)</th>
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<td>N (%)</td>
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<tr>
<td>α</td>
<td>9 (32.1)</td>
<td>3 (25)</td>
<td>0.725</td>
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<tr>
<td>β</td>
<td>9 (32.1)</td>
<td>3 (25)</td>
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<td>γ</td>
<td>24 (85.7)</td>
<td>1 (8.3)</td>
<td>&lt;0.001</td>
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Northern Blot Analysis. Thirty µg of total RNAs were denatured and electrophoresed in a 1% agarose gel containing formaldehyde. The gel was blotted to a Hybond-N+ nylon membrane (Amersham, Buckinghamshire, United Kingdom) by capillary action using 20× SSC and prehybridized in 0.25 M NaCl, 7% SDS, 1 mM EDTA, 0.25 M sodium phosphate (pH 7.0), 150 mg/ml salmon sperm DNA, and 50% formamide at 42°C. The LAMC2 probe was generated by RT-PCR amplification using the RNA of the HT1080 human fibrosarcoma cell line as template, and the product was purified according to the manufacturer’s instructions using the Microcon PCR column (Millipore, Bedford, MA); this purified product was quantified by spectrophotometry. The probe spans positions 2412–2724 on the GenBank sequence NM008485 and was labeled by random priming using a commercially available kit (Amersham) and [32P]dCTP (specific activity, 3000 Ci/mmol). The probe (106 cpm/ml) was added to 7 ml of prehybridization solution, and the blot was hybridized overnight at 42°C. The membrane was washed once in 2× SSC, 0.1% SDS at room temperature for 5 min; washed once in the same buffer at 55°C for 30 min; and washed in 1× SSC, 0.01% SDS at 55°C for an additional 30 min. Once dry, the membrane was exposed to a Kodak X-OMAT film with intensifying screens at ~70°C.

PCR analysis was performed as described previously (20). PCR products were resolved on 1.6% agarose gels stained with ethidium bromide, and the digital images were captured and quantified using the software image analysis program Image Master 1D Prime (Pharmacia Biotech). LAM3, LAMB3, and LAMC2 gene expression was measured in comparison with glyceraldehyde-3-phosphate dehydrogenase expression. The oligonucleotides used as primer for amplification were purchased from Genset (Genset SA, Paris, France). All primers were tested for specificity using the Blast program available at the National Center for Biotechnology Information web site. Primer sequences and product size are as follows: (a) for glyceraldehyde-3-phosphate dehydrogenase, 5’-TCACCATCCTCCAGGAGCGAGA-3’ and 5’-CTTCTGGGTGCAGTGATG-3’ with a product of 337 bp; (b) for LAMA3, 5’-ACATCTCTGTCTTGTTTCTT-3’ and 5’-CAAGGCTCCACTTCAGTTGTG-3’ with a product of 531 bp; (c) for LAMB3, 5’-CGGAGATCTGCTTCGTCTAGCCCAT-3’ and 5’-GGAATTCTCACAACGCA-2412 to 2724 on GenBank sequence NM008485 and was labeled by random priming using a commercially available kit (Amersham) and [32P]dCTP (specific activity, 3000 Ci/mmol). The probe (106 cpm/ml) was added to 7 ml of prehybridization solution, and the blot was hybridized overnight at 42°C. The membrane was washed once in 2× SSC, 0.1% SDS at room temperature for 5 min; washed once in the same buffer at 55°C for 30 min; and washed in 1× SSC, 0.01% SDS at 55°C for an additional 30 min. Once dry, the membrane was exposed to a Kodak X-OMAT film with intensifying screens at ~70°C.

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**Statistical Analysis.** Fisher’s exact test was used to determine the statistical significance of the association between each Ln-5 chain and the presence of metastasis in HCC patients. Student’s test was used to determine the 99% confidence intervals for the survival of HCC patients with or without expression of the Ln-5 γ2 chain in the primary lesion tissues.

**RESULTS**

**Ln-5 Expression in Normal and HCC Tissue.** The immunohistochemical staining was defined as positive or negative based on accurate microscopic observation, although in some cases a very weak pink staining could be observed as a background. Therefore, all sections were further examined with image analysis software (see “Materials and Methods”), so that the background staining constituted a cutoff for the determination of positive and negative results (19).

Extensive investigation of the expression and localization of Ln-5 in HCC, peritumoral, and normal liver tissues on serial sections showed that in all of the tissues of both metastatic and nonmetastatic HCC patients, Ln-5 was distributed along the BM of the blood vessels, as confirmed by the overlapping distribution of the staining of α3, β3, and γ2 chains. A similar pattern was observed in the normal liver tissues, where blood vessels were stained in the portal space (data not shown).

On the contrary, the distribution of Ln-5 in the liver parenchyma showed a striking difference among normal, peritumoral, and tumoral liver tissues and between the tissues of metastatic and nonmetastatic HCC samples. In particular, in the normal tissues, none of the Ln-5 chain was stained in the parenchymal area (data not shown), in agreement with other studies (15). In the tissues of HCC patients, Ln-5 chains were stained differentially according to the presence or absence of metastasis. As shown in Table 1, the percentage of Ln-5 chain expression varied depending on the chain. Positive staining of the γ2 chain, which was the most frequently observed chain, was found in 25 of 40 patients (62.5%) whereas in the same patients, α3 and β3 chains were positive in only 12 of 40 patients (30%). Interestingly, the γ2 chain was present in 24 of 28 (85.7%) patients with metastatic HCC but only in 1 of 12 patients (8.3%) with nonmetastatic HCC. Staining for the γ2 chain was confirmed using two different monoclonal antibodies (D4B5 and GB3), both of which yielded similar results.

Furthermore, 15 of 28 patients with a metastatic HCC were positive for the γ2 chain but not for the α3 or β3 Ln-5 chains,

![Fig. 1 Differential expression of Ln-5 chains in the primary lesion tissue of a patient with a metastatic HCC. a–c show positive staining for γ2 in serial sections but negativity for the α3 and β3 Ln-5 chains in the primary lesion of a patient with a metastatic HCC (scale bar, 40 μm). d–f show higher magnification of a different microscopic field in the same patient. The γ2 chain is present in the parenchyma, in the cytoplasm of HCC cells, and also in the extracellular space, whereas expression of the α3 and β3 chains is limited to the BM of the blood vessels (scale bar, 20 μm).](image-url)
suggesting that Ln-5 chains could be differentially expressed. In particular, the association between the γ2 chain and the presence of metastasis in HCC patients was statistically significant (P < 0.001), as calculated with Fisher’s exact test, whereas the presence of the α and β chain was not associated with the presence or absence of metastasis (P = 0.725).

To determine the potential role of positive staining for the Ln-5 γ2 chain, we compared the histological diagnosis of micrometastasis in the peritumoral tissue with the staining in the HCC primary tissue. As shown in Table 1, the HCC patients with metastatic nodules in the peritumoral tissue (as documented by histology) showed positive staining for the Ln-5 γ2 chain in 24 of 28 (85.7%) cases. These results suggest that positive staining for the Ln-5 γ2 chain in the primary nodule might facilitate the identification of metastatic HCC nodules.

Fig. 1 shows a representative case of a patient with a metastatic HCC, where the γ2 chain was strongly expressed in the parenchyma of the HCC primary tissue, whereas in serial sections, α3 and β3 were virtually negative. The Ln-5 γ2 staining appeared diffuse in the HCC tissues (unevenly spotted with a “leopard skin” pattern). However, the α3 and β3 chains, when present, appeared colocalized with a distribution and intensity similar to those of γ2, as shown in Fig. 2.

The differential expression pattern of Ln-5 chains between HCC patients with and without metastasis was confirmed by investigating LAMA3, LAMB3, and LAMC2 gene expression by RT-PCR analysis in the primary lesion tissues of 20 of the 40 HCC patients (14 patients with metastasis and 6 patients without metastasis). As shown in Fig. 3, 5 of 14 patients with metastatic HCC expressed α3, β3, and γ2 chains, whereas the remaining 9 of 14 patients were positive for the γ2 chain only. Among the patients with nonmetastatic HCC, two of six patients were positive for α3 and β3 but negative for the γ2 chain, whereas the remaining four of six patients were negative for all of the chains tested.

The differential expression of the Ln-5 γ2 chain in metastatic and nonmetastatic HCC tissues was further confirmed by Northern blot analysis in the same patients studied by RT-PCR. As a positive control, we used RNA harvested from the HT1080 cell line, which is known to express high levels of the Ln-5 γ2 chain (21, 22). As shown in Fig. 4, the Ln-5 γ2 chain mRNA was strongly expressed in all of the primary nodule tissues of the patients with metastatic HCC but was absent in that of patients without metastasis. Overall, the results of the RT-PCR and Northern blot analysis were consistent with the immunohistochemical staining, showing differential expression of Ln-5 chains in the primary lesion tissues of metastatic and nonmetastatic HCC and, moreover, preferential expression of the Ln-5 γ2 chain in the tissues of patients with metastatic HCC.

Ln-5 Expression in Metastatic and Nonmetastatic Peritumoral Tissues. The peritumoral tissues were investigated with immunohistochemistry and processed under the same experimental conditions as those used for the HCC tissues. As in the controls, in the peritumoral tissues of patients with nonmetastatic HCC, Ln-5 staining appeared to be confined to the BM of blood vessels present in the portal space and in the fibrotic tissues and was absent in the parenchyma. On the contrary, in the peritumoral tissues of patients with metastatic HCC, in which the presence of micrometastasis was documented by routine histology (data not shown), Ln-5 was also localized along tumor nodules (Fig. 5). In these tissues, the Ln-5 chains were stained with the same expression pattern as that observed in the tissue of the HCC primary lesions in the same patient. Furthermore, Ln-5 was absent in the metastatic nodules of the patients (4 of 28 patients) whose primary HCC did not show any positive staining for Ln-5. Fig. 5 shows a sample of peritumoral tissue with a metastatic HCC nodule featuring positive staining.
for all of the Ln-5 chains, expressed and codistributed along the advancing edge of the invasive tissue, whereas the remaining peritumoral surrounding tissue was completely negative.

Ln-5 γ2 Chain Correlation with HCC Patient Survival.
We retrospectively analyzed the survival of HCC patients over a 4-year period, according to the presence or absence of the Ln-5 γ2 chain. As shown in Fig. 6, survival was significantly shorter in the HCC patients with a detectable Ln-5 γ2 chain compared with those in whom it was undetectable (18.0 ± 8.4 versus 32.1 ± 10.0 months; P < 0.01). This result suggests a potential role of the Ln-5 γ2 chain as a prognostic marker in patients with HCC.

DISCUSSION
In Mediterranean countries as well as in North America, HCC usually develops on cirrhotic livers, and its frequency is constantly increasing with the spread of the hepatitis C virus infection (1, 2). The occurrence of metastasis in the course of HCC is the most serious problem because it severely affects prognosis and survival (1, 3). Many studies have recently reported that Ln-5 is implicated in cancer invasion and metastasis, although its role is still unknown (6, 7).

This is the first study to demonstrate the presence of Ln-5 in the liver adopting three different experimental approaches; the presence of Ln-5 was found to be strongly associated with a more aggressive and invasive HCC phenotype. We base our conclusions on the following observations: (a) Ln-5 was absent in normal and cirrhotic liver but present in the HCC tissues, with differences in expression among the Ln-5 chains; (b) in the HCC primary lesions, the Ln-5 γ2 chain was present in 85.7% versus 8.3% of the patients with and without metastasis, respectively; (c) in the peritumoral specimens, Ln-5 staining was confined to the advancing edge of the metastatic tissues; and (d) survival was shorter in the HCC patients expressing the Ln-5 γ2 chain in the primary lesions than in those without the chain.

Ln-5 is an ECM molecule that promotes motility and migration of different epithelial cells after their cleavage by MMP-2 and/or MT1-MMP (9, 23); both enzymes are strongly implicated in cancer invasion and metastasis (24–26). On this basis, many studies have suggested that this molecule may be involved in a number of different malignancies, although the mechanism is still unclear (6, 7).

Ln-5 is widely expressed in human tissues, but although it has been reported to promote migration and scattering of rat...
liver cells, it has never before been detected in the liver (15, 16). Our study showing the presence of Ln-5 in liver cancer is only in apparent contrast with previous reports, because all of the studies reported thus far have investigated the presence of Ln-5 in normal but not in cancerous liver (15, 27). Consistent with other studies showing differential regulation of the \( LAMA3 \), \( LAMB3 \), and \( LAMC2 \) genes by cytokines (28), the differential expression among the Ln-5 chains in the HCC tissues reported here could be caused by the altered cytokine network that occurs in HCC patients.

The finding that the Ln-5 \( \gamma2 \) chain is concentrated, in some cases together with the other Ln-5 chains, at the advancing edge of HCC metastatic nodules in the peritumoral tissue is in agreement with other reports showing overexpression of the Ln-5 \( \gamma2 \) chain together with MMPs along the invasive front of different highly metastatic cancer tissues of the colon, breast, and stomach as well as melanoma (10–13, 29). In these situations, it has been proposed that proteolytic cleavage of the \( \gamma2 \) chain of Ln-5 could facilitate crossing of the BM and spread into the adjacent tissues by cancer cells (9).

In the liver, Ln-5 and in particular, the \( \gamma2 \) chain is expressed only in HCC tissues and not in cirrhotic peritumoral liver, despite the accumulation of ECM components (5). We have previously shown that “invasive” but not “noninvasive” HCC cell lines secrete high levels of MMP-2 and MT1-MMP, express \( \alpha_5 \) integrin, and efficiently migrate on Ln-5 cleaved by MMPs (18). Therefore, we cannot rule out the possibility that in this case, too, MMPs cleave the Ln-5 \( \gamma2 \) chain and thus trigger HCC invasiveness. This is consistent with our description in a previous study of increased levels of MMP-2 and MT1-MMP in patients with metastatic HCC (17).

Another possibility is that HCC cell migration and invasion are supported by the presence of a migratory element located in the \( LAMC2 \) gene, as suggested by other studies (30). In any case, the strong correlation (85.7%) between the presence of the Ln-5 \( \gamma2 \) chain and the occurrence of metastasis in HCC patients tends to confirm its implication in the invasion process.

Another intriguing possibility is that Ln-5 \( \gamma2 \) is not functionally implicated in HCC invasiveness. If this is the case, then it could represent a downstream effect of the activation of the \( LAMC2 \) gene by various oncoproteins such as \( \beta \)-catenin in synergism with the hepatocyte growth factor (31) that is known to be increased in HCC patients (32). In several malignancies, overexpression of the \( \gamma2 \) chain has been reported in the cancers as compared with normal tissues (12, 33). Thus, Ln-5 and, in particular, the \( \gamma2 \) chain could represent a marker of HCC invasion, serving to detect the presence of micrometastasis, as
already proposed for other epithelial cancers (14, 34). The fact that Ln-5 is synthesized “de novo” in HCC because it is absent in normal and pathological liver tissues further emphasizes its role as a possible tumor-associated antigen and hence as a prognostic factor. In fact, our data are consistent with another study showing that Ln-5 γ2 chain-positive patients with squamous cell carcinoma of the tongue exhibit poor prognosis (35).

In conclusion, our data suggest that Ln-5 is expressed in HCC tissues and also that the presence of the γ2 chain is correlated with worse prognosis. In these circumstances, Ln-5 could play a role in favoring metastatic diffusion due to proteolytic remodeling of the γ2 chain and/or as a protein stimulating cell motility. In addition, detection of the Ln-5 γ2 chain could be a helpful tool for use as a prognostic marker to identify the presence of micrometastasis in surrounding tissues. More studies are needed to better characterize the role of this protein in HCC patients.

REFERENCES
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