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Abstract

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KEYWORDS: type IV collagen, collagen-degrading enzyme, hepatocellular carcinoma, tumor invasion

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Type IV Collagen-Degrading Enzyme Activity in Hepatocellular Carcinoma

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Type IV collagen-degrading enzyme activity was measured in liver homogenate obtained from 10 patients with hepatocellular carcinomas. Type IV collagen, the enzyme substrate, was extracted from human placenta with pepsin digestion, and labeled with [1-14C] acetic anhydride. The homogenate was preincubated with p-aminophenylmercuric acetate to activate the latent form of the enzyme, and then the enzyme activity was measured at pH 7.5 by adding a substrate mixture. Referring to previous reports, the enzyme measured seemed to be a neutral metalloprotease. The enzyme activity of the homogenate was markedly reduced by omitting the p-aminophenylmercuric acetate pretreatment, indicating that the enzyme was present mainly in the latent form. The activity seemed to be higher in the peripheral portion of hepatocellular carcinoma than in the center. Further the activity was found to be the highest in a hepatocellular carcinoma patient with many metastatic nodules in the lung. The results might suggest that type IV collagen-degrading enzyme participates in tumor invasion and intrahepatic or remote metastasis.

Key words: type IV collagen, collagen-degrading enzyme, hepatocellular carcinoma, tumor invasion

Invasion and metastasis are the most embarrassing problems in malignant tumors. During growth, invasion and metastasis of neoplasma, some proteases may play very important roles in degrading and dissolving the surrounding interstitial matrices, which consist mainly of collagens and other proteins at the tumor penetration front (1). Invasion of malignant tumor cells into the vessels is the starting point of metastasis, and extravasation of these cells is an important requirement for establishment of both hematogenous and lymphogenous metastases.

Hepatocellular carcinoma (HCC), which is commonly accompanied with liver cirrhosis, invades the surrounding fibrotic tissue as it grows. Nakashima et al. (2) found by investigating many autopsied cases that HCC frequently invaded the portal (64.7%) and major hepatic veins (23.3%), and that HCC metastasized (64%) via hematogenous (56%) and lymphogenous (27%) routes to other organs.

Type I and III collagens are the main component of the interstitial matrices of cirrhotic liver (3). Tissue collagenase (EC 3. 4. 24. 7), which cleaves type I, II, and III collagens at a specific site of the molecule, does not degrade type IV collagen (4). Type IV collagen is the main structural component of basement membrane and is digested by some proteases (5, 6) besides tissue collagenase. When HCC grows, the surrounding type I and III collagens may be dissolved by tissue collagenase allowing the tumor to invade the circumferential tissues. On the occasion of intrahepatic or distant metastasis through the portal or hepatic vein,
the basement membrane of the vessel wall is
degraded mainly by type IV collagen-degrading
enzyme.

In the present study, type IV collagen-
degrading enzyme activity was investigated
in cases of HCC to elucidate the relation-
ship between this enzyme activity and the
potential of tumor invasion or metastasis.

Materials and Methods

Cases. Livers were obtained from 9 patients
with HCC within 6 to 10 h of death and from 8
control patients with the following diseases: rupture
of aortic aneurysma (2 cases), lower pharyngeal
cancer (1 case), lung cancer (2 cases) and schizo-
phrenic malignant syndrome (3 cases). All control
patients had neither liver disease nor metastasis.
Liver tissue was also obtained within 2 h after
hepatic resection from a patient with HCC. These
specimens were obtained from Okayama University
Hospital, Okayama Red Cross Hospital and Oka-
yama Saiseikai General Hospital, from January
1984 to September 1985. Schizophrenic cases
were obtained from Okayama Prefectural Hospital.

Clinical features of 10 cases with HCC are de-
scribed in Table 1. Cases MY, TI, and KK suffered
from hepatic failure. The cause of death of TS,
MS, KM and FT was gastrointestinal bleeding due
to gastric ulcers and erosions, and that of Case
GI was rupture of HCC. Macroscopic and histol-
agic gradings of HCCs were performed according to
Nakashima et al. (2) and Edmondson et al. (7),
respectively. Case TK was classified in grade IV.
Cases MY, TS, TI, and KM in grade III, and Cases
KK, MS, GI, FT and YM in grade II. Metastases
to the lungs were demonstrated semi-quantitatively.
Cases TS, KK, KM and YM had no metastasis to
the lungs, and other cases besides Cases TK, GI
and FT showed a few metastatic nodules in their
lungs. Case TK revealed an expanding type of
HCC with a partial capsule, and histologic find-
gings of this tumor indicated Edmondson-Steiner’s grade
IV. The patient’s lungs were violated by multiple
metastases which thereafter induced respiratory
failure.

Preparation of substrate. Type IV collagen
was purified according to the method of Sage et
al. (6). All following procedures were carried out
at 4°C. Human placentas, from which the
amnion and chorion were removed, were minced
and washed sequentially with water, 50 mM Tris-

Table 1 Clinical features of the patients with hepatocellular carcinoma

<table>
<thead>
<tr>
<th>Case</th>
<th>Age</th>
<th>Sex</th>
<th>HBS</th>
<th>Alcohol*</th>
<th>AFP</th>
<th>CEA</th>
<th>LC</th>
<th>Cause of death</th>
<th>Gross type</th>
<th>Tumor size</th>
<th>Edmondson’s grade</th>
<th>Metastasis to the lung</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autopsied cases</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TK 66 F</td>
<td>56</td>
<td>-/-</td>
<td>-</td>
<td>24600</td>
<td>5</td>
<td>+</td>
<td>RF</td>
<td>Expanding (±)</td>
<td>7.5 x 4.5</td>
<td>IV (+III)</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>MY 59 M</td>
<td>59</td>
<td>-/-</td>
<td>-</td>
<td>16400</td>
<td>2</td>
<td>+</td>
<td>HF</td>
<td>Spreading (-)</td>
<td>13.2 x 9.3</td>
<td>III (+IV)</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>TS 68 M</td>
<td>68</td>
<td>-/-</td>
<td>±</td>
<td>62400</td>
<td>40</td>
<td>+</td>
<td>GI-B</td>
<td>Expanding (-)</td>
<td>8.5 x 7.5</td>
<td>III</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>TI 51 M</td>
<td>51</td>
<td>-/-</td>
<td>+</td>
<td>1</td>
<td>2</td>
<td>+</td>
<td>HF</td>
<td>Expanding (+)</td>
<td>8.2 x 5.3</td>
<td>III</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>KM 56 M</td>
<td>56</td>
<td>-/-</td>
<td>++</td>
<td>ND</td>
<td>2</td>
<td>+</td>
<td>GI-B</td>
<td>Expanding (-)</td>
<td>4.0 x 4.0</td>
<td>III</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>KK 73 M</td>
<td>73</td>
<td>+/-</td>
<td>±</td>
<td>534</td>
<td>ND</td>
<td>+</td>
<td>HF</td>
<td>Expanding (-)</td>
<td>2.0 x 3.0</td>
<td>II</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>MS 50 M</td>
<td>50</td>
<td>-/+</td>
<td>+</td>
<td>32300</td>
<td>1</td>
<td>-</td>
<td>GI-B</td>
<td>Multinodular</td>
<td>2.0 x 3.0</td>
<td>II</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>GI 71 M</td>
<td>71</td>
<td>-/+</td>
<td>+</td>
<td>2230</td>
<td>ND</td>
<td>+</td>
<td>Rupture of HCC</td>
<td>Expanding (-)</td>
<td>14.0 x 10.0</td>
<td>II</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>FT 53 M</td>
<td>53</td>
<td>+/-</td>
<td>±</td>
<td>43000</td>
<td>ND</td>
<td>+</td>
<td>GI-B</td>
<td>Diffuse</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surgically resected case</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YM 56 M</td>
<td>56</td>
<td>-/-</td>
<td>-</td>
<td>17</td>
<td>5</td>
<td>-</td>
<td>alive</td>
<td>Expanding (+)</td>
<td>5.0 x 3.5</td>
<td>II</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

a: patient did not drink; ±, sometimes drank small amounts; +, drank about 80 g ethanol daily over 10 years;
+++, drank more than "+" patients.
b: Liver cirrhosis.
c: RF, respiratory failure; HF, hepatic failure; GI-B, gastrointestinal bleeding.
d: -, negative; +, a few metastatic nodules; ++++, multiple.
e: Not detected.
HCl buffer (pH 7.5) and 0.5 M acetic acid. The placentas were thoroughly digested for 24 h with pepsin in a pepsin to placenta weight ratio of 1 to 400. After several steps of salt precipitation under acidic and neutral conditions, type IV collagen was purified. The purified collagen was lyophilized and kept at −70°C until use.

**Amino acid analysis of type IV collagen.** Samples for amino acid analysis were hydrolyzed at 105°C for 22 h with 6 M HCl in sealed and nitrogen-flushed tubes. An amino acid analyzer (JLC-6AH analyzer: Nihpondenshi Co.) was used for analysis with a double-column program. The amino acid composition of purified type IV collagen is described in Table 2. High ratios of hydroxyproline to proline and hydroxylysine to lysine, and low contents of alanine and arginine were demonstrated.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Residues/1000 amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>43</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>113</td>
</tr>
<tr>
<td>Threonine</td>
<td>27</td>
</tr>
<tr>
<td>Serine</td>
<td>35</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>83</td>
</tr>
<tr>
<td>Glycine</td>
<td>321</td>
</tr>
<tr>
<td>Alanine</td>
<td>37</td>
</tr>
<tr>
<td>Proline</td>
<td>98</td>
</tr>
<tr>
<td>Valine</td>
<td>26</td>
</tr>
<tr>
<td>Methionine</td>
<td>4</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>31</td>
</tr>
<tr>
<td>Leucine</td>
<td>49</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>7</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>27</td>
</tr>
<tr>
<td>Hydroxylysine</td>
<td>52</td>
</tr>
<tr>
<td>Lysine</td>
<td>8</td>
</tr>
<tr>
<td>Histidine</td>
<td>8</td>
</tr>
<tr>
<td>Arginine</td>
<td>32</td>
</tr>
</tbody>
</table>

**Table 2 Amino acid composition of type IV collagen**

**Labeling of type IV collagen.** Type IV collagen was radiolabeled at 4°C by the modified method of Cawston et al. (8). Purified type IV collagen was thoroughly dissolved at a concentration of 2 mg/ml in 5 mM acetic acid overnight. After adding 1 M CaCl₂ drop by drop up to a final concentration of 0.2 M, 0.1 M sodium borate (pH 9.4) was added until the pH of the solution reached 8.0. Then [1-¹⁴C] acetic anhydride (New England Nuclear Co.: 0.125 mCi/0.75 ml in dry benzene) was slowly added and incubated at 10°C for 1 h. Radiolabeled type IV collagen was extensively dialized against 5 mM acetic acid. The radiolabeled substrate (specific radioactivity was 7.2 × 10⁴ dpm/mg collagen) was stored in small aliquots at −70°C until use.

**Preparation of enzymes.** Livers from 7 autopsied patients with HCC were separated into the following four parts (Fig. 1): 1) tumor-bearing liver without cancer tissue and far from the HCC (Tumor-bearing), 2) non-malignant circumferential tissue neighbouring the HCC, about 3 to 5 mm thick (Border), 3) peripheral portion of cancer tissue in about 3 to 5 mm thickness (Cancer front), 4) cancer tissue inside the cancer front (Cancer). The liver of Case FT was not divided because it contained a diffuse type of HCC. The livers from Cases GI and YM were divided into 2 parts, Tumor-bearing and Cancer, because there was very little tissue. These tissues and control livers were extensively washed with cold saline, and homogenized with 4 volumes of 50 mM Tris-HCl buffer (pH 7.5), containing 5 mM CaCl₂, 200 mM NaCl and 0.1% of Triton X-100, in a polytron homogenizer for 2 min at a speed of 18,000 rpm.

**Assay procedures.** Type IV collagen-degrading enzyme activity was assayed by the modified method of Liotta et al. (5). A hundred μl of homogenate was activated by 1 mM p-aminophenylmercuric acetate (APMA: Wako Chemical Co.) at 37°C for 2 h, and then incubated with 40 μg of ¹¹C-labeled type IV collagen at a final volume of 840 μl in 50 mM Tris-HCl buffer (pH 7.5), containing 5 mM CaCl₂, 200 mM NaCl and 1 mM diisopropyl fluorophosphate (DFP; Sigma Chemical Co.) for 4 h at 37°C. Then 10 mM ethylenediaminetetraacetic acid (EDTA) was added to the reaction mixture of each control. The reaction was terminated by adding trichloroacetic acid (final 2%) and tannic acid (0.1%) with 20 μg bovine serum albumin as a carrier. After precipitation at 4°C for 30 min, each tube was centrifuged at 3,000 rpm for 30 min, and then the radioactivity of the supernatant was counted in a liquid scintillation counter (LSC-700: Aloka Co.). Each assay was run in duplicate.

One unit of type IV collagen-degrading enzyme activity was defined as 1 μg of collagen degraded at 37°C per min. The protein concentration of the homogenate was determined by the folin-phenol method (9).
Polyacrylamide gel electrophoresis (PAGE). PAGE at an acid pH and sodium dodecyl sulfate (SDS)-PAGE were carried out by the method of Nagai et al. (10) and Laemmli (11), respectively, in order to identify the components of type IV collagen and its degradation products.

Results

SDS-PAGE of purified type IV collagen showed 4 bands corresponding to the molecular weights of 170K, 145K, 105K, and 70K (Fig. 2-A). The results of SDS-PAGE and amino acid analysis were similar to those of Sage et al. (6). When ¹⁴C-labeled type IV collagen was incubated at 37°C for 4 to 9 h with homogenate of cirrhotic liver under the same assay condition, 4 bands of degradation products were seen on PAGE (lane 3-7). The degradation products were further degraded after 12 h incubation (lane 8). The band of the 105K polypeptide remained on PAGE even after the enzyme digestion. EDTA, a chelating agent, inhibited the enzyme activity (lane 9) (Fig. 2-B). The homogenate dose dependency and kinetics of type IV collagen-degrading enzyme activity were linear up to 300 µl of homogenate and up to 6 h, respectively (Fig. 3). Type IV collagen-degrading enzyme activity of liver homogenate was significantly reduced by the omission of APMA pretreatment, and some APMA-independent activity remained. The ratio of the APMA-dependent enzyme to the APMA-independent enzyme activity was variable from sample
to sample. Trypsin activated the latent enzyme, but the extent of activation was less than that by APMA (data not shown).

Type IV collagen-degrading enzyme activities in cases of HCC are shown in Table 3. The APMA-independent and total (APMA-independent and -dependent) enzyme activities in 8 cases of control livers were 0.57 ± 0.57 mU/mg protein (Mean ± SD) and 4.62 ± 1.76, respectively. The activity of the APMA-independent enzyme was low except for Case TK, and when the homogenate was pretreated with APMA, some increase in activity was seen in every portion of the liver in each case. Prominently high activity was found in Case TK in whom many metastatic nodules were observed in her lungs. Furthermore, enzyme activities at the Border and Front were much higher than those of other portions of tumor-bearing liver or cancer tissue. Case TS showed a similar tendency. Total activity was high at the Border compared to other portions in Cases TS and KK. The total activity of Border tissue was higher than that of other parts in Cases MY and KK. The activity of the Front was the highest in Case KM. Patients with the multinodular type of HCC
Fig. 3 The homogenate-dose dependency and time course of type IV collagen-degrading enzyme activity. Each assay was done in duplicate.

Table 3 Type IV collagen-degrading enzyme activity in cases of hepatocellular carcinoma

<table>
<thead>
<tr>
<th>Case</th>
<th>Enzyme activity (mU/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tumor-bearing</td>
</tr>
<tr>
<td>Autopsied</td>
<td></td>
</tr>
<tr>
<td>TK</td>
<td>2.00*</td>
</tr>
<tr>
<td></td>
<td>5.06*</td>
</tr>
<tr>
<td>MY</td>
<td>1.78</td>
</tr>
<tr>
<td></td>
<td>6.28</td>
</tr>
<tr>
<td>TS</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2.62</td>
</tr>
<tr>
<td>TI*</td>
<td>1.16</td>
</tr>
<tr>
<td></td>
<td>2.40</td>
</tr>
<tr>
<td>KM</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>1.50</td>
</tr>
<tr>
<td>KK</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4.04</td>
</tr>
<tr>
<td>MS</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.66</td>
</tr>
<tr>
<td>GI</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>2.49</td>
</tr>
<tr>
<td>FT</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>NT</td>
</tr>
<tr>
<td>Surgically resected</td>
<td></td>
</tr>
<tr>
<td>YM</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3.01</td>
</tr>
</tbody>
</table>

* The activity without p-aminophenylmercuric acetate.
* The activity with p-aminophenylmercuric acetate.
* Not detected.
* Cancer front tissue from Case TI could not be obtained.
* Not tested.

The ratios of the APMA-independent and -dependent enzyme activities were not significantly different between Tumor-bearing liver and Cancer tissue. The differences in the levels of type IV collagen-degrading enzyme activity between cirrhotic and non-cirrhotic liver in tumor-bearing tissue were not clear because there were only two HCC patients without cirrhosis in the present study. There was no relation between type IV collagen-degrading enzyme activity and either Edmondson's classification of HCC or serum alpha-fetoprotein levels. There was also no significant difference in type IV collagen-degrading enzyme activities between autopsied and surgically resected livers.

Discussion

The basement membrane, a resilient component of the extracellular matrix, forms structures separating the organ parenchymal cells from the interstitial tissue. Type IV collagen is one of the main structural components of the basement membrane and is different from other types of collagen in
physiological, chemical and immunological properties (12, 13). When tumor cell invasion occurs in blood vessels, the basement membrane may be a mechanical barrier to metastasis. Profound changes occur in the distribution and quantity of the basement membrane underneath the epithelial cells during the transition from benign tissue to invasive carcinoma (14). Furthermore, when the basement membrane is broken, metastasis of cancer is initiated.

Proteolytic enzymes may play an important role in tumor growth and invasion (15). The concept that invasive tumor cells secrete matrix-degrading enzymes has been demonstrated by many reporters (16, 17). Tissue collagenase specifically cleaves interstitial collagens types I, II and III at a quarter of the way from the carboxyl terminus of each molecule, but does not cleave type IV collagen (4). Many types of human and murine tumor cells can degrade basement membrane in vitro (18). Human breast carcinoma cells exhibit positive immunoreactivity for "type IV collagenase"(14). Up to date, however, most of the experimental works on type IV collagen degradation were conducted by using tissue cell lines passaged for a long period and it is unclear how different their properties and characteristics are from the primary tumor. There has been no study of a quantitative assay of type IV collagen degradation in view of the invasiveness of human tumors.

Cathepsin G and elastase act proteolytically on type IV collagen(19). Furthermore, two or more enzymes are known to degrade type IV collagen, one being a serine protease and the other(s) a metalloprotease(s)(20). These enzymes may facilitate tumor metastasis. Degradation of type IV collagen is regulated by many inhibitors of these proteases and also by host (macrophage)-tumor interactions (21). Type IV collagen-degrading enzyme in this study was thought to be metalloprotease, because EDTA completely inhibited the enzyme activity. "Type IV-specific collagenase" was recently demonstrated as a neutral metalloprotease (molecular weight, about 60,000 to 70,000), and it was inhibited by both metal chelators and serum but not by serine protease inhibitors (22). Type IV collagen was cleaved into two fragments of approximate mass ratio of 3 (the mass of the C-procollagen IV segment) : 1 (that of the N-segment) by this enzyme.

While type IV collagen-degrading enzyme activity was detected from extracts of human tissue, the cell source of this enzyme is still unknown. The liver consists of many kinds of cells: hepatocytes, endothelial cells, Kupffer cells, fibroblasts, leukocytes, and fat-storing cells. All of these cells may have potential for producing type IV collagen-degrading enzyme. Furthermore, cell-cell interactions may be important in the production and activation of type IV collagen-degrading enzyme in vitro. Type IV collagen-degrading enzyme activity was very low in both cirrhotic liver and HCC tissues in the absence of an activation such as APMA. By incubating with APMA, the activity increased. Tissue collagenase in the latent form is reported to be an enzyme-inhibitor complex and to be activated by some proteases in vitro (23). It is not known whether the latent form of type IV collagen-degrading enzyme is an enzyme-inhibitor complex or proenzyme. Type IV collagen-degrading enzyme was activated by both APMA and trypsin in vitro, but the mechanism of the activation of this enzyme in vivo is not clear yet. Activation of "basement membrane (type IV)-latent collagenase" in vivo might be facilitated by a plasminogen activator through the conversion of plasminogen to plasmin (18).

Cirrhotic livers have large amounts of interstitial matrix which mainly consists of type I and III collagens. Type IV collagen also accumulates in cirrhotic liver (24).
The liver sinusoid becomes similar to a capillary, because type IV collagen deposits in Disse's space with the progression of liver fibrosis. Synthesis and degradation of type IV collagen is thought to occur in normal liver, because type IV collagen-degrading enzyme activity has been revealed in it, and the degree of the enzyme activity is higher in on-going fibrosis of the liver. Our previous data showed that the activity of the tissue collagenase which cleaves type I and III collagens is increased in parallel with collagen accumulation, and that the enzyme activity is decreased when liver fibrosis reaches an irreversible stage in experimental fibrosis of the liver (data not shown). Not only the degradation of type I and III collagens, but also of type IV collagen, is essential for HCC invasion. HCC frequently spreads over other portions of the liver through portal vein invasion. Therefore, type IV collagen-degrading enzyme may have a very important role in invasation to the hepatic blood vessels. The present study revealed that it is possible to measure type IV collagen-degrading enzyme activity in liver homogenate. Type IV collagen-degrading enzyme activity tended to be higher in the peripheral portion (Cancer front) and adjacent tissue (Border) of the tumor than in the center portion of the tumor or tumor-bearing tissue. The highest activity was found in a HCC case with many metastatic nodules to the lungs. These results support the hypothesis that type IV collagen-degrading enzyme is essential to the destruction of the basement membrane, which makes it possible for the HCC to invade into the hepatic blood vessels and to metastasize. The level of type IV collagen-degrading enzyme activity was related to neither the gross type, size, presence of capsules and Edmondson's classification of HCC nor serum alpha-fetoprotein level. Whereas this study has been done from the macroscopic viewpoint, cancer cell invasion in blood vessels is a microscopic phenomenon and thus microscopic investigation is necessary to explain the exact relationship between type IV collagen-degrading enzyme activity and HCC invasion in hepatic blood vessels.

Type IV collagen-degrading enzyme activity in surgically resected HCCs are examined further to avoid the effect of postmortem change on the activity.

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