

Association of E-cadherin, matrix metalloproteinases, and tissue inhibitors of metalloproteinases with the progression and metastasis of hepatocellular carcinoma

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Molecular markers can provide additional information to traditional histomorphological evaluation for the assessment of tumor progression and predicting the likelihood of invasion and metastasis in various types of malignancies. We studied the association of E-cadherin, matrix metalloproteinases (MMPs), and tissue inhibitors of metalloproteinase with the progression and metastasis of hepatocellular carcinoma. Tissue microarray including six normal livers, 14 cirrhotic livers, 39 macroregenerative nodules, 16 dysplastic nodules, 22 grade I hepatocellular carcinomas, 43 grade II hepatocellular carcinomas, seven grade III hepatocellular carcinomas, and 10 metastatic hepatocellular carcinomas were stained immunohistochemically with antibodies against MMPs -1, -2, -3, -7, -9, tissue inhibitors of metalloproteinase-1, -2, -3, and E-cadherin. The intensities of staining were scored manually by two pathologists and verified by the Chromavision Automated Cellular Imaging System. Compared with normal liver, cirrhotic liver had significantly lower E-cadherin and tissue inhibitors of metalloproteinase-1 but higher MMP-1 and -7, which suggest a more favorable environment for tumor invasion and metastasis. Grade I and grade II hepatocellular carcinomas demonstrated high E-cadherin and decreased MMP-3 and -9, which may explain the rarity of extrahepatic metastasis in low-grade hepatocellular carcinomas despite the high circulatory volume of the liver. The histological progression from dysplastic nodule to well-differentiated hepatocellular carcinoma and to less differentiated tumors was associated with a gradual decrease in tissue expression of E-cadherin, tissue inhibitors of metalloproteinase-2 and -3. Metastatic hepatocellular carcinomas showed significantly lower level of tissue inhibitors of metalloproteinase-1, -2, -3 but higher level of MMP-7. These data suggest that tissue expression of E-cadherin, certain MMPs, and tissue inhibitors of metalloproteinases could be useful markers to predict the progression and metastasis of hepatocellular carcinoma.

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Hepatocellular carcinoma is the sixth most common cancer worldwide in terms of numbers of cases (626 000/year) but because of the very poor prognosis, it is the third most common cause of death from cancer (598 000/year).¹ Intrahepatic metastasis and recurrence of the neoplasm after surgical removal remains high.² However, extrahepatic

metastasis is unusual considering the high proliferative activity of hepatocellular carcinoma and rich blood circulation of the liver.^{3,4} The molecular mechanisms that underline the clinical behavior of hepatocellular carcinoma are still poorly understood. Knowledge of the pathogenesis of tumor progression and metastasis of hepatocellular carcinoma could help us to predict the prognosis and to make decisions on adjuvant therapies for those patients.

Dispersion of tumor cells from the primary tumor is considered one of the key events for metastatic progression. Tumor cell dispersion relies on the loss of homotypic cell–cell adhesion, which is largely mediated by E-cadherin/catenin complex.⁵

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E-cadherin, a transmembrane glycoprotein, mediates Ca^{2+} -dependent cell–cell adhesion through its intracytoplasmic interaction with β and α -Catenin. α -Catenin connects the cadherin–catenin complex to actin filament networks, leading to increased adhesive strength. Changes in adhesion complexes lead to alterations of cell polarity, proliferation, mobility, and differentiation.⁶ In a variety of cancers such as lobular breast carcinoma, diffuse gastric carcinoma, endometrial and ovarian carcinoma as well as hepatocellular carcinoma, reduced expression of E-cadherin because of genetic mutations, in combination with loss of heterozygosity at the E-cadherin gene (*CDH1*), has been correlated with disruption of cell–cell contacts, epithelial–mesenchymal transition, invasiveness, and metastatic potential.^{7–11} However, this concept has been challenged in some studies of hepatocellular carcinoma.^{12–14}

Invasion through basement membrane and interstitial extracellular matrix is another key event for metastatic progression, which requires the action of a series of proteolytic enzymes named matrix metalloproteinases (MMPs).^{15–17} MMPs are a group of zinc-dependent endopeptidases that share many structural and functional properties but with different substrate specificities.^{15,18,19} They are historically divided on the basis of their specificity for extracellular matrix components into collagenases (MMP-1, -2, -9), gelatinases (MMP-2, -3, -9), stromelysins (MMP-3, -10, -11), and matrilysin (MMP-7).^{20,21} As the list of MMP substrates grow (21 human MMPs have been identified thus far), they have been grouped into eight distinct structural classes, five are secreted and three are membrane-type.²² Enhanced MMP expressions have been reported in various human malignant tumors.^{16,17} Most clinical data show a correlation between MMP expression with advanced tumor stage, invasion, metastasis, and shortened survival. However, there are a few cases in which increased expression of specific MMPs reflects a favorable prognosis.^{23,24} The activity of MMPs is regulated by a group of molecules named tissue inhibitors of MMP (TIMPs) that reside in the normal tissue and counter react with MMPs with a 1:1 stoichiometry.^{15,19,20,25} There are four members of the TIMP family designated TIMP-1, -2, -3 and -4. TIMPs have been proposed to act selectively on different MMPs: TIMP-2, -1 and -3 preferentially bind to MMP-2, -9 and MMP-9/membrane type 1-MMP, respectively.²⁶ The recently cloned TIMP-4 has been shown to act on numerous MMPs.²⁷

To gain insight into the involvement of MMPs, TIMPs, and E-cadherin in the progression and metastasis of hepatocellular carcinoma, we studied the expression patterns of E-cadherin, MMP-1, -2, -3, -7, -9, and TIMP-1, -2 and -3 in tissue samples of normal liver, cirrhotic liver, macroregenerative nodule, dysplastic nodule, hepatocellular carcinoma of various grade of differentiation, and metastatic hepatocellular carcinoma. The results

elucidated the predictive value of immunohistochemical evaluation of MMPs, TIMPs, and E-cadherin expression for the progression and metastasis of hepatocellular carcinoma. The data also offered a possible explanation for the rarity of extrahepatic metastasis of low to moderate grade hepatocellular carcinoma and the effects of cirrhosis on tumor invasion and metastasis.

Materials and methods

Tissue Samples

With the approval of the institutional review board at the University of Chicago, we retrieved the following tissue samples from the Department of Pathology, University of Chicago Hospitals: six normal livers (two men and four women; age range 12–69 years), 14 cirrhotic livers (seven men and seven women; age range 46–90 years), 39 macroregenerative nodules (19 men and 20 women; age range 43–73 years), 16 dysplastic nodules (10 men and six women; age range 43–72 years), 22 grade I hepatocellular carcinomas (14 men and eight women; age range 46–85 years), 43 grade II hepatocellular carcinomas (27 men and 16 women; age range 47–85 years), seven grade III hepatocellular carcinomas (two men and five women; age range 53–68 years), and 10 metastatic hepatocellular carcinomas (five men and five women; age range 18–80 years). The tissue samples of normal liver, cirrhotic liver, and hepatocellular carcinoma were initially obtained from resection specimens performed for metastatic tumors, explanted HCV-related cirrhotic livers, and surgically removed HCV-related hepatocellular carcinomas, respectively. The routine H&E-stained tissue sections were reviewed by two pathologists and the tumors were graded using WHO grading system.²⁸ The diagnostic criteria devised by the international working group were used for the selection of macroregenerative nodule and dysplastic nodule, with the latter one composed exclusively of small cell dysplasia.²⁹ Representative areas were selected for the construction of the tissue microarray blocks using 1.5 mm punchers on the manual tissue arrayer MTA-1 (Beecher Instruments, Sun Prairie, WI, USA). Because heterogeneity of tumor differentiation is common, especially in large sized tumors, grading was based on the worst area and this area was selected when making the tissue microarray. Owing to the small number of cases with high-grade tumors, the grade III (poorly differentiated) and grade IV (undifferentiated) hepatocellular carcinomas were combined and designated as grade III in this study. All metastatic hepatocellular carcinomas were histologically high-grade tumors.

Immunohistochemical Analysis

Immunohistochemical staining was performed on 4 μm sections obtained from formalin-fixed,

Table 1 List of antibodies against MMPs, TIMPs, and E-cadherin

	MMP-1	MMP-2	MMP-3	MMP-7	MMP-9	E-cadherin	TIMP-1	TIMP-2	TIMP-3
Isotype	Poly	IgG1	IgG2b	IgG2b	IgG1	IgG1	IgG1	IgG2a	IgG2b
Clone	—	42-5D11	SL-1	ID2	23C	4A2C7	2A5	3A4	18D12b
Species	Rabbit	Mouse	Mouse	Mouse	Mouse	mouse	mouse	mouse	mouse
Dilution	1:750	1:100	1:20	1:25	1:30	1:100	1:20	1:20	1:50
Source	Neomarkers	Oncogene Research Products	Neo-markers	Neo-markers	Novo-castra	Zymed	Novo-castra	Novo-castra	Novo-castra

paraffin-embedded tissue microarray blocks. After deparaffinization and rehydration, tissue sections were incubated with monoclonal antibodies against MMP-1, -2, -3, -7, -9, TIMP-1, -2, -3, and E-cadherin (Table 1). A subsequent reaction was performed with biotin-free HRP enzyme labeled polymer of EnVision plus detection system (DakoCytomation, Carpinteria, CA, USA). A positive reaction was visualized with diaminobenzidine solution followed by counterstaining with hematoxylin. Positive controls were selected according to the manufacturer's recommendations: squamous epithelium for E-cadherin; placenta, bladder, breast, and ovarian carcinomas for MMPs -1, -2, -3 -7; macrophages in tonsil tissue for MMP-9 and TIMP-1; colonic adenocarcinoma for TIMP-2; and placenta for TIMP-3. Negative controls were prepared by using nonimmune mouse or rabbit IgGs. The intensity of membrane staining for E-cadherin and cytoplasmic staining for MMPs and TIMPs was graded blindly by two pathologists (ZG, WL) independently at different times using a 4-tiered (0–3) grading system. Discrepancies in grading were resolved by simultaneous grading at a multihead microscope in the presence of a third pathologist (JH). The grading results were further verified by the automated Chromavision Cellular Imaging System (Clariant Inc., San Juan Capistrano, CA, USA).

Statistical Analysis

Nonparametric multiple analysis of variance was used to see the effect of a categorical variable (normal liver, cirrhotic liver, macroregenerative nodule, dysplastic nodule, etc) on multiple dependent variables (MMP-1, -2, -3, etc). The result shows that the effect can be regarded as significant ($\chi^2 = 294.02$, $P < 0.0001$). Subsequently, a series of Kruskal–Wallis test on each dependent variables followed by Dunn's *post hoc* test were used to determine which tissue types differ significantly from others.

Results

The results are illustrated in Table 2, Figures 1–3.

Compared with normal liver, cirrhotic liver had significantly increased expression of MMP-1 ($P < 0.001$), -7 ($P < 0.001$) and decreased expression of E-cadherin ($P < 0.05$) and TIMP-1 ($P < 0.05$);

macroregenerative nodule and dysplastic nodule had significantly increased expression of MMP-1 ($P < 0.001$); grade I hepatocellular carcinoma had significantly increased expression of MMP-7 ($P < 0.01$) and decreased expression of MMP-9 ($P < 0.05$); grade II hepatocellular carcinoma had significantly decreased expression of MMP-3 ($P < 0.05$) and -9 ($P < 0.01$); metastatic hepatocellular carcinoma had significantly decreased expression of E-cadherin ($P < 0.05$), TIMP-1 ($P < 0.01$), -2 ($P < 0.05$), -3 ($P < 0.05$) but increased expression of MMP-7 ($P < 0.001$).

Compared with cirrhotic liver, macroregenerative nodule had significantly decreased expression of MMP-3 ($P < 0.01$) and -7 ($P < 0.001$); grade I hepatocellular carcinoma had significantly increased expression of E-cadherin ($P < 0.01$), but decreased expression of MMP-3 ($P < 0.05$); grade II hepatocellular carcinoma had significantly increased expression of E-cadherin ($P < 0.01$), but decreased expression of MMP-1 ($P < 0.01$), -3 ($P < 0.001$), and -7 ($P < 0.01$); metastatic hepatocellular carcinoma had significantly decreased expression of MMP-1 ($P < 0.001$) and -2 ($P < 0.01$).

Compared with macroregenerative nodule, grade I hepatocellular carcinoma had significantly decreased expression of MMP-1 ($P < 0.05$) and TIMP-3 ($P < 0.01$), but increased expression of MMP-7 ($P < 0.01$); grade II hepatocellular carcinoma had significantly decreased expression of MMP-1 ($P < 0.001$), -9 ($P < 0.05$), TIMP-2 ($P < 0.05$) and -3 ($P < 0.001$); grade III hepatocellular carcinoma had significantly decreased expression of MMP-1 ($P < 0.05$) and TIMP-2 ($P < 0.05$); metastatic hepatocellular carcinoma had significantly decreased expression of MMP-1 ($P < 0.001$), -2 ($P < 0.01$), TIMP-1 ($P < 0.01$), -3 ($P < 0.001$), but significantly increased expression of MMP-7 ($P < 0.001$).

Compared with dysplastic nodule, grade I hepatocellular carcinoma had significantly decreased expression TIMP-2 ($P < 0.001$) and -3 ($P < 0.01$); grade II hepatocellular carcinoma had significantly decreased expression of MMP-1 ($P < 0.01$), -3 ($P < 0.001$), TIMP-2 ($P < 0.001$) and -3 ($P < 0.001$); grade III hepatocellular carcinoma had significantly decreased expression of MMP-1 ($P < 0.001$) and TIMP-2 ($P < 0.001$); metastatic hepatocellular carcinoma had significantly decreased expression of MMP-2 ($P < 0.01$), TIMP-1 ($P < 0.01$), -2 ($P < 0.001$) and -3 ($P < 0.001$).

Table 2 Comparison of tissue expression of MMPs, TIMPs, and E-cadherin

	MMP-1	MMP-2	MMP-3	MMP-7	MMP-9	E-cadherin	TIMP-1	TIMP-2	TIMP-3
Normal:cirrhosis	$P < 0.001$			$P < 0.001$		$P < 0.05$	$P < 0.05$		
Normal:MRN	$P < 0.001$								
Normal:DN	$P < 0.001$								
Normal:HCC-I				$P < 0.01$	$P < 0.05$				
Normal:HCC-II			$P < 0.05$		$P < 0.01$				
Normal:HCC-III									
Normal:HCC-M				$P < 0.001$		$P < 0.05$	$P < 0.01$	$P < 0.05$	$P < 0.05$
Cirrhosis:MRN			$P < 0.01$	$P < 0.001$			$P < 0.05$		
Cirrhosis:DN									
Cirrhosis:HCC-I			$P < 0.05$			$P < 0.01$			
Cirrhosis:HCC-II	$P < 0.01$		$P < 0.001$	$P < 0.01$		$P < 0.01$			
Cirrhosis:HCC-III									
Cirrhosis:HCC-M	$P < 0.001$	$P < 0.01$							
MRN:DN									
MRN:HCC-I	$P < 0.05$			$P < 0.01$					$P < 0.01$
MRN:HCC-II	$P < 0.001$				$P < 0.05$			$P < 0.05$	$P < 0.001$
MRN:HCC-III	$P < 0.05$							$P < 0.05$	
MRN:HCC-M	$P < 0.001$	$P < 0.01$		$P < 0.001$			$P < 0.01$		$P < 0.001$
DN:HCC-I								$P < 0.001$	$P < 0.01$
DN:HCC-II	$P < 0.01$		$P < 0.001$					$P < 0.001$	$P < 0.001$
DN:HCC-III	$P < 0.001$							$P < 0.001$	$P < 0.001$
DN:HCC-M		$P < 0.01$					$P < 0.01$	$P < 0.001$	$P < 0.001$
HCC-I:II									
HCC-I:III									
HCC-I:M									
HCC-II:III									
HCC-II:M									
HCC-III:M									

Blank space indicates no statistical significance. DN, dysplastic nodule; HCC, hepatocellular carcinoma; HCC-I (-II, -III), grade I (II, III) hepatocellular carcinoma; HCC-M, metastatic hepatocellular carcinoma; MRN, macroregenerative nodule.

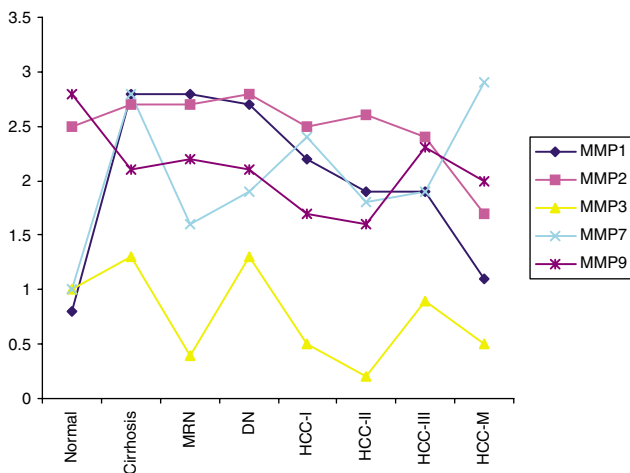


Figure 1 Expression of MMPs in different liver tissues. DN, dysplastic nodule; HCC, hepatocellular carcinoma; HCC-I (-II, -III), grade I (II, III) hepatocellular carcinoma; HCC-M, metastatic hepatocellular carcinoma; MRN, macroregenerative nodule.

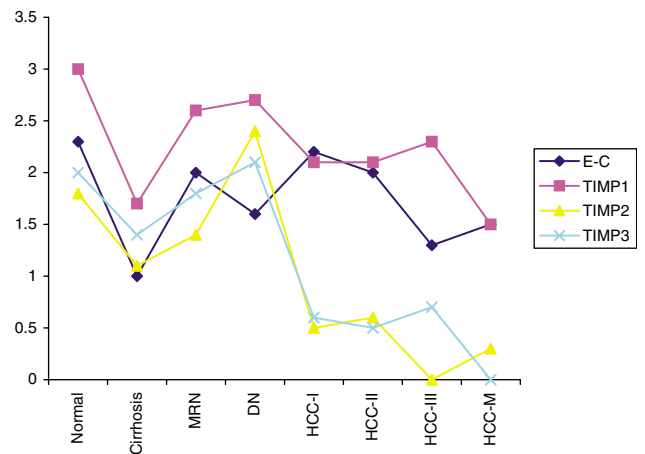


Figure 2 Expression of E-cadherin and tissue inhibitor of MMPs in different liver tissues. DN, dysplastic nodule; HCC, hepatocellular carcinoma; HCC-I (-II, -III), grade I (II, III) hepatocellular carcinoma; HCC-M, metastatic hepatocellular carcinoma; MRN, macroregenerative nodule.

There is no statistically significant difference in the tissue expression of MMPs, TIMPs, and E-cadherin between grade I and grade II hepatocellular carcinoma, grade I and grade III hepatocellular carcinoma, grade I and metastatic hepatocellular carcinoma, grade II and grade III hepatocellular carcinoma, grade III and metastatic hepatocellular carcinoma.

From grade I, to grade II, to grade III and to metastatic hepatocellular carcinoma, there is progressive decrease of E-cadherin expression. From dysplastic nodule to grade I, to grade II, to grade III and to metastatic hepatocellular carcinoma, there is progressive decrease of TIMP expression, especially TIMP-2 and -3.

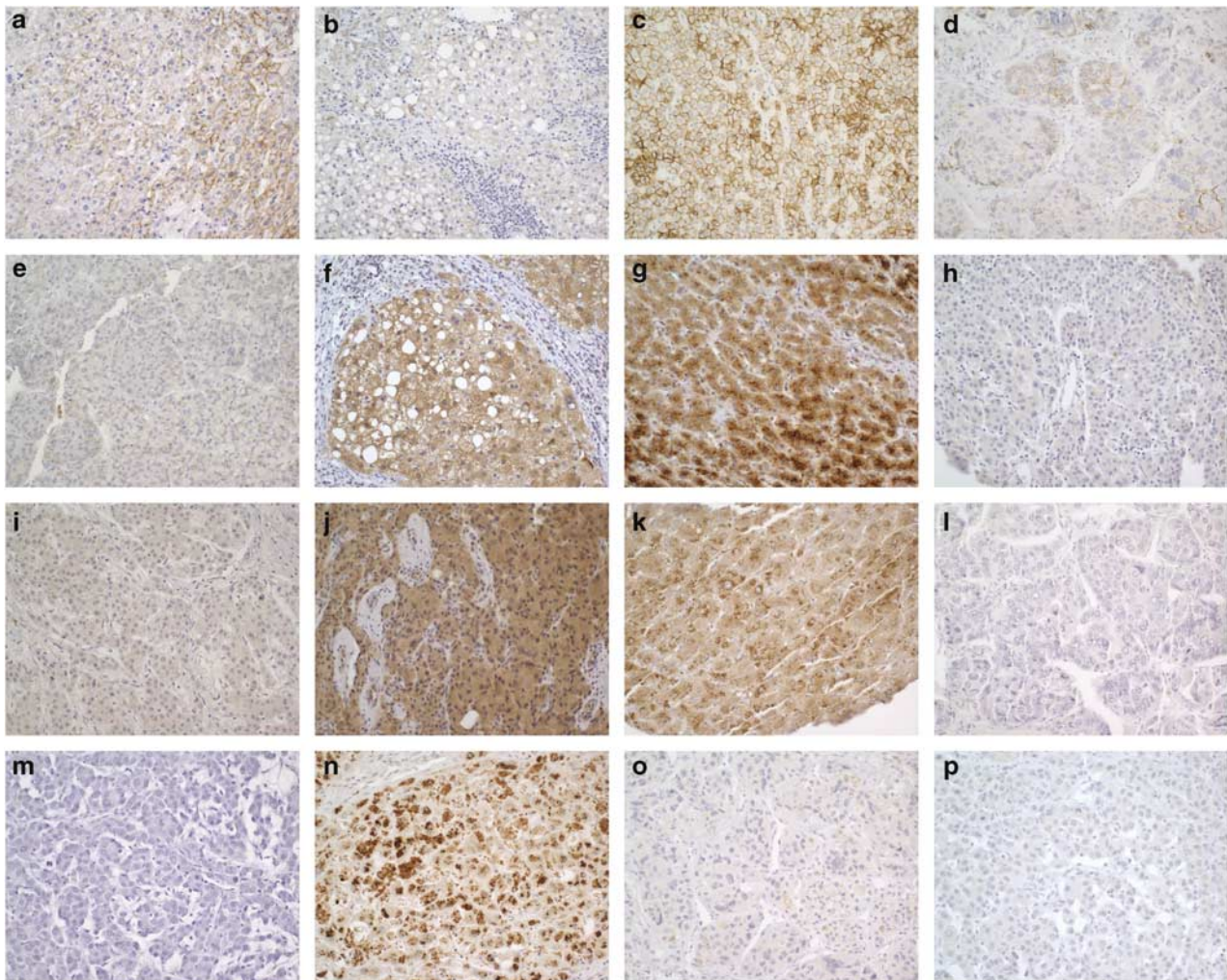


Figure 3 Representative expression patterns of E-cadherin, MMPs and TIMPs in different liver tissue (200 \times , immunoperoxidase). (a) E-cadherin expression in normal liver; (b) E-cadherin expression in cirrhotic liver; (c) E-cadherin expression in grade I hepatocellular carcinoma; (d) E-cadherin expression in grade III hepatocellular carcinoma; (e) E-cadherin expression in metastatic hepatocellular carcinoma; (f) MMP-1 expression in cirrhotic liver; (g) MMP-7 expression in cirrhotic liver; (h) MMP-3 expression in grade I hepatocellular carcinoma; (i) MMP-9 expression in grade I hepatocellular carcinoma; (j) MMP-7 expression in metastatic hepatocellular carcinoma; (k) TIMP-2 expression in dysplastic nodule; (l) TIMP-2 expression in grade III hepatocellular carcinoma; (m) TIMP-2 expression in metastatic hepatocellular carcinoma; (n) TIMP-3 expression in dysplastic nodule; (o) TIMP-3 expression in grade III hepatocellular carcinoma; (p) TIMP-3 expression in metastatic hepatocellular carcinoma.

Discussion

Hepatocellular carcinoma is multifactorial in etiology and complex in pathogenesis. The major risk factor is liver cirrhosis associated with chronic HBV and HCV infections, aflatoxin B exposure, and various metabolic disorders.^{30–32} Survival advantages of hepatocellular carcinomas arising from noncirrhotic liver over hepatocellular carcinomas arising from cirrhotic liver have been well documented.^{33–35} This survival difference is believed to be because of the poor liver functional reserve in cirrhotic liver and the tendency towards development of a new primary after surgery.^{36,37} However, it is yet unknown whether there is any effect of cirrhosis on the metastatic potential of hepatocellular carcinomas. Significant increases of MMP-2,

MMP-9, TIMP-1 and TIMP-2 have been observed in an experimental hepatic fibrosis model.^{20,38,39} The difference in E-cadherin expression between cirrhotic liver and noncirrhotic normal liver has not been observed in previous studies.⁴⁰ Our study demonstrated a significantly lower E-cadherin expression and higher expression of MMP-1, -7 in cirrhotic liver tissue in comparison to noncirrhotic liver tissue. This observation suggests that cirrhosis provides a favorable environment for the invasion and intrahepatic metastasis of primary hepatocellular carcinoma. The balance between cirrhosis, in which abundant extracellular matrix is accumulated, and extracellular matrix degradation by factors secreted from tumor cells, might be one of the essential molecular process associated with the invasion and intrahepatic metastasis of hepatocellular carcinoma

in cirrhotic liver. The finding of increased expression of MMP1 in macroregenerative nodule and dysplastic nodule in this study indicate common genetic alterations among cirrhotic liver, macroregenerative nodule, and dysplastic nodule.

Despite the high proliferative activity of hepatocellular carcinoma and rich blood circulation of the liver, extrahepatic metastasis is unusual^{3,4} especially for low-grade hepatocellular carcinomas. One of the proposed theories was that the increase in concentration of TIMP-1 in hepatocellular carcinoma cause increased type I collagen accumulation and consequent prevention of cellular detachment.³ In this study, we have found that expression of E-cadherin in grade I and grade II hepatocellular carcinomas is almost as high as normal liver. There was decreased expression of MMP-3 and -9 in both grade I and grade II hepatocellular carcinomas. The combinations of these findings provide, at least in part, an explanation for the rarity of extrahepatic metastasis of low-grade hepatocellular carcinoma. Liver transplantation might be able to cure those patients with well-differentiated hepatocellular carcinoma that have high E-cadherin expression and low level of MMP expression because of the low incidence of extrahepatic metastasis.

It has been well documented in several studies that as hepatocellular carcinoma progress from low to high histological grade, there is gradual loss of E-cadherin expression, which further correlates with vascular invasion and metastasis.^{41,42} In this study, hepatocytes in normal liver showed uniform high expression of E-cadherin. There is progressive decreased expression of E-cadherin from grade I through grade II and grade III to metastatic hepatocellular carcinoma. The expression of E-cadherin in metastatic hepatocellular carcinoma is statistically significantly lower than that of normal liver. These data reflect the importance of the adhesion junction system in the progression of hepatocellular carcinoma from low grade to high grade, and to metastatic carcinoma.

Studies about the association of MMPs with hepatocellular carcinoma have generally focused on the following three aspects: (1) those associated with carcinogenesis including overexpression of MMP-2 and MT1-MMP; (2) those associated with tumor progression including overexpression of MMP-2, -3, -9 and MT1-MMP; and (3) those associated with invasion and metastasis including overexpression of MMP-2, -3, -9.⁴³⁻⁴⁷ In this study, we were unable to document progressive changes of MMPs as hepatocellular carcinoma progress from low to high grade except a significantly higher level of expression of MMP-7 in metastatic hepatocellular carcinoma. MMP-7, also known as matrilysin, is the smallest MMP. Overexpression of MMP-7 has been shown to be associated with metastatic progression of colorectal carcinoma and cholangiocarcinoma, as well as hepatocellular carcinoma.⁴⁸⁻⁵¹ The observation of increased expression of MMP-7 in HCV-

associated cirrhotic liver by us (see above) and others further emphasizes the role of this molecule in hepatocellular carcinoma carcinogenesis and metastatic progression.⁵²

Overexpression of TIMP-1 but underexpression of TIMP-2 and -3 have been reported to be associated with invasion and metastasis in hepatocellular carcinoma.^{25,53,54} Early studies using recombinant TIMPs or basic gene transfer system (plasmids or retrovirus) have demonstrated that inhibition of MMPs by TIMPs blocks both tumor growth and local invasion. However, the use of TIMPs in clinical trials has proven largely disappointing.^{25,55} In this study, a low level of TIMP-1 expression was seen in cirrhotic liver tissue, but a high level of TIMP expression was observed in macroregenerative nodule (TIMP-1) and dysplastic nodule (TIMP-1, -2 and -3) tissue. However, the clinical implication of these observations is unclear. The progressive decrease of TIMP-2 and -3 tissue expression from dysplastic nodule to grade I, to grade II, to grade III and to metastatic hepatocellular carcinoma provides evidence for the involvement of TIMPs in hepatocellular carcinoma development and progression.

In summary, we have studied the tissue expression patterns of E-cadherin, MMP-1, -2, -3, -7, -9, and TIMP-1, -2 and -3 in tissue samples of normal liver, cirrhotic liver, macroregenerative nodule, dysplastic nodule, and hepatocellular carcinoma of various grade of differentiation. The increased expression of MMP-1, -7 and decreased expression of E-cadherin in cirrhotic liver suggests a more favorable environment for invasion and metastasis of hepatocellular carcinoma in comparison to non-cirrhotic liver. Preserved E-cadherin and lower levels of MMP-3 and -9 may explain the rarity of extrahepatic metastasis in low-grade hepatocellular carcinoma despite the high circulatory volume of the liver. Decreased expression of E-cadherin, TIMP-2, -3 and increased expression of MMP-7 could be useful markers for the prediction of tumor progression and metastasis. Once verified by larger scale studies, these observations are critical for the development of therapeutic strategies such as gene therapy to block tumor progression and to suppress invasion and metastasis.

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