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Expression of Matrix Metalloproteinase 2 and Extracellular Matrix Metalloproteinase Inducer are Unfavorable Postoperative Prognostic Factors in Intrahepatic Cholangiocarcinoma

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Abstract Many investigators have indicated that over-expression and amplification of matrix metalloproteinase 2 (MMP-2) and extracellular matrix metalloproteinase inducer (EMMPRIN) are independent prognostic factors for primary tumors. We studied expression of them in tissues from intrahepatic cholangiocarcinoma (IHCCA) and normal bile ducts, and discussed the occurrence and development of IHCCA. Another goal was to explore possible association of MMP-2 and EMMPRIN with clinicopathologic parameters and prognosis of IHCCA. MMP-2 and EMMPRIN expression in 106 cases of IHCCA tissues and 15 cases of normal bile ducts were examined by immunohistochemical staining. Then, the association of MMP-2 and EMMPRIN expression with clinicopathologic parameters and patients' prognosis was analyzed. The positive expression levels of MMP-2 and EMMPRIN associated significantly with various clinicopathologic risk factors, such as poor histologic differentiation (p=0.03, 0.02), higher TNM stages (p=0.02, 0.01) and decreased tumor-specific survival. In particular, the tumor-specific survival rate of the patients with MMP-2+/ EMMPRIN+expression was the lowest (p< 0.01). Using Cox regression analysis of the 89 patients, the conjoined expressions of MMP-2-/ EMMPRIN-, MMP-2+/ EMMPRIN +, histologic differentiation, and the clinical TNM stages of tumorous tissues were independent prognostic indicators of IHCCA (p<0.01, p<0.01, p=0.02, p=0.01 and p=0.01, respectively). MMP-2 and EMMPRIN expression in primary tumor predicts an unfavorable prognosis in IHCCA, suggesting a crucial role of the two markers in progression of human IHCCA.

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Abbreviation

IHCCA	intrahepatic cholangiocarcinoma
EMMPRIN (EMMPRIN)	extracellular matrix metalloproteinase inducer
MMP	matrix metalloproteinases
ECM	extracellular matrix

Introduction

Cholangiocarcinoma is a malignant epithelial tumor derived from the bile duct epithelium and is increasing in incidence in the last two decades, with approximately 800~1000 new cases presenting each year in China [1, 2]. Surgical resection is the main modality of treatment for extrahepatic cholangiocarcinoma including hilar bifurcation cholangiocarcinoma and intrahepatic cholangiocarcinoma (IHCCA), which is less frequent than the former but the second most common primary hepatobiliary cancer, only after hepatocellular carcinoma [3]. However, of those patients who undergo operation, only 10% receive a 'curative' resection. Mean survival of patients with unresectable IHCCA is 8 months when jaundice is relieved by operative palliation and 5 months in those treated with a biliary endoprosthesis [4, 5]. Although several clinicopathologic factors have been reported to significant in the prognosis of IHCCA, such as age at diagnosis, local tumor size and distant metastasis [6], the markers which could identify patients with a potentially favourable outcome from those with a higher risk of suffering a poor outcome are lacking. Therefore, it is

important to find biological factors that affect disease recurrence and the survival of patients with IHCCA.

The evolution and progression of carcinoma is a multi-step process, which requires the degradation or remodeling of extracellular matrix (ECM) macromolecules by proteolytic enzymes. Among these proteinases, matrix metalloproteinases (MMPs), a family of zinc- and calcium-dependent enzymes, are particularly implicated because of their specific spectrum of substrates and central mediators of tumor metastasis [7, 8]. Extracellular matrix metalloproteinase inducer (EMMPRIN), also known as EMMPRIN or HAb18G, is highly expressed on the outer surface of carcinoma cells, but not on normal mucosal cells. EMMPRIN stimulates adjacent interstitial normal cells to produce MMPs [9]. Thus, carcinoma cells can interact with adjacent normal cells to produce MMPs via EMMPRIN on their surface, and, in turn, invade lymphatic tissue and blood vessels and penetrate through the ECM to adjacent organs with the help of MMPs [10]. The roles of EMMPRIN and MMPs in tumor invasiveness have been confirmed immunohistochemically in several types of cancer cells and surrounding tissue, including astrocytomas and melanomas [11]. Moreover, the expression of MMPs is reported to correlate with the clinical prognosis of patients with breast carcinoma and other types of cancers [12]. However, there seems to be a paucity of research concerned with MMPs and EMMPRIN expression in IHCCA. For this reason, the goal of the present study was to investigate the immunohistochemical expression of MMP-2 and EMMPRIN in IHCCA tissues and their prognositic values.

Materials and methods

Patients and Tissue Samples

Surgical specimens were obtained from 106 patients with IHCCA, who underwent liver resection for IHCCA at the Department of Surgery, Taihe Hospital Affiliated with the Yunyang Medical College, Shiyan, Hubei, P.R.China from January 1996 to January 2002. They included 50 men and 56 women with aging from 31 to 88 years (66.16± 18.92 years). The resected liver tissues had been macroscopically examined to determine the location and size of the tumors. Histological sections, cut at 4 μm, were stained with hematoxylin and eosin, and immunoperoxidase procedures (avidin-biotin complex method). Histological sections were independently reclassified by two experienced pathologists according to histological typing of the WHO as IHCCA to examine the clinical TNM stages and histological subtype.

After surgery, all patients were given a follow-up with different time ranging from 1 to 60 months (follow-up was performed by the time of December 2007). In addition, tissues from normal bile ducts were offered by Pathology Department of Taihe Hospital Affiliated with the Yunyang Medical College, Shiyan, Hubei, P.R.China. All the patients who died of other diseases rather than IHCCA and unexpected events were excluded from the case collection. The study was approved by the Research Ethics Committee of Taihe Hospital Affiliated with the Yunyang Medical College, Shiyan, Hubei, P. R. China. Informed consent was obtained from all of the patients. All specimens were handled and made anonymous according to the ethical and legal standards.

Immunohistochemical Staining and Assessment

For immunohistochemical staining, tissues were fixed in 10% buffered formalin and embedded into paraffin. Immunohistochemical examination was carried out on tissue microarray (TMA) sections using a commercially available kit (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA), mouse anti-human MMP-2 monoclonal antibody (dilution, 1: 200; Catalog # sc-53630, Santa Cruz Biotechnology, California) and mouse anti-human EMMPRIN monoclonal antibody (dilution, 1: 200; Catalog

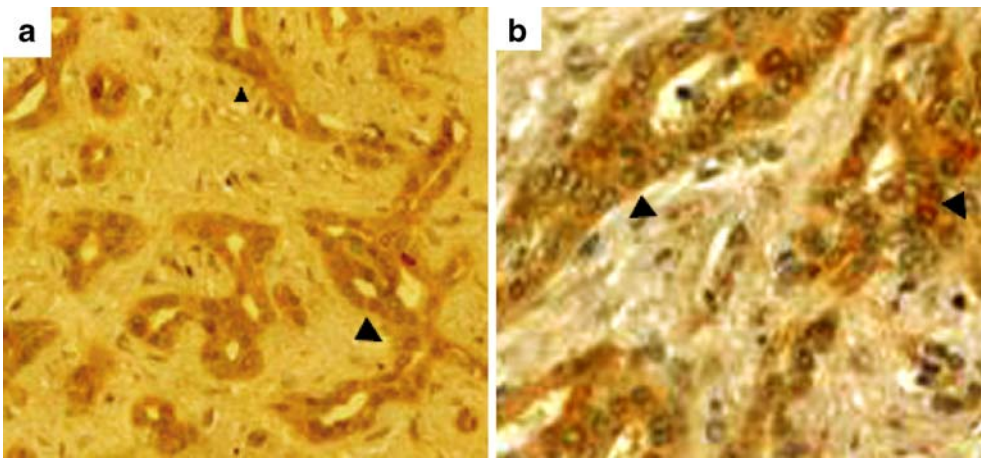

Fig. 1 Immunohistochemical staining for MMP-2 and EMMPRIN in IHCCA tissues (Original magnification×200). A, EMMPRIN positive cells of IHCCA and EMMPRIN mainly seen in cell membrane and cytoplasm at various levels; (B) MMP-2 positive cells of IHCCA and MMP-2 mainly seen in cell membrane at various levels. The arrow indicates the intermediate and intensive positive area in the tumor tissues

sc-71038 Santa Cruz Biotechnology, California). Briefly, for enhancement of the immunoreactivity of MMP-2 and EMMPRIN, sections were pretreated in a microwave oven; and deparaffinized sections were transferred to 0.01 M citrate buffer at pH 6 and heated in a 500-W microwave oven for 20 min. To inactivate endogeneous peroxidase, all sections were treated in methanol containing 0.3% hydrogen peroxidase for 10 min. After washing with PBS, the sections were incubated overnight with primary monoclonal antibodies MMP-2 and EMMPRIN at 4°C. They were then washed with PBS and incubated with biotinylated goat anti-mouse IgG antibody (Catalog # BA-9200, Vector Laboratories, Inc., Burlingame, CA), followed by incubation with avidin-biotin-peroxidase complex for 40 min at room temperature and incubation with a combination of 3,3′-diaminobenzidine (DAB) for 5 min at room temperature. The nuclei were counterstained with hematoxylin. Reproducibility of staining was confirmed by reimmunostaining via the same method in multiple, randomly selected specimens (five for each staining). As a negative staining control, the tissue section was treated in an identical manner with the omission of primary antibody. The following positive controls were used: (i) hepatocellular carcinoma tissues known to be MMP-2-protein-positive for MMP-2; (ii) hepatocellular carcinoma tissues known to be EMMPRIN-protein-positive for EMMPRIN. The same operator should perform all studies and should be blinded to tissue type.

TMA immunostaining was assessed for staining intensity (grades 0~3) using light microscopy, and three cores/ case were scored. The criteria used for assessment were as previously reported [13–15], where: 0 (negative, <5%); 1+ (low, 6~25%); 2+ (intermediate, 26~50%); 3+ (high, >51%) of the tumor cells stained. This analysis is comparable with whole section analysis [16]. All cores (3/3) constituted positive staining for each case. Many tissues showed heterogeneity between cores; an average score was, determined from specimens scored blindly by three observers, taken. Where discordant, divergences were resolved by joint review after consulting with a fourth observer.

Statistical Analysis

SPSS12.0 software for Windows (SPSS Inc, USA) and SAS 9.1 (SAS Institute, NC) was used for statistical analysis. Continuous variables were expressed as $\overline{X} \pm s$. Statistical analyses were performed with Fisher's exact test for any 2×2 tables, Pearson χ^2 test for non- 2×2 tables. Survival curves were constructed by the Kaplan-Meier method, and differences in survival curves were compared by the log-rank (Mantel-Cox) test. The p values of less than 0.05 were considered to be statistically significant.

Table 1 Association of MMP-2 and EMMPRIN expression with clinicopathologic parameters of IHCCA tissues

Clinicopathologic parameters	NO.	MMP-2 expression (%, n)				p	EMMPRIN expression (%, n)				p
		0	1	2	3		0	1	2	3	
Gender											
Male	50	20.00 (10)	34.00 (17)	26.00 (13)	20.00 (10)	0.08*	16.00 (8)	32.00 (16)	30.00 (15)	22.00 (11)	0.07*
Female	56	19.64 (11)	26.79 (15)	26.79 (15)	26.79 (15)		17.86 (10)	30.36 (17)	25.00 (14)	26.79 (15)	
Tumor size (cm)											
<4.0	61	19.67 (12)	31.15 (19)	26.23 (16)	22.95 (14)	0.19**	16.39 (10)	31.15 (19)	27.87 (17)	24.59 (15)	0.18**
≥4.0	45	20.00 (9)	31.11 (14)	28.89 (13)	20.00 (9)		17.78 (8)	31.11 (14)	28.89 (13)	22.22 (10)	
Differentiated type											
Well	37	35.14 (13)	40.54 (15)	24.32 (9)	0.00 (0)	0.03#	29.73 (11)	43.24 (16)	27.03 (10)	0.00 (0)	0.02#
Moderate	35	20.00 (7)	17.14 (6)	34.29 (12)	28.57 (10)		14.29 (5)	14.29 (5)	48.57 (17)	22.86 (8)	
Poor	34	2.94 (1)	5.88 (2)	20.59 (7)	70.59 (24)		8.82 (3)	2.94 (1)	8.82 (3)	82.35 (27)	
Clinical TNM stage											
I~II	60	28.33 (17)	35.00 (21)	31.67 (19)	5.00 (3)	0.02##	25.00 (15)	33.33 (20)	36.67 (22)	5.00 (3)	0.01##
III~IV	46	8.70 (4)	4.35 (2)	19.57 (9)	67.39 (31)		6.52 (3)	4.35 (2)	17.39 (8)	71.74 (33)	

'*' refers to the comparison between male and female groups

'**' refers to the comparison between cancerous tissues with <4.0 cm size and cancerous tissues with ≥4.0 cm size

'#' refers to the comparison among three groups with well, moderate and poor differentiated type

'##' refers to the comparison between TNM I~II and TNM III~IV groups

Table 2 Postoperative survival rate of MMP-2 and EMMPRIN expression in IHCCA tissues by Kaplan-Meier method

Clinicopathologic parameters	NO.	5-year survival rate (%)		*p*	5-year survival rate (%)		*p*
		MMP-2-	MMP-2+		EMMPRIN -	EMMPRIN +	
Differentiated type							
Well	35	33.33 (n=4)	21.74 (n=5)	0.01 *	37.14 (n=4)	20.00 (n=5)	0.01 *
Moderate	29	28.57 (n=2)	9.09 (n=2)		20.00 (n=1)	8.33 (n=2)	
Poor	25	0.00 (n=0)	0.00 (n=0)		0.00 (n=0)	0.00 (n=0)	
Clinical TNM stage							
I~II	50	29.41 (n=5)	21.21 (n=7)	<0.01 #	33.33 (n=5)	20.00 (n=7)	<0.01 #
III~IV	39	25.00 (n=1)	0.00 (n=0)		0 (n=0)	0.00 (n=0)	

'*' refers to the comparison among three groups with well, moderate and poor differentiated type

'#' refers to the comparison between TNM I~II and TNM III~IV groups

Results

Immunohistochemical Detection of MMP-2 and EMMPRIN in IHCCA Tissues

MMP-2 was mainly seen in the cell membrane and EMMPRIN in the cytoplasm and cell membrane, expressed as dark yellow granules (Fig. 1). The two markers were highly expressed in IHCCA tissues, in 80.19% (85/106) and 83.02% (88/106) of cases, respectively, but normal bile duct tissues were negative as the proteins were not expressed. In particularly, MMP-2 and EMMPRIN immunoreactivities were detected with high intensity in most of the neoplasic cells of IHCCA tissues. There were 23 in 106 (21.70%) and 22 in 106 (20.75%) cases showed low MMP-2 and EMMPRIN expression, 28 in 106 (26.42%) and 30 in 106 (28.30%) showed intermediate expression, and 34 in 106 (32.08%) and 36 in 106 (33.96%) showed high expression, respectively. We observed that primary tumours showed a higher percentage of staining cells or cells with higher intensity than matching peritumoral normal tissues.

Association of MMP-2 and EMMPRIN Expression with Clinicopathologic Parameters of IHCCA Tissues

In IHCCA tissues, the expression of MMP-2 and EMMPRIN was directly proportional to the histological differentiation and TNM stages also had a significant association (Table 1). The expression rates of MMP-2 and EMMPRIN in the tumors with poor differentiation were significantly higher than in the tumors with well/ intermediate differentiation (p= 0.03 and 0.02, respectively). The expression rates of MMP-2 and EMMPRIN in the tumors with TNM stage III~IV were significantly higher than in the tumors with TNM stage I~II (p=0.02, 0.01). The expression of them were not correlated with gender of patients and size of tumor (p>0.05). Additionally, the Spearman correlation (rs) was 0.88 (p= 0.01) indicating that the expression level of MMP-2 was postively correlated with that of EMMPRIN significantly.

Prognostic Implications of MMP-2 and EMMPRIN Expression in IHCCA Tissues

Survival data were available for 89 patients with IHCCA. The observation period ranged from 1 to 60 months, with a median survival of 22 months. At the most recent follow-up, 31 patients were alive and 18 had died.

The association between 5-year survival rate and the expression levels of MMP-2 and EMMPRIN was analyzed using Kaplan-Meier method. When the patients were classified based on clinical TNM stages, those with low MMP-2 and EMMPRIN expression had significantly higher 5-year survival rates than those with high expression. The p values were lower than 0.01 (Table 2). In all three groups for differentiation levels of IHCCA tissues, patients with intensive MMP-2 and EMMPRIN positive expression had the poorest prognosis (Table 2).

According to the conjoined expressions of MMP-2/EMMPRIN, the patients were categorized into four groups: MMP-2-/EMMPRIN-, MMP-2-/EMMPRIN+, MMP-2+/EMMPRIN- and MMP-2+/EMMPRIN+. The Chi-square

Table 3 Postoperative survival rate and conjoined expression of MMP-2/EMMPRIN

Type	Total N	5-year survival rate	
		n	Percent (%)
MMP-2-/EMMPRIN-	27	18	66.67
MMP-2-/EMMPRIN+	22	5	22.73
MMP-2+/EMMPRIN-	21	7	33.33
MMP-2+/EMMPRIN+	19	1	5.26
Overall	89	31	34.83

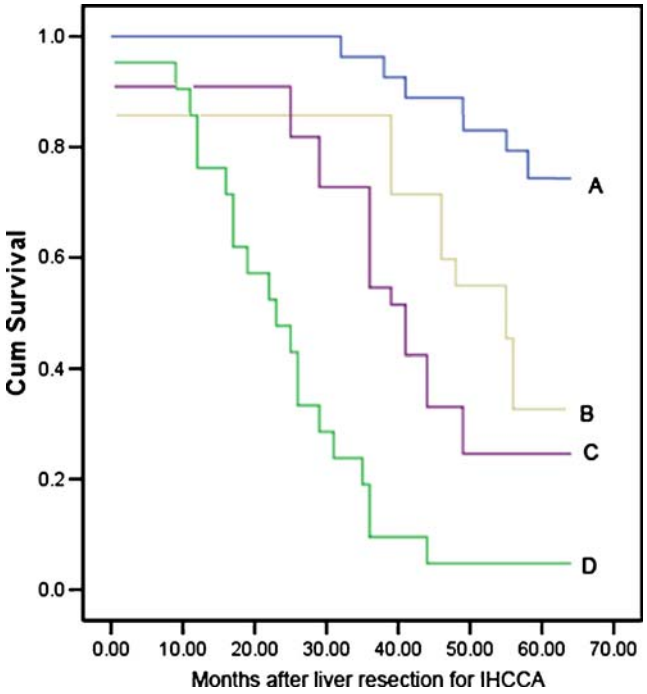

Fig. 2 Kaplan-Meier survival curves for MMP-2 and EMMPRIN expression in IHCCA tissues. (**A**), for MMP-2-/EMMPRIN-; (**B**), for MMP-2+/EMMPRIN; (**C**), for MMP-2-/EMMPRIN+; (**D**), for MMP-2+/EMMPRIN+; Survival was significantly better for patients with MMP-2-/EMMPRIN expression than those with positive expression ($p<0.01$)

value by Mantel-Cox indicated a significant difference among different groups with regard to the conjoined expression status of MMP-2/EMMPRIN ($p<0.01$, Table 3). The results by pairwise comparisons showed that the statistically significant difference of survival rates existed between MMP-2+/EMMPRIN+patients and any of other three groups ($p<0.01$). In all four groups, MMP-2+/EMMPRIN+patients had the poorest prognosis (Fig. 2). Using Cox regression analysis of the 89 patients, conjoined expressions of MMP-2+/EMMPRIN+and MMP-2-/EMMPRIN-, histologic differentiation, and the clinical TNM stages of tumorous tissues were independent prognostic indicators of IHCCA ($p<0.01$ $p<0.01$, $p=0.02$, $p=0.01$ and $p=0.01$, respectively, Table 4).

Discussion

Cholangiocarcinoma has no obvious symptoms and metastasizes early, because of its anatomical and physiological characteristics. Thus it is diagnosed mostly at an advanced stage and its excision rate is low with poor prognosis. In the last decades, considerable efforts have been devoted to the analysis for molecular mechanisms of the invasion and metastasis of IHCCA. For instance, Romani AA, et al [16], have demonstrated that the combined expression of the Maspin and Bax proteins appears to be a predictor of survival in IHCCA likely influencing the susceptibility of tumor cholangiocytes to apoptosis. Li Qiang, et al. [17], have shown that the aPKC-ι and E-cadherin expression may be correlated with invasion and prognosis of cholangiocarcinoma. In the present study, we indicate that the immunohistochemical staining of MMP-2 and EMMPRIN in tumor tissue significantly correlates with patients' survival and might be regarded as a novel potential prognostic factor in IHCCA.

To our knowledge, tumor metastasis and invasion is a complicated process with many steps including basement membrane disruption, stromal infiltration, intravasation and extravasation, and invasion of a target organ by tumor cells. MMPs are particularly implicated in the metastastic cascade [18]. Even though quiescent fibroblasts usually produce relatively low amounts of MMPs, it is likely that tumor-associated fibroblasts are stimulated to produce the elevated levels of MMPs usually present in malignant tumors [19, 20]. EMMPRIN has been demonstrated to stimulate *in vitro* the fibroblast production of various MMPs such as interstitial collagenase (MMP-1), gelatinase A (MMP-2), and stromelysin-1 (MMP-3) [21–23]. Recently, overexpression of MMPs and EMMPRIN has been identified in breast cancers as well as in gastrointestinal, pulmonary, and genitourinary tumors [24]. It has been indicated that overexpressed MMPs and EMMPRIN play direct roles in the pathogenesis and aggressiveness of tumors through several lines of experimental evidence: transfection of MMPs and EMMPRIN into nonneoplastic cells effects malignant transformation; transgenic mice expressing

Table 4 Prognostic value of MMP-2/EMMPRIN conjoined expression in multivariate analysis by Cox Regression

	Wald	df	*p*	Exp(B)	95.0% CI for Exp(B)	
					Lower	Upper
MMP-2-/EMMPRIN-	30.12	3	<0.01			
MMP-2-/EMMPRIN+	0.91	1	0.40	0.98	0.76	2.01
MMP-2+/EMMPRIN-	0.65	1	0.32	0.451	0.34	1.67
MMP-2+/EMMPRIN+	26.47	1	<0.01	5.13	2.18	8.42
Differentiated type	5.01	1	0.02	2.89	1.02	3.85
Clinical TNM stage	13.72	1	0.01	2.73	1.60	5.16

MMPs and EMMPRIN develop mammary tumors; and the presence of MMPs and EMMPRIN overexpression may be associated with the development of metastatic disease [25].

The aberrant expression of MMP-2 and EMMPRIN during development of IHCCA was addressed in this study through an immunohistochemical examination in 106 IHCCA tissues, which is a method which has been used widely to evaluate molecular status in clinical laboratories. As the results, there is significant correlation of MMP-2 and EMMPRIN expression with histologic differentiation, and the TNM stages of IHCCA tissues, but not with gender of patients and tumor size. This suggests that their expression may associate with tumor progression of IHCCA. Although Ishibashi et al [26] reported that EMMPRIN expression was not associated with the recurrence-free survival of oesophageal squamous cell carcinoma, Davidson et al [27] found that EMMPRIN was a good prognostic marker in ovarian carcinoma, and Sillanpää S et al [28] demonstrated that EMMPRIN and MMP-2 in cancer cells are significant indicators of a favorable prognosis of epithelial ovarian cancer. To further clarify the clinicopathological significance, we analyzed the correlation of MMP-2 and EMMPRIN expression with survival of 89 patients with IHCCA. The results revealed a link between loss and favourable survival.

In summary, our study revealed that aberrant expression of MMP-2 and EMMPRIN were found in considerable proportion of patients with IHCCA. These results support the concept that the two markers may participate in IHCCA. Additionally, MMP-2 and EMMPRIN could thus be considered as an objective and effective marker to predict the invasion and prognosis of IHCCA. Further studies on the role of this proto-oncogene in IHCCA will demonstrate whether MMP-2 and EMMPRIN can also be used as targets for therapy.

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