Tissue invasive macrophage density is correlated with prognosis in cholangiocarcinoma

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Abstract. Cholangiocarcinoma (CCA) is a high metastatic cancer with no effective treatment. Here, the pro-metastatic action of tissue macrophages in CCA is demonstrated and suggested as a prognostic marker and novel target for the therapeutic intervention of CCA. Fifty CCA tissues were immunohistochemically stained with a marker for reactive/infiltrating monocytes/macrophages (MAC387) and matrix metalloproteinase (MMP)-9. The antigenic densities in positively-stained cells along the leading edge of tumors were scored. Correlations between the densities of MAC387, MMP-9-positive cells, clinicopathological features and patient survival were investigated. High densities of MAC387-positive cells were detected in more than 60% of the CCA tissues. This was significantly associated with poor prognosis parameters (non-papillary and mass-forming type CCA). Overall survival was worst in patients with high-density MAC387-positive cells. Double immunofluorescent staining indicated that MAC387-positive cells co-expressed MMP-9. Immunohistochemical staining of MMP-9 in serial sections of CCA tissues indicated that MMP-9 was rarely expressed in CCA tumor cells, but highly expressed in MAC387-positive cells and polymorphonucleated infiltrating cells. Patients with high tissue expression levels of MAC387 in combination with MMP-9-expressing cells had the worst survival. These factors were found to be independent predictors of the post-resectional survival of CCA patients. Since CCA tumor cells rarely expressed MMP-9, it is likely that tissue macrophages are critical for degrading the extracellular matrix and for facilitating tumor metastasis. They may therefore serve as a prognostic marker for poor clinical outcome, and represent novel targets for the therapeutic intervention of CCA.

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Introduction

Carcinogenesis and tumor progression are multistep processes in which genetic alterations and modifications in malignant cells may help to establish tumor-supporting microenvironments. A variety of soluble mediators (e.g., chemokines and cytokines) have been implicated in the recruitment of leukocytes to the tumor microenvironment, a process that has either been associated with tumor progression or inhibition in different types of tumors. Although all classes of leukocytes are found within tumors, the most abundant cells are tumor associated macrophages (TAMs) (1).

TAMs are diffusely found throughout tumors in localized zones, tumor edges, around the ductal areas and in the tumor stromal areas (2,3). Although macrophages under certain conditions can kill tumor cells (antitumor activity), several studies have highlighted their potential role as tumor promoters. The balance between the pro-tumorigenic and antitumorigenic properties of macrophages may depend on tumor type and organ site. TAMs are derived from circulating monocytes that infiltrate the tumor and differentiate to macrophages (1,4). MAC387, which is expressed in circulating neutrophils and monocytes, is a macrophage marker that distinguishes resident tissue macrophages from newly arrived blood migrants (5,6).

Cholangiocarcinoma (CCA), a slow growing but highly metastatic tumor, is highly prevalent in northeast Thailand. Both epidemiologic and experimental evidence implicates chronic inflammation resulting from liver fluke (*Opisthorchis viverrini*; OV) infection as the major risk factor for CCA in Thailand (7,8). The role of infiltrating leukocytes in carcinogenesis and the progression of experimental CCA is supported by the high levels of infiltrating leukocytes within the tumor tissues of OV-associated CCA hamsters (9). No study has been performed on human CCA to test whether this class of tumor contains tumor infiltrating macrophages. Whether the presence of these macrophages plays a role in pro- or anti-tumorigenic activities is unclear.

In this study, we report the association of newly infiltrated tissue macrophages (MAC387-positive cells) and TAM expressed factors – namely matrix metalloproteinase (MMP)-9

– with poor prognosis in CCA patients. To date, inflammatory TAMs have not been targets for therapeutic intervention. However, in for example rheumatoid arthritis, anti-inflammatory therapeutic approaches have significantly improved patient clinical outcomes. The goal of the present study was to test whether CCA, an aggressive form of tumor arising from an inflammatory environment, contains inflammatory TAMs, and to determine whether TAMs play a role in pro-tumorigenic activities and whether they may serve as important cell targets for future therapeutic intervention.

Materials and methods

Subjects and tissues. Fifty paraffin-embedded blocks were obtained from the specimen bank of the Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University. Informed consent was obtained from each subject, and the Human Research Ethics Committee of Khon Kaen University approved the research protocol (HE471214 and HE480312). Age, gender, tumor location, histological grading and pTNM stage were evaluated by reviewing the medical charts and pathological records.

Immunohistochemistry studies. Specimens were fixed in 10% neutral formalin buffer, embedded in paraffin and cut into 5-μm-thick sections. Immunohistochemical staining was performed by an immunoperoxidase method using mouse monoclonal anti-human myeloid/histocyte antigen (MAC387 clone; Dako, Glostrup, Denmark) and rabbit monoclonal anti-human MMP-9 (Dako). Each section was deparaffinized and re-hydrated with antigen retrieval citrate buffer (pH 6), and endogenous peroxidase was blocked with hydrogen peroxide in methanol. The serial sections were incubated with 1:200 MAC387 for 30 min or 1:100 anti-human MMP-9 overnight, followed by the addition of Envision labeled polymer peroxidase (Dako) for 30 min. After washing, the sections were reacted with liquid 3,3'-diaminobenzidine tetrahydrochloride (DAB) substrate chromogen system (Dako). All slides were counterstained with Mayer's hematoxylin. Paraffin-embedded sections of human tonsil and colon cancer tissue sections were used as positive controls for MAC387 staining and MMP-9 staining, respectively.

The densities of MAC387 and MMP-9-positive cells at the leading edge of invasive tumor were semi-quantitatively classified into four scoring categories: 0, negative; 1^+, 1-25%; 2^+, 26-50%; and 3^+, >50%. The specimens were evaluated by two researchers without any knowledge of prognosis or clinicopathologic variables. For statistical analysis, the scores 0 and 1^+ were categorized as low expression (or negative), and the scores 2^+ and 3^+ as high expression (or positive).

Double immunofluorescence-labeling method. A modified double immunofluorescence-labeling method was performed to examine the co-localization of human myeloid/histocyte antigen with MMP-9. Heat-mediated antigen retrieval was performed in a pressure cooker with citrate buffer (pH 6) and 0.05% Tween-20 for 3 min. The sections were cooled to room temperature for 30 min then incubated with the primary antibodies at room temperature overnight. The co-localization of MMP-9 and MAC387 in CCA tissue was assessed using 1:100 of rabbit polyclonal anti-human MMP-9 and 1:200 of mouse monoclonal anti-MAC387. The sections were incubated for a further 3 h with 1:400 of Alexa 488-labeled goat antibody against rabbit IgG and an Alexa 568-labeled goat antibody against mouse IgG (Molecular Probes Inc., Eugene, OR, USA). The stained sections were examined using a fluorescence microscope.

Statistical analysis. Statistical analysis was performed using SPSS statistical software version 16.0.1 (SPSS Inc., Chicago, IL, USA) and STATA version 8. Cross tabulations were analyzed with the χ^2-test for associations between MAC387 and MMP-9 expression and the clinicopathological features of CCA patients. Kaplan-Meier survival analysis was used to estimate disease-specific survival, and comparisons between groups were performed using the log-rank test. Multivariate survival analysis was used to investigate the importance of MAC387 and MMP-9 expression in comparison to other prognostic parameters. $P<0.05$ was considered statistically significant.

Results

The mean age of the CCA patients recruited to the study (n=50) was 56 years (range 33-75). The ratio of men to women was 1.17 to 1. Tissues were obtained during liver resection for CCA. None of the patients received radiotherapy or chemotherapy prior to the surgery. Tumor staging was based on the American Joint Committee on Cancer classification (10). The clinicopathological features of the patients are shown in Table I. Patients with CCA stages I-III (n=36) received a resection with curative intention, whereas patients with stage IV CCA (n=14) underwent palliative surgery to relieve jaundice, debulk the tumor and achieve an improved quality of life. The survival of each CCA patient was recorded from the date of surgery to the date the patient succumbed to the disease or to June 13, 2008. Only patients with CCA stages I-III were included in the survival analysis. The median duration for follow-up was 299 days. The median overall survival was 386 days and the overall survival rate was 52% at one year post-surgery.

MAC387-positive cells in CCA tissues and clinical significance. Immunohistochemistry of 50 CCA tissues revealed that MAC387-positive cells were distributed throughout the tumor-involved liver tissue, including the leading edge of invasive tumor, tumor parenchyma (Fig. 1A) and perivascular areas. The majority of MAC387-positive cells were located in apparent direct contact with or immediately adjacent to tumor cells at the edge of invasive tumor (Fig. 1B). MAC387-positive cells were also present in regions of necrosis. Although the highest density of MAC387-expressing cells was at the tumor edge, they were also frequently present in perivascular areas of tumors (Fig. 1C). As MAC387 stains blood-derived macrophage migrants, these perivascular cells may represent the most recent blood-derived monocytes in CCA tissues.

Since MAC387-positive cells were found in high quantities at the leading edge of the tumor, we quantitatively analyzed the correlation between the density of MAC387-positive cells in this region and the clinicopathological parameters using a univariate analysis. High numbers of MAC387-positive cells were significantly associated with poor survival outcomes, namely

Table I. Density of MAC387-positive cells in cholangiocarcinoma (CCA) tissues in relation to the clinicopathological features of the patients.

Variable	MAC387-positive cells			
	No.	Low (%)	High (%)	P-value
Age				0.608
<56	26	9 (34.6)	17 (65.4)	
≥56	24	10 (41.7)	14 (58.3)	
Gender				0.879
Female	23	9 (39.1)	14 (60.9)	
Male	27	10 (37.0)	17 (63.0)	
Tumor location				0.610
Intrahepatic CCA	32	13 (40.6)	19 (59.4)	
Extrahepatic CCA	18	6 (33.3)	12 (66.7)	
Gross morphology				0.047
Mass forming type	24	7 (29.2)	17 (70.8)	
Periductal infiltrating type	20	7 (35.0)	13 (65.0)	
Intraductal growth type	6	5 (83.3)	1 (16.7)	
Tumor stage				0.091
I-II	13	8 (61.5)	5 (38.5)	
III	23	8 (34.8)	15 (65.2)	
IV	14	3 (21.4)	11 (78.6)	
Histological type				0.006
Non-papillary	35	9 (25.7)	26 (74.3)	
Papillary	15	10 (66.7)	5 (33.3)	
Vascular invasion				0.198
Absent	37	14 (32.4)	23 (67.6)	
Present	13	3 (23.1)	10 (76.9)	

the non-papillary (P=0.006), mass forming and periductal infiltrating types of CCA (P=0.047) (Table I). There was no correlation between the density of MAC387-positive cells and gender, age, tumor location, tumor staging or vascular invasion.

High density of MAC387-expressing cells and poor patient survival. Kaplan-Meier and log-rank tests were used to determine the overall survival of CCA patients with low vs. high expression of MAC387 at the tumor edge. No patients with perioperative death (survival <30 days) were included in the analysis. Overall, the post-resectional survival of patients with CCA stages I-III was significantly reduced in patients with high levels of MAC387-positive cells (mean survival 380 days; 95% CI 228-531 days) compared to those with a low density of MAC387-positive cells (mean survival 679 days; 95% CI 541-817 days; P=0.01) (Fig. 2). The overall survival rates were 35 and 75% at 1 year post-surgery for patients with high and low levels of tissue MAC387-positive cells, respectively.

Age, gender, tumor location, tumor type and vascular invasion had no influence on survival. However, overall survival was significantly decreased in patients with advanced stage tumors (stages III and IV), non-papillary type CCA or a high density of MAC387-positive cells (Table II).

MMP-9 expression in MAC387-positive cells. Since the significance of the high density of MAC387-expressing cells at the leading edge of invasive tumor was observed to be associated with poor survival parameters and shorter survival in CCA patients, we further analyzed whether MAC387-positive cells co-expressed MMP-9, a factor known to play a significant role in tumor invasion. Double immunofluorescent analyses were performed on CCA tissues positive for the MMP-9 antibody together with MAC387. MAC387-positive cells with co-expression of MMP-9 were found in the highest quantities at the leading edge of the tumor and tumor-involved tissue areas (Fig. 3A and B). Scattered MAC387-positive cells with MMP-9 expression were also observed in non-tumor tissue.

Serial sections of CCA tumor tissues were examined for MAC387 and MMP-9 expressing cells by immunohistochemistry. Although MMP-9 was observed in cells with tissue invasion characteristics, the antigen was rarely observed in CCA cells. Only 6% (3/50) of CCA tissues were positive, with weak immunostaining, for MMP-9. By contrast, MMP-9 was significantly expressed in tissue-infiltrating leukocytes, especially monocyte-macrophages and polymorphonucleated cells. The distribution of MMP-9-positive cells within CCA tissues was similar to that of

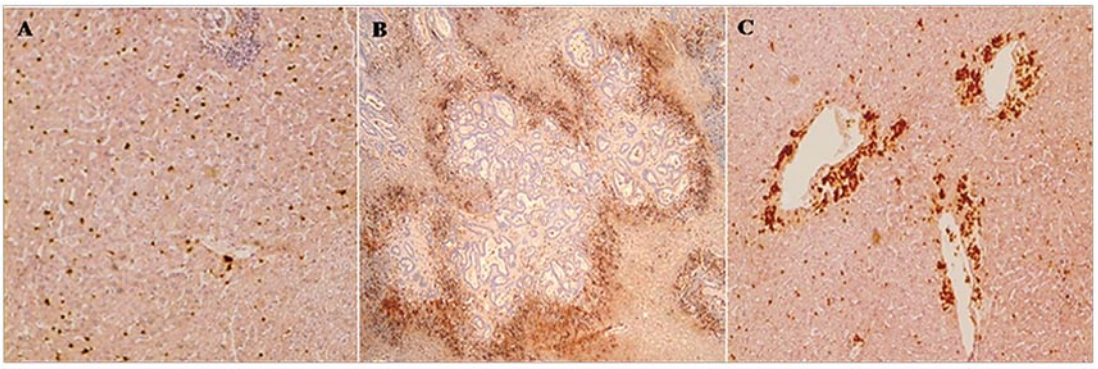

Figure 1. Distribution and density of MAC387-positive cells in CCA tissue detected by immunostaining. (A) Non-malignant liver tissue (x10 HP). (B) Leading edge of invasive tumor (x4 HP). (C) Perivascular areas (x10 HP).

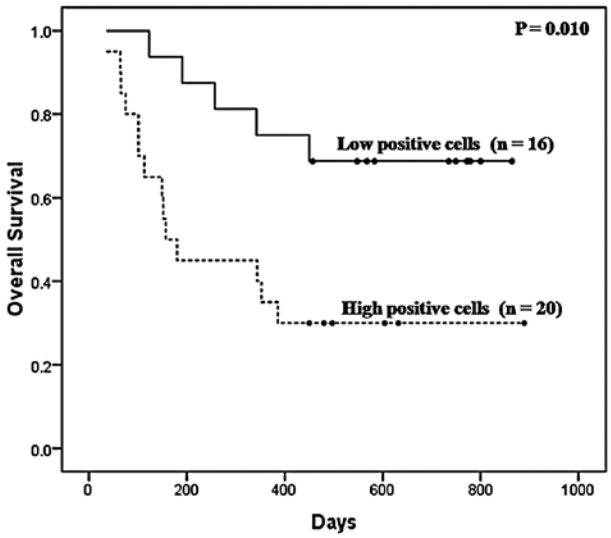

Figure 2. Survival curves using Kaplan-Meier analysis for patients with CCA stages I-III. CCA patients with a low density of MAC387-positive cells had a better survival than those with a high density of MAC387-positive cells. Black circles indicate censor cases.

MAC387-positive cells. High levels of MMP-9-positive cells were found at the CCA tumor invasive edge. A high density of MMP-9-expressing cells was found in 44% (22/50) of the CCA patients, and was significantly associated with a high density of MAC387-positive cells (P<0.001).

Multivariate Cox proportional hazard analysis. Finally, we statistically assessed the overall post-resectional survival of CCA patients in relation to the cell density of MAC387 and MMP-9-positive cells. Multivariate analysis was performed to explore the importance of monocyte/macrophage (MAC387-expressing cells) in comparison to other prognostic parameters. Cox proportional hazards analysis in the multivariate analysis model was performed, taking into account age, histological tumor type, tumor staging, MAC387-positive cell density and the density of cells co-expressing MAC397 and MMP-9 ($MAC387^{+}$ $MMP\text{-}9^{+}$). Only age, tumor type and the density of cells expressing MAC387 and MMP-9 were identified as independent prognostic markers for patients with CCA (Table III).

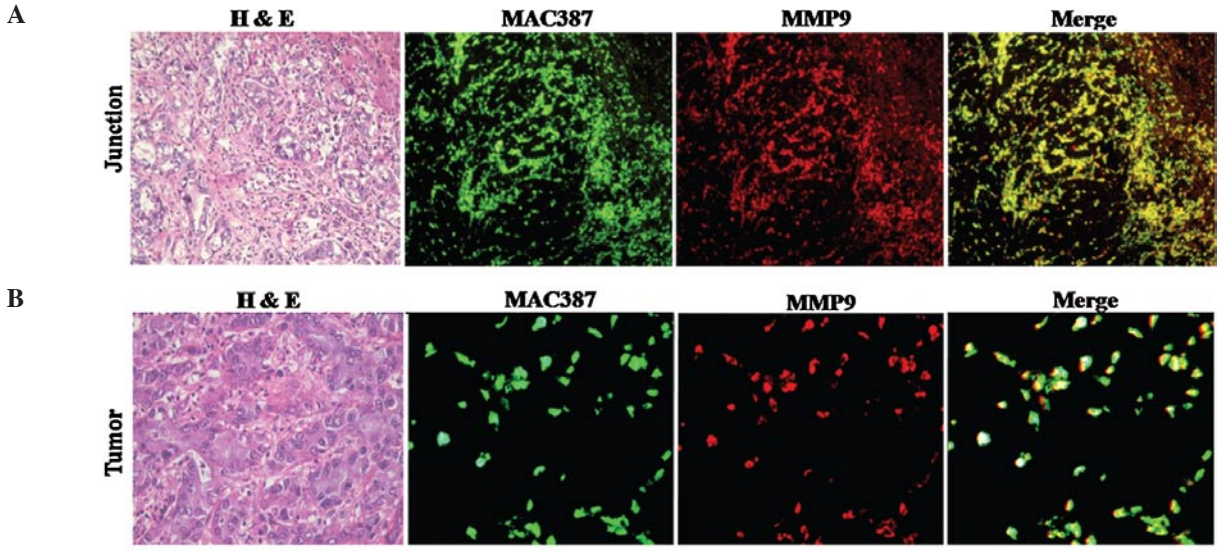

Figure 3. Co-expression of MAC387-positive cells with MMP9 detected by immunostaining at the (A) leading edge junction (x20 HP) and (B) tumor area of cholangiocarcinoma tissue (x40 HP).

Table II. Factors influencing overall survival of cholangiocarcinoma (CCA) patients.

Variable	Hazard ratio	P-value	95% CI
Age			
<56	1		
≥56	1.645	0.161	0.819, 3.301
Gender			
Female	1		
Male	1.079	0.829	0.538, 2.163
Tumor location			
Extrahepatic CCA	1		
Intrahepatic CCA	0.990	0.979	0.484, 2.067
Gross morphology			
Mass forming type	1		
Periductal infiltrating type	0.894	0.757	0.441, 1.815
Intraductal growth type	0.145	0.061	0.019, 1.093
Tumor stage			
I-II	1		
III	4.201	0.023	1.219, 14.471
IV	8.549	0.001	2.390, 30.567
Histology type			
Non-papillary	1		
Papillary	0.262	0.006	0.1001, 0.685
Vascular invasion			
Absent	1		
Present	1.455	0.326	0.688, 3.078
MAC387-positive cells			
Low	1		
High	2.821	0.012	1.259, 6.323

Table III. Multivariate analysis by a Cox proportional hazards regression model in cholangiocarcinoma (CCA) patients.

Variable	Crude hazard ratio	Adjusted hazard ratio	95% CI	P-value[a]
Age				
<56	1	1		
≥56	1.645	2.34	1.09, 5.00	0.02
Histology type				
Non-papillary	1	1		
Papillary	0.262	0.36	0.12, 1.06	0.04
Tumor stage				
I-II	1	1		
III	4.201	2.11	0.55, 8.02	0.08
IV	8.549	3.89	0.99, 15.25	
MAC387-positive cells				
Low	1	1		
High	2.821	0.61	0.18, 2.12	0.43
MAC387 + MMP-9-positive cells				
Low	1	1		
High	5.11	5.55	1.07, 28.76	0.03

[a]Partial likelihood ratio test.

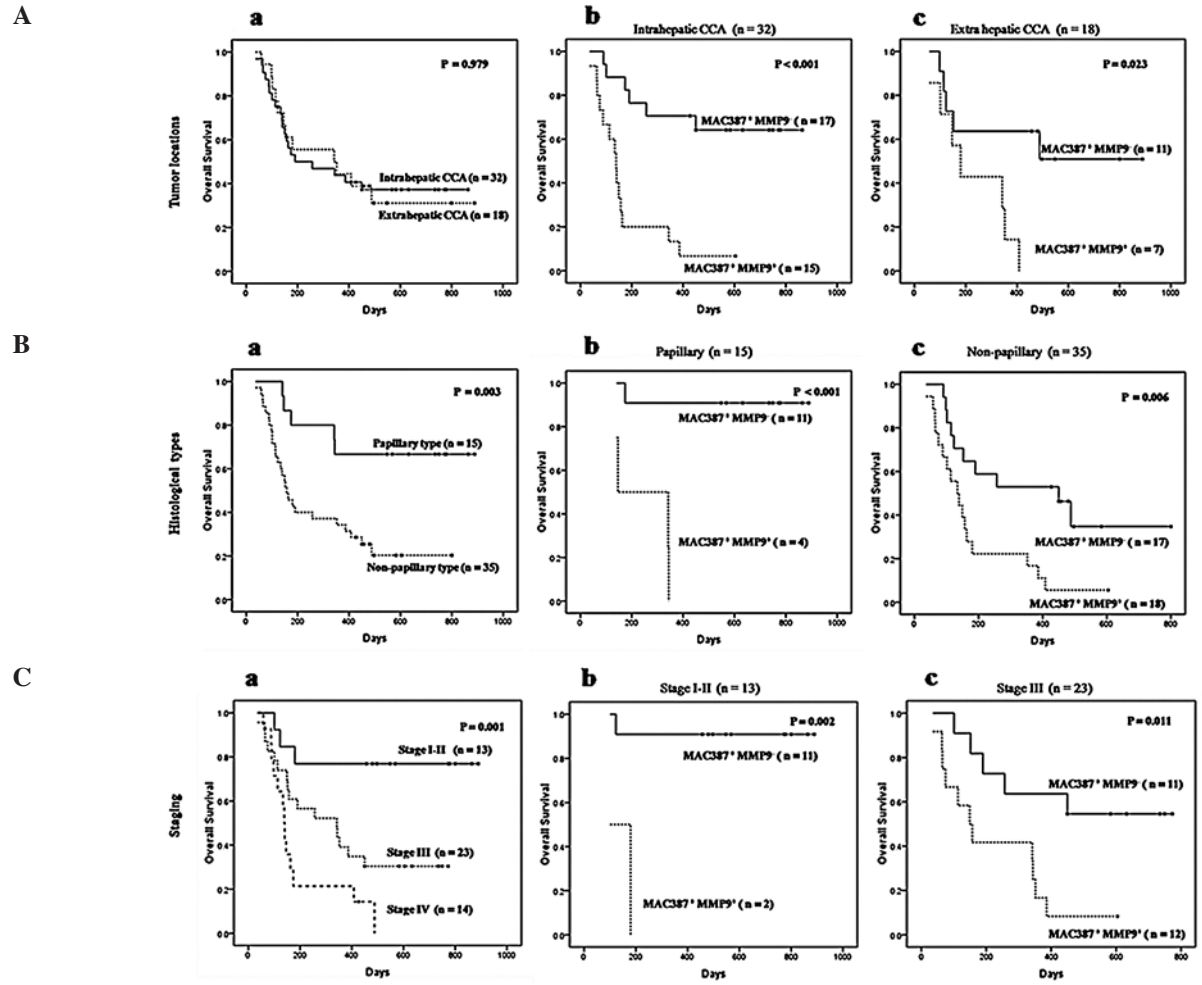

Figure 4. Prognostic significance of MAC387$^+$ MMP-9$^+$ cells in CCA tissue. Cumulative overall survivals was determined using Kaplan-Meier analysis for CCA patients with MAC387$^+$ MMP-9$^+$ cells vs. those with MAC387$^+$ MMP-9$^-$ cells in relation to (A) tumor location, (B) histological type and (C) tumor stage. Black circles indicate censor cases.

MAC387 and MMP-9 expression and poor prognosis. CCAs are heterogeneous. Tumor location, histological type and staging have been shown to have prognostic significance in determining the overall survival of patients. In the present study, multivariate analysis by a Cox proportional hazards regression model indicated that a high density of cells expressing MAC387 in conjunction with MMP-9 in CCA tissue is an independent factor in poor patient outcome. For further analysis, Kaplan-Meier survival curves were plotted in order to investigate the influence of the MAC387$^+$ MMP-9$^+$ cells on the overall survival of patients in relation to each prognostic parameter. Log-rank statistical analysis was used to compare the survival rate in patients with MAC387$^+$ MMP-9$^+$ cells to that in patients with MAC387$^+$ MMP-9$^-$ cells. Regardless of tumor location, histological type and tumor staging, patients with a high density of MAC387$^+$ MMP-9$^+$ cells had a significantly shorter survival than those with MAC387$^+$ MMP-9$^-$ cells (Fig. 4). Mean survival times stratified according to MAC387$^+$ MMP-9$^+$ cells vs. MAC387$^+$ MMP-9$^-$ cells are summarized in Table IV. There was an inverse association between MAC387$^+$ MMP-9$^+$ cells and the survival of patients with intrahepatic CCA. Similar associations were found for patients with extrahepatic CCA, papillary or non-papillary type CCA, or CCA stages I-II and III. However, MAC387$^+$ MMP-9$^+$ cells had no prognostic significance in stage IV patients.

Discussion

Tissue macrophages are the major class of infiltrating leukocytes in solid tumors (1) and are known to play an inhibitory or promotional role in tumorigenesis (1,11,12). In the present study, we determined the degree of MAC387 expression in CCA tissue, which represented a subset of recent blood-derived reactive/infiltrating monocytes/macrophages, but not Kupffer cells (6,13,14). The relationship of these cells to clinicopathological factors and post-operative survival was also examined.

The data demonstrate that a high density of MAC387-positive cells at the leading edge of invasive tumor was significantly associated with the non-papillary, mass forming and periductal infiltrating types of CCA. These observations suggest that a high density of MAC387-positive cells is correlated with an undesirable outcome, since the present study (Table II) and previous reports show that these parameters are poor prognostic indicators of CCA (15-17). The correlation between high levels of MAC387 expression and poor prognosis was again emphasized with the finding that an incremental increase in the density of MAC387-positive cells was inversely related to CCA patient survival. Patients with a high density of MAC387 cells had a significantly worse

Table IV. Comparison of mean survival times of cholangiocarcinoma (CCA) patients according to the presence of MAC387-positive cells with or without co-expressing MMP-9.

Variable	No.	Mean survival time (days)		P-value
		Mean	95% CI	
Intra and extra-hepatic CCA				
Intra-hepatic CCA				
MAC387$^+$ MMP-9$^-$	17	631	478-784	<0.001
MAC387$^+$ MMP-9$^+$	15	177	102-251	
Extrahepatic CCA				
MAC387$^+$ MMP-9$^-$	11	558	344-773	0.023
MAC387$^+$ MMP-9$^+$	7	226	124-329	
Histological types				
Papillary type				
MAC387$^+$ MMP-9$^-$	11	824	702-945	<0.001
MAC387$^+$ MMP-9$^+$	4	243	130-356	
Non-papillary type				
MAC387$^+$ MMP-9$^-$	17	430	282-578	0.006
MAC387$^+$ MMP-9$^+$	18	181	112-250	
Tumor stage				
Stage I-II				
MAC387$^+$ MMP-9$^-$	11	819	689-949	0.002
MAC387$^+$ MMP-9$^+$	2	140	63-217	
Stage III				
MAC387$^+$ MMP-9$^-$	11	526	359-693	0.011
MAC387$^+$ MMP-9$^+$	12	223	127-320	
Stage IV				
MAC387$^+$ MMP-9$^-$	6	242	84-399	0.259
MAC387$^+$ MMP-9$^+$	8	159	86-233	

overall post-resectional survival than those with a low tissue density of MAC387. This indicates the pro-tumorigenic role of these recent blood-derived migrant-macrophages in CCA. The association of TAMs with poor patient outcome has been reported in patients with various types of cancer (18,19).

The pro-tumorigenic functions of TAMs in certain types of cancer are related to their differentiation state as M2-polarized macrophages releasing various factors supporting tumor growth, metastasis, angiogenesis, tissue remodeling and the suppression of adaptive immunity (19-23). One key mechanism by which macrophages promote invasion and metastasis involves the production of protease enzymes for the digestion of the extracellular matrix. This tissue digestion subsequently facilitates cancer cell migration and tissue invasion (23). Serine proteases and MMPs are thought to be the principle activators of MMP precursors *in vivo*. Normally, the proteolytic activities of MMPs are regulated via MMP synthesis balancing between activators and inhibitors of precursor proenzymes. The proteinase-proteinase cascade is initiated by the activation of plasmin by uPA, which then activates proMMP-3 (MMP-3 precursor) to MMP-3. MMP-3 in turn activates MMP-9 (24,25). The association of MMP-9 expression within tumors with tumor invasion, metastasis and poor prognosis for CCA patients has been demonstrated in numerous studies. A similar association and shorter survival of patients with high MMP-9 expression has been observed in hepatocellular and colorectal carcinoma and renal cancers (16-28). The cellular location of these tissue-invasion molecules has always been assumed to be within cancer cells or tissue macrophages. However, in the present study, these factors localized primarily with tissue-invasive MAC387-expressing recent blood-derived monocytes/macrophages.

There is now a large body of evidence implicating tissue macrophages as an important site for the production of MMP-9 during tumor metastasis. Our finding, that CCA specimens with a high density of MAC387-positive cells at the tumor invasive edge was associated with poor prognostic parameters and metastatic stages in CCA, encouraged us to investigate the involvement of MAC387-positive cells in enhancing protease bioactivity – namely MMP-9 expression in CCA tissues. In the present study, we observed a very strong correlation between the presence of high levels of MAC387-positive cells in CCA regions and the high expression of MMP-9. Double immunofluorescence studies confirmed that, in most cases, MMP-9 immunoreactivities co-localized with MAC387-positive cells, indicating that MAC387-expressing macrophages were the major site of tumor-associated MMP-9 expression (Fig. 3).

The recent blood-derived migrant macrophages (MAC387-positive cells) may have direct activity in promoting tissue invasion in CCA. The co-expression of MAC387 with MMP-9 may be responsible for tissue remodeling by degrading the extracellular matrix of surrounding tissue, resulting in a microenvironment favourable to tumor cell invasion. This assumption is supported by the observation that CCA tumor cells rarely expressed tissue invasion molecules (MMP-9) as compared to the MAC387-positive cells. In addition, $MAC387^+$ $MMP\text{-}9^+$ cells were observed in non-tumor tissue adjacent to the tumor front.

In this study, the presence of high levels of MAC387 and MMP-9 in the leading edge of the tumor was an indicator of an unfavorable outcome, with a 3- to 5-fold shorter survival time. Notably, patients whose tissues were negative for these markers had the best survival. The influence of $MAC387^+$ $MMP\text{-}9^+$ cells on the unfavorable outcome of CCA patients was again emphasized in the multivariate analysis, which showed that the presence of $MAC387^+$ $MMP\text{-}9^+$ cells in CCA tissue was an independent prognostic marker in a Cox proportional hazards analysis for the post-resectional survival of CCA patients (Table III). These data favor a model wherein MAC387-positive recent blood-derived macrophages promote tumor invasion by degrading the extracellular matrix via MMP-9 activities. The pro-metastatic role of $MAC387^+$ cells is further supported by our finding that $MAC387^+$ $MMP\text{-}9^+$ cells are an independent prognostic marker for an unfavorable outcome in a CCA regardless of tumor location, type or stage.

Metastasis is a multistep process in which cancer cells spread within local organs and subsequently throughout the body. To facilitate this tissue-invasiom process, tumor cells must have associated tissue degradative enzymes. In the present study, we identified the predominant pro-tumorigenic cell in CCA to be recent blood-derived macrophages co-expressing the macrophage marker MAC387 and the tissue-invasion associated molecule MMP-9. These cells, through their proteolytic activities, may play the most important role in tumor progression in patients with CCA, signified by the shortened survival of patients expressing high levels of these two markers. This is the first study to directly implicate recent blood-derived macrophage expression of tissue invasion molecules in the pathogenesis of metastatic cancer. The study also suggests that approaches directed at this population of tissue invasion-promoting macrophages may be useful as an adjunctive therapy for this class of highly metastatic cancer. For example, recent studies on breast cancer implicated osteopontin (a major macrophage attractant) in metastasis, and suggested that the knockout of osteopontin expression may block metastasis. However, previously osteopontin had mainly been implicated in tissue invasion/inflammatory diseases such as rheumatoid arthritis, another disease highly associated with MAC387 invasion (29-31). Clearly, if CCA (an incurable highly-invasive cancer) is associated with MAC387 invasion, novel immunomodulatory approaches such as those being examined in inflammatory diseases should be studied. As a final point, determining the level of MAC387-expressing cells and their protease product (MMP-9 expression) may be a useful metastatic stage prognostic marker in CCA patients, and lead to the development of novel approaches to address this important tumor invasion-associated cell population.

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