CHAPTER IV

TISSUE INVASIVE MACROPHAGE DENSITY CORRELATES WITH PROGNOSIS IN CHOLANGIOCARCINOMA

4.1 Introduction

Carcinogenesis and tumor progression are multistep processes in which the genetic alterations and modification in malignant cells may help establish tumor-supporting microenvironments. A variety of soluble mediators (e.g., chemokines, cytokines) have been implicated in recruitment of leukocytes into tumor microenvironment, a process that has been associated either with tumor progression or inhibition in different classes of tumor. Although all classes of leukocyte are found within tumors, the most abundant cells are tumor associated macrophages (TAMs) (Balkwill, Mantovani, 2001)

TAMs are diffusely found throughout tumors, in localized zones, tumor edges, around the ductal areas and in the tumor stromal areas (Goede et al., 1999; Lee et al., 1997). Although macrophages under certain conditions can kill tumor cells (antitumor activity), several studies have highlighted their potential role as tumor promoters. The balance between protumorigenic and antitumorigenic properties of macrophages may depend on tumor type and organ site. A close association between a dense macrophage infiltration at the tumor front and a positive prognosis in colorectal and prostate cancer has been reported (Shimura et al., 2000; Sickert et al., 2005). In contrast, high TAM content in breast, gastric, endometrial, oral squarmous cell and ovarian cancers (Li et al., 2002; Ohno et al., 2004; Orre, Rogers, 1999; Tsutsui et al., 2005; Valkovic et al., 2002) have been associated with poor prognosis.

TAMs derive from circulating monocytes that infiltrate the tumor and differentiate to macrophages (Balkwill, Mantovani, 2001; Crowther et al., 2001). MAC387, a marker expressed in circulating neutrophils and monocytes, is a macrophage marker that distinguishes resident tissue macrophages from newly arrival blood migrants (Brandtzaeg et al., 1988; Chilosi et al., 1990; Goebeler et al., 1994). Antibodies to this marker recognize the L1 protein identical with myeloid related

protein (mrp8/14) complex. Most chronic inflammatory lesions including rheumatoid arthritis synovial membranes, sarcoid granulomas, and perivascular (peri-portal) macrophages associated with chronic hepatitis C infection contain significant numbers of MAC387 positive cells (Chilosi et al., 1990; McGuinness et al., 2000).

Cholangiocarcinoma (CCA), a slow growing but highly metastatic tumor, is highly prevalent in Northeast Thailand (Sripa, Pairojkul, 2008). 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 (Bhamarapravati, Thamavit, 1978; Vatanasapt et al., 1990). The role of infiltrating leukocytes in carcinogenesis and progression of experimental CCA is supported by the high levels of infiltrating leukocytes within tumor tissues of OV-associated CCA hamsters (Pinlaor et al., 2004). Human intrahepatic CCA has been associated with high expression of granulocyte colony stimulating factor (G-CSF) and granulocyte/macrophage colony stimulating factor (GM-CSF) in tumor epithelium, suggesting at least one mechanism for recruitment of neutrophile/monocytes to sites of tumor (Sasaki et al., 2003).

No study has been performed on CCA to test whether this class of tumor contains tumor infiltrating macrophages. Whether these macrophages play role in protumorigenic or antitumorigenic activities are not clear. In the current study, we report the association of newly infiltrated tissue macrophages, (MAC387 positive cells), and TAM expressed factors (plasminogen activator urokinase (uPA) and matrix metalloproteinase-9; MMP-9) that are associated poor prognosis in patients with CCA. These data suggest that in contrast to models implicating tumor cells directly in tissue invasion, in patient with CCA, it appears that the TAMs directly facilitate this invasive process.

4.2 Materials and Methods

4.2.1 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 subjects and the Human Research Ethics Committee, Khon Kaen University approved the research

protocol (HE471214 & HE480312). The age, gender, tumor location, histological grading, and pTNM stage (Greene, 2002) were evaluated by reviewing the medical charts and pathological records. Survival of each CCA patient was recorded from the date of surgery to the date of death or to June 13, 2008.

4.2.2 Immunohistochemistry studies

All 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), rabbit polyclonal anti-human plasminogen activator urokinase; uPA (Abcam, Cambridge,USA) and rabbit monoclonal anti-human matrix metalloproteinase-9; MMP-9 (Dako, Glostrup, Denmark). 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 uPA or 1:100 anti-human MMP-9 overnight followed by the addition of Envision labeled polymer peroxidase (Dakocytomation, Glostrup, Denmark) for 30 min. After washing, the sections were reacted with liquid 3,3'-diaminobenzidine tetrahydrochloride (DAB) substrate chromogen system (Dakocytomation, Glostrup, Denmark). All slides were counterstained with Mayer's hematoxylin. Paraffin embedded sections of human tonsil were used as a positive control for MAC387 staining, whereas colon cancer tissues sections were used as positive controls for uPA and MMP-9 staining.

The densities of MAC387, uPA and MMP-9 positive cells at the lead edge of invasive tumor were semi-quantitatively classified into 4 scoring categories: 0 = negative; 1^+ = 1-25%; 2^+ = 26-50%; and 3^+ = >50% (Figure 4.1). The specimens were evaluated by 2 researchers without any knowledge about prognosis or clinicopathologic variables. For statistical analysis, the scores 0 and 1^+ were categorized as low expression or negative and score 2^+ and 3^+ were summed as high expression or positive.

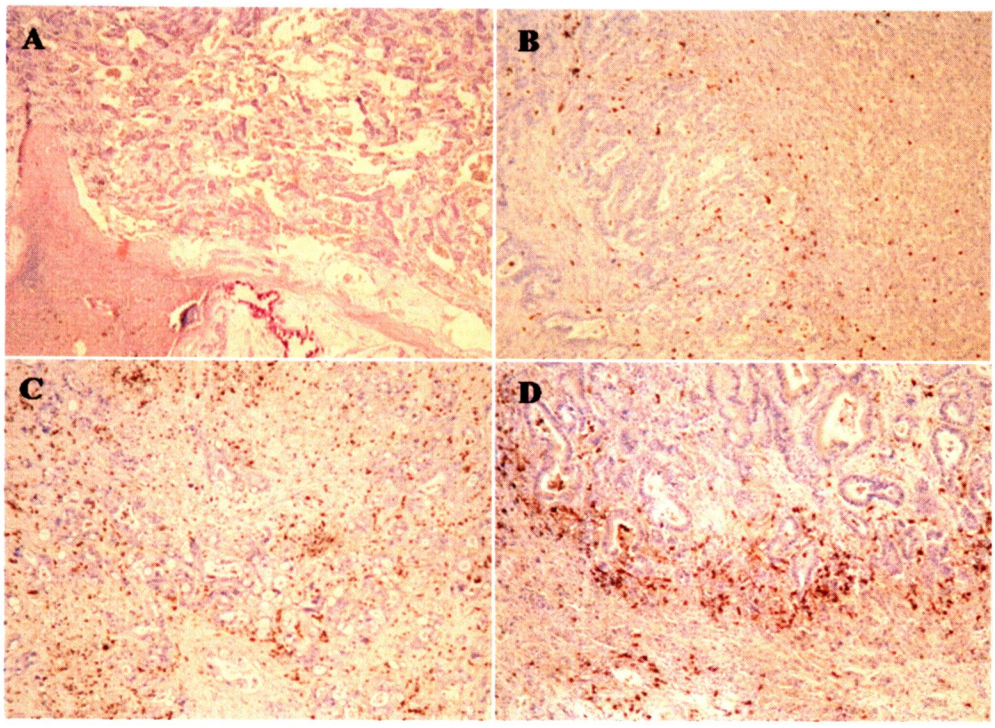

Figure 4.1 Examples of MAC387 positive cells at leading edge of invasive CCA tumor tissues, representing the different grades of macrophage infiltration; (A), 0 = negative; (B), 1^+ = 1-25%; (C), 2^+ = 26-50% and(D) 3^+ = >50% (100X HP).

4.2.3 Double immunofluorescence-labeling method

To verify the expression of human myeloid/histocyte antigen (MAC387) co-localized with uPA or MMP9, we performed a modified double immunofluorescence-labeling method to examine the co-localization of human myeloid/histocyte antigen (MAC387) with uPA or MMP-9. Heat mediated antigen retrieval was performed in a pressure cooker with citrate buffer pH 6, 0.05% tween20, for 3 min. The sections were cooled down to room temperature for 30 min and incubated with the primary antibodies at room temperature, overnight. The co-localization of MMP-9 and MAC387 in CCA tissue was assessed by using 1:100 of rabbit polyclonal anti-human MMP-9 and 1:200 of mouse monoclonal anti-MAC387. Co-localization of uPA and MAC387 was detected with 1:100 of rabbit polyclonal anti-human uPA and 1:100 of mouse monoclonal anti-MAC387. The sections were incubated for a further 3 hrs 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, Oregon, USA). The stained sections were examined using a fluorescence microscope.

4.2.4 Statistical analysis

Statistic analyses were done using SPSS statistical software version16.0.1 (SPSS Inc., Chicago, IL) and STATA version 8. Cross tabulations were analyzed with χ^2-test for the associations between MAC387, uPA and MMP-9 expression with clinic-pathological features of CCA patients. Kaplan-Meier survival analysis was used to estimate the disease-specific survival and comparison between groups were done with a log-rank test. Multivariate survival analysis was used to investigate the importance of MAC387, uPA and MMP-9 expression in comparison with other prognostic parameters. $P < 0.05$ was considered statistically significant.

4.3 Results

Of the 50 CCA tissues examined, the mean age of the CCA patients was 56 years (range: 33 to 75 years). The ratio of men to women was 1.17 to 1. The studied tissues were obtained from CCA patients who underwent surgical resection of hepatogastrointestinal cancer, of which 64% were intrahepatic CCA, and 36% were extrahepatic CCA, including perihilar and distal common bile duct CCA. Based on the classification of American Joint Committee on Cancer (Greene, 2002), 74% (37/50) of the patients were at stages III and IV.

4.3.1 MAC387 positive cells in CCA tissues and clinical significance

MAC387 positive cells were distributed throughout the tumor involved liver tissue including the leading edge of invasive tumor, tumor parenchyma and perivascular areas (Figure 4.2). 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 (Figure 4.2B). MAC387 positive cells also appeared at regions of necrosis. Although the highest frequency of MAC387 expressing cells were at the tumor edge, MAC387 positive cells were also frequently present in perivascular areas of tumors (Figure 4.2C). As MAC387 stains blood derived macrophage migrants, these perivascular cells may represent the most recent blood derived monocyte into CCA tissues

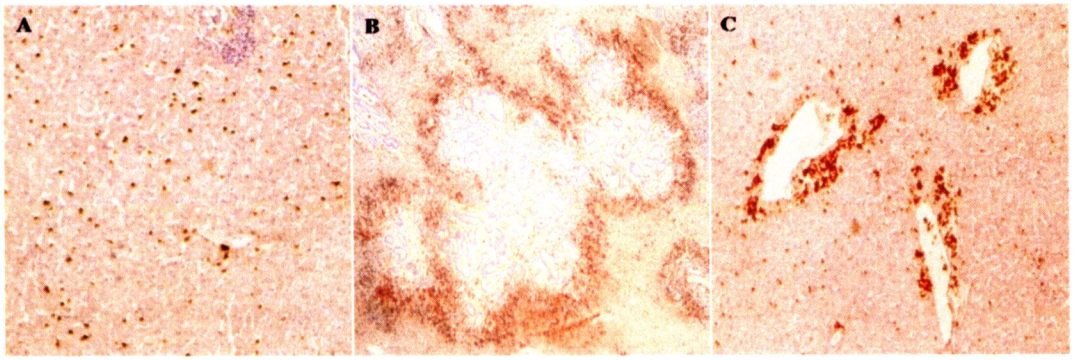

Figure 4.2 Distribution and density of tissue MAC387 positive cells. Immunostaining of MAC387 positive cells at different areas of CCA tissue: (A) non tumorous liver tissue (100X HP), (B) leading edge of invasive tumor (40X HP), and (C) perivascular areas (100X HP).

Univariate analysis was used to investigate the correlation between the density of MAC387 positive cells and clinicopathological parameters. High numbers of MAC387 positive cells were significantly association with: the non-papillary type of CCA ($P < 0.05$), mass forming and periductal infiltration type of CCA ($P = 0.047$), high uPA expression ($P < 0.001$) and high MMP-9 expression ($P < 0.001$) (Table 4.1). There was no correlation between density of MAC387 positive cells and gender, age, tumor location, tumor staging and vascular invasion. A trend of increasing MAC387 positive cells with increasing tumor stage was observed, however this trend was not statistically significant ($P = 0.09$).

Table 4.1 Density of MAC387 positive cells in CCA tissues in relation to clinicopathological features of patients

Variable		Number	MAC387 positive cells (%)				*P* value
			Low		High		
Age:	< 56	26	9	(34.6)	17	(65.4)	0.608
	≥ 56	24	10	(41.7)	14	(58.3)	
Sex:	Female	23	9	(39.1)	14	(60.9)	0.879
	Male	27	10	(37.0)	17	(63.0)	
Tumor location:	Intrahepatic CCA	32	13	(40.6)	19	(59.4)	0.61
	Extrahepatic CCA	18	6	(33.3)	12	(66.7)	
Tumor type:	Mass forming type	24	7	(29.2)	17	(70.8)	0.047
	Periductal infiltrating type	20	7	(35.0)	13	(65.0)	
	Intraductal Growth type	6	5	(83.3)	1	(16.7)	
Tumor Stage:	I-II	13	8	(61.5)	5	(38.5)	0.054
	III-IV	37	11	(29.7)	26	(70.3)	
Histology type:	Non-Papillary	35	9	(25.7)	26	(74.3)	0.006
	Papillary	15	10	(66.7)	5	(33.3)	
Vascular invasion:	Absent	37	14	(43.2)	21	(56.8)	0.198
	Present	13	3	(23.1)	10	(76.9)	
MMP9 positive cells:	low	28	18	(64.3)	10	()35.7	< 0.001
	High	22	1	(4.5)	21	()95.5	
uPA positive cells:	low	27	17	(63.0)	10	(37.0)	< 0.001
	High	23	2	(8.7)	21	(91.3)	

4.3.2 uPA and MMP-9 expressing cells in CCA tissues

Although both uPA and MMP-9 identify cells with tissue invasion characteristics, these antigens were rarely observed in CCA cells. Only 6% (3/50) of CCA tissues were positive with weakly immunostaining of MMP-9. However, uPA and MMP-9 antigens were significantly expressed within CCA tumor tissue infiltrating leukocytes, especially polymorphonucleated cells and monocyte-macrophages. The distribution of uPA and MMP-9 positive cells within CCA tissues was similar to that of MAC387 positive cells. High levels of uPA and MMP-9 positive cells were found at the CCA tumor invasive edge. High densities of uPA and MMP-9 expressing cells in CCA tissues were found in 46% (23/50) and 44% (22/50) of the patients, respectively. No correlation between the expression levels of uPA or MMP-9 positive cells with clinicopathological feature of CCA patients were observed except for high density MMP-9 expressing cells correlating with advanced-metastatic stage tumors (stages III and IV). Notably, high uPA and MMP-9 expression was significantly associated with high densities of MAC387 positive cells (Tables 4.2 and 4.3).

4.3.3 Co-expression of uPA and MMP-9 with MAC387 positive cells in CCA tissues

Double immunofluorescent analyses were performed on CCA tissues with the MMP-9 or uPA antibodies together with a macrophage specific antibody that identifies recent blood derived tissue migrants (MAC387). The uPA and MMP-9 were expressed in mononuclear cells located at the tumor leading edge and in tumor regions involving MAC387 positive cells (Figure 4.3 A and B). Serial sections of CCA tissues stained with all three antibodies demonstrated the co-expressions of uPA and MMP-9 with MAC387 positive cells. MAC387 positive cells with co-expression of uPA and MMP-9 were found most intensely at the tumor leading edge and tumor involved tissue areas (Figure 4.4). Scattered MAC387 positive cells with MMP-9 but not uPA were observed in non-tumor tissue.

Table 4.2 Density of uPA positive cells in CCA tissues in relation to clinicopathological features of patients

Variable		Number	uPA positive cells (%)				*P* value
			Low		High		
Age:	< 56	26	14	(53.8)	12	(46.2)	0.982
	≥ 56	24	13	(54.2)	11	(45.8)	
Sex:	Female	23	11	(47.8)	12	(52.2)	0.419
	Male	27	16	(59.3)	11	(40.7)	
Tumor location:	Intrahepatic CCA	32	19	(59.4)	13	(40.6)	0.309
	Extrahepatic CCA	18	8	(44.4)	10	(55.6)	
Tumor type:	Mass forming	24	13	(54.2)	11	(45.8)	0.255
	Periductal infiltrating type	20	9	(45.0)	11	(55.0)	
	Intraductal Growth type	6	5	(83.3)	1	(16.7)	
Tumor Stage:	I-II	13	9	(69.2)	4	(30.8)	0.338
	III-IV	37	27	(18.0)	66.6	(19.0)	
Histology type:	Non-Papillary	35	16	(45.7)	19	(54.3)	0.073
	Papillary	15	11	(73.3)	4	(26.7)	
Vascular invasion:	Absent	37	22	(59.5)	15	(40.5)	0.191
	Present	13	5	(38.5)	8	(61.5)	
MAC387 positive cells:	low	19	17	(89.5)	2	(10.5)	<0.0001
	High	31	10	(32.3)	21	(67.7)	
MMP9 positive cells:	low	28	24	(85.7)	4	(14.3)	<0.0001
	High	22	3	(13.6)	22	(46.0)	

Table 4.3 Density of MMP9 positive cells in CCA tissues in relation to clinicopathological features of patients

Variable		Number	MMP9 positive cells (%)				*P* value
			Low		High		
Age:	< 56	26	14	(53.8)	12	(46.2)	0.749
	≥ 56	24	14	(58.3)	10	(41.7)	
Sex:	Female	23	12	(52.2)	11	(47.8)	0.615
	Male	27	16	(59.3)	11	(40.7)	
Tumor location:	Intrahepatic CCA	32	17	(53.1)	15	(46.9)	0.585
	Extrahepatic CCA	18	11	(61.1)	7	(38.9)	
Tumor type:	Mass forming type	24	10	(41.7)	14	(58.3)	0.107
	Periductal infiltrating type	20	13	(65.0)	7	(35.0)	
	Intraductal Growth type	6	5	(83.3)	1	(16.7)	
Tumor Stage:	I-II	13	11	(84.6)	2	(15.4)	0.036
	III-IV	37	17	(46.0)	20	(54.0)	
Histology type:	Non-Papillary	35	37	(48.6)	38	(52.4)	0.106
	Papillary	15	11	(73.3)	4	(26.4)	
Vascular invasion:	Absent	37	21	(56.8)	16	(43.2)	0.856
	Present	13	7	(53.3)	6	(46.2)	
MAC387 positive cells:	low	19	18	(94.7)	1	(5.3)	<0.0001
	High	31	10	(32.3)	21	(67.7)	
uPA positive cells:	low	27	24	(88.9)	3	(11.1)	<0.0001
	High	23	4	(56.0)	19	(44.0)	

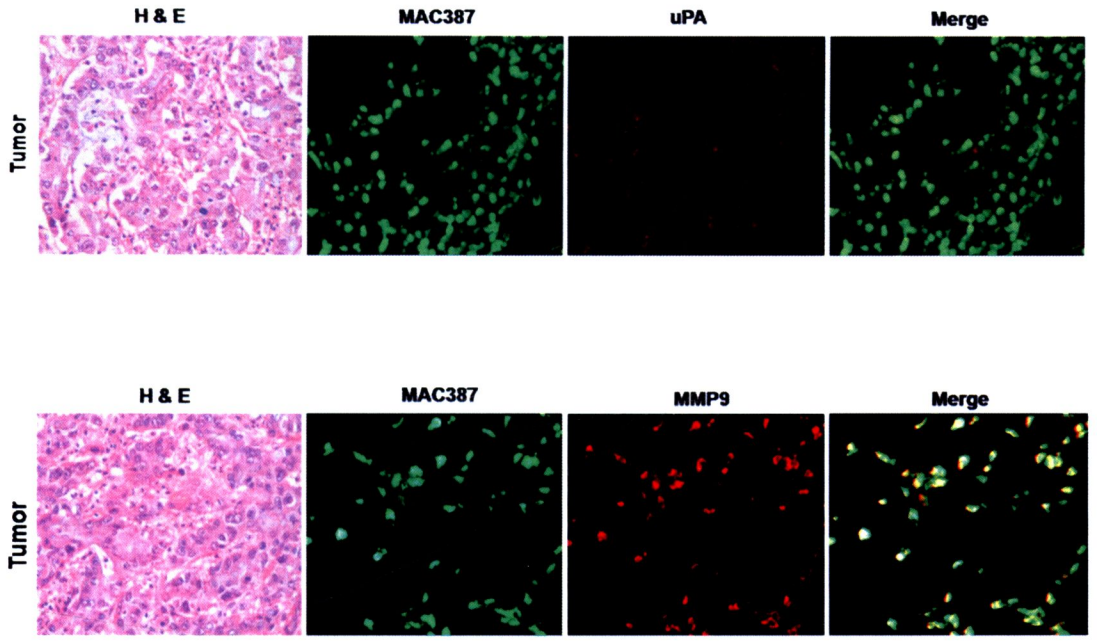

Figure 4.3 Immunofluorescent staining indicates co-expression of MAC387 positive cells with uPA (A) and MMP-9 (B) in Cholangiocarcinoma tissue (20 HP).

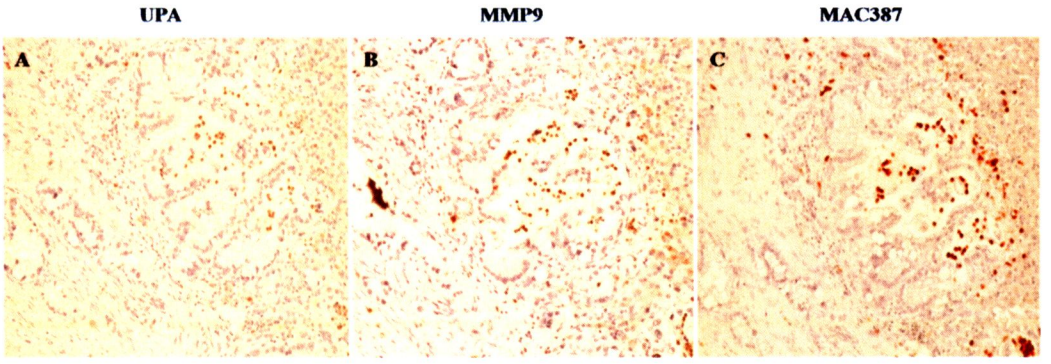

Figure 4.4 Immunostainings of MAC387, MMP-9, and uPA positive cells in the serial sections of cholangiocarcinoma tissues. MAC387 (A), uPA (B) and MMP-9 (C) expressing cells were found in the same tumor area. Immunoreactivity of uPA and MMP-9 was rarely detected in CCA cells.

4.3.4 MAC387, uPA and MMP-9 expressing cells and correlation with overall survival of CCA patients

Kaplan-Meier and log-rank tests were used to determine the overall survival of CCA patients who had low vs. high expression of MAC387, or uPA or MMP-9 at the tumor edge in CCA tissues. No patient with perioperative death (survival under 30 days) was included in this analysis. Overall, post-resectional survival of CCA patients was significantly reduced in patients with high levels of CCA tissue MAC387 positive cells (mean survival of 326 days; 95% CI, 245-438 days) when compared with those who had a low density of tissue MAC387 positive cells (mean survival of 590 days; 95% CI, 442–739 days) ($P = 0.009$, Figure 4.5A). Similar outcomes were also observed for patients whose tumor contained high tissue uPA or MMP-9 expressing cells (Figure 4.5 B and C). Log Rank analysis indicated that CCA patients with high tissue uPA had an unfavorable survival with mean survival of 259 days (95% CI, 151–367 days) compared to those with the low tissue uPA (mean survival of 571 days; 95% CI; 444–698 days) ($P < 0.0001$). Patients with high tissue MMP-9 expressing cells had shorter survival with mean survival of 192 days; (95% CI; 132–252 days), than those with low tissue MMP-9 positive cells (mean survival of 614 days; 95% CI, 486–743 days) ($P < 0.0001$). Finally, the overall post-resectional survival of CCA patients was statistically assessed with cell densities of MAC387 in combination to those of uPA and MMP-9 (Figure 4.5D). Patients with high tissue expression levels of MAC387, uPA and MMP-9 ($MAC387^+$ and uPA^+ and $MMP\text{-}9^+$; $n = 18$) had the worst survival with the mean survival of 187 days (95% CI, 119–254 days) comparing with those whose tumor tissue exhibited either high MAC387 and high uPA or high MAC387 and high MMP-9 ($MAC387^+$ with uPA^+ or $MMP\text{-}9^+$; $n = 15$) with a mean survival of 457 days; 95% CI, 277–637 days) or those who had low cell density of MAC387, uPA and MMP-9 ($MAC387^-$, uPA^- and $MMP\text{-}9^-$; $n = 17$) with a mean survival of 634 days (95% CI, 483-785 days) ($P < 0.0001$).

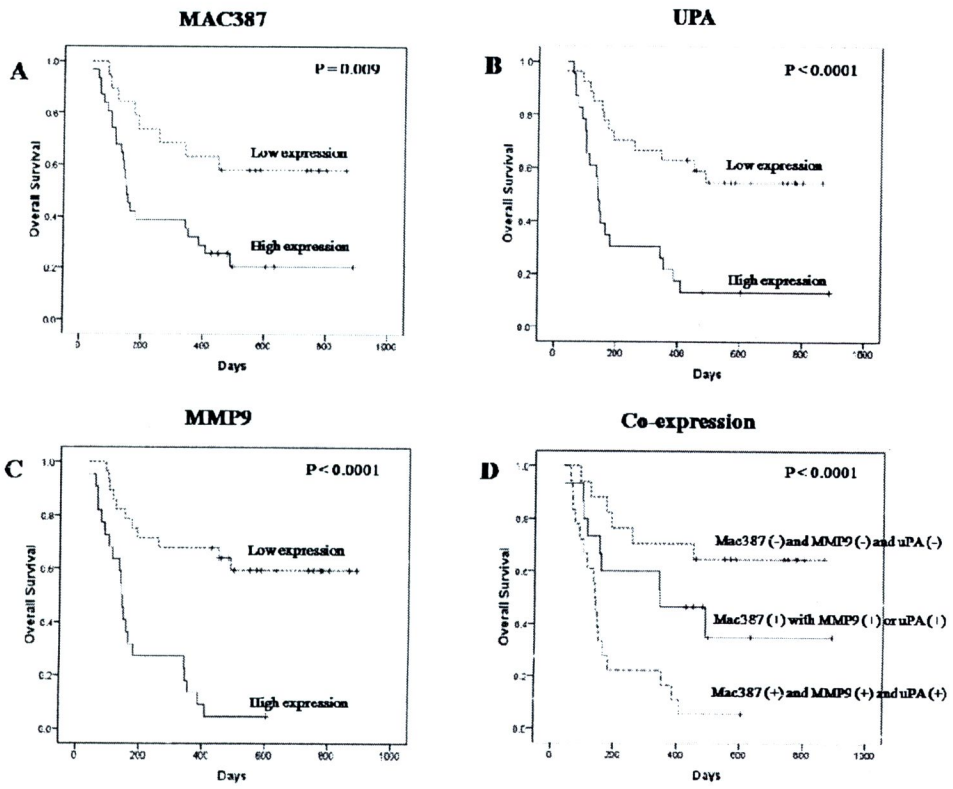

Figure 4.5 Survival curves using Kaplan-Meier analysis for CCA patients with low vs high densities of cells with positive immunoreactivity of MAC387 (A); uPA (B) MMP-9 (C) and different combinations of MAC387, uPA and MMP-9 positive cells (D).

4.3.5 Univariate and Multivariate Cox proportional hazard analysis

Of the 50 patients included in survival analysis, age, sex, tumor location, tumor type and vascular invasion had no influence on survival. However, overall survival was decreased significantly in patients with non-papillary type CCA, advanced stage tumors (stages III and IV), or high density of MAC387, uPA and MMP-9 (Table 4.4). Multivariate analysis was performed on all of the data obtained in the current study to explore the importance of MAC387, uPA and MMP-9 expression in comparison with other prognostic parameters. Cox proportional hazard analyses in multivariate analysis model was done including age, histological type of tumor, tumor staging, densities of cells with MAC387, uPA and MMP-9 positive cells at the invasive front. Only age, tumor type and density of MMP-9 positive cells were identified as independent prognostic markers for patients with CCA (Table 4.5).

Table 4.4 Factors influencing overall survival of CCA patients

Variable	Hazard ratio	*P* value	95% CI
Age: < 56	1		
≥ 56	1.645	0.161	0.819, 3.301
Sex: 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
Tumor type: 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	2.39, 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
uPA positive cells: low	1	0.001	1.705, 7.366
High	3.544		
MMP9 positive cells: low	1		
High	5.11	<0.0001	2.373, 11.014

Table 4.5 Multivariate analysis by a Cox proportional hazard regression model in CCA patients

Variable		Crude HR	Adjusted HR	95%CI	*P* value*
Age: < 56		1	1	1.09, 5.00	0.0288
≥ 56		1.645	2.34		
Histology type:	Non-Papillary	1	1	0.12. 1.06	0.0475
	Papillary	0.262	0.36		
Tumor Stage:	I-II	1	1		
	III	4.201	2.11	0.55, 8.02	0.0851
	IV	8.549	3.89	0.99, 15.25	
MAC387 positive cells:	low	1	1	0.18, 2.12	0.4335
	High	2.821	0.61		
uPA positive cells:	low	1	1	0.25. 3.97	0.985
	High	3.544	0.98		
MMP9 positive cells:	low	1	1	1.07, 28.76	0.0325
	High	5.11	5.55		

* = partial likelihood ratio test

4.4 Discussion

Tissue macrophages are known to be the major class of infiltrating leukocytes in solid tumors (Balkwill, Mantovani, 2001) and are accepted as playing either inhibitory or promotional roles in tumorigenesis.(Balkwill, Mantovani, 2001; Mantovani et al., 2004; Van Ginderachter et al., 2006). In the present study, we investigated the degree of MAC387 positive cells (marker for a subset of recent blood derived reactive/infiltrating monocytes/macrophages, but not Kupffer cells) (Bardadin et al., 1991; Brandtzaeg et al., 1988; Pulford et al., 1989) in CCA tissues and the relationship of these cells to clinicopathological factors and postoperative survival.

Our data demonstrated that a high frequency of MAC387 positive cells at the leading edge of invasive tumor was associated significantly with nonpapillary-, mass forming- and periductal infiltrating- types of CCA. Although not significant, a higher density of MAC387 positive cells were found in advanced-stage tumors than in early stage tumors ($P = 0.054$). These observations implied that a high frequency of MAC387 positive cells was correlated with an undesired outcome as these parameters have previously been reported to be poor prognostic parameters of CCA (Isa et al., 2001; Trishe Y.-M. Leong et al., 2007; Uenishi et al., 2001). The linkage between high level of MAC387 expression with poor prognosis was again emphasized with the finding that an incremental increase in the density of MAC387 positive cells was inversely related to CCA patients' survival. Patients with a high tissue density of MAC387 had a significantly worse overall post-resectional survival than those with a low tissue density of MAC387. This result is consistent with previous studies reported for patients with breast, pancreatic, gastric and endometrial cancers (Hashimoto et al., 2000; Ishigami et al., 2003; Mantovani et al., 2002; Ohno et al., 2005; Ohno et al., 2004; Tsutsui et al., 2005; Valkovic et al., 2002) and indicates the protumorigenic role of these newly blood derived migrant-macrophages in CCA.

The pro-tumorogenic functions of TAMs in certain cancers are related to their differentiation state as M2-polarized macrophages (Mantovani et al., 2004; Mantovani et al., 2002) that release various factors supporting tumor growth, angiogenesis, tissue remodeling and suppress adaptive immunity.(Ben-Baruch, 2003; Bingle et al., 2002; Leek, Harris, 2002; Mantovani et al., 2002; Pollard, 2004).

The role of macrophages in promoting cancer metastasis has also been described. One key mechanism by which macrophages promote invasion and metastasis involves production of protease enzymes for digestion of extracellular matrix. This tissue digestion then facilitates cancer cells migration and tissue invasion (Pollard, 2004). 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 synthesis of MMPs balancing between activators and inhibitors of precursor proenzymes. The proteinase-proteinase cascade starts from the activation of plasmin by uPA which then activates proMMP3 (MMP3 presursor) to MMP3, and then MMP3 activates MMP-9 (Morodomi et al., 1992; Ogata et al., 1995). Activation of MMP-9 via a plasmin cascade was shown in breast cancer cell lines to enhance tumor cell invasion (Ramos-DeSimone et al., 1999). The association of MMP-9 expression within tumors with tumor invasion, metastasis and poor prognosis of CCA patients has been demonstrated in many studies (Jo Chae et al., 2004; Mon et al., 2006; Shirabe et al., 1999; Terada et al., 1996). Similarly, high uPA and MMP-9 expression within tumors has been shown to be associated with increasing tumor metastasis in hepatocellular carcinoma, colorectal carcinoma and renal cancers (Illemann et al., 2006; Inuzuka et al., 2000; Kim et al., 2006) and with shorter survival of CCA patients as shown in the present study.

There is now a large body of evidence implicating tissue macrophages as an important site for the production of uPA and MMP-9 during tumor metastasis.(Khan, Falcone, 1997). Our findings that CCA specimens with a high frequency of MAC387 positive cells at the tumor invasive edge was associated with poor prognostic parameters and metastatic stages of CCA (stages III and IV) encouraged us to investigate the involvement of MAC387 positive cells in enhancing protease bioactivity-namely uPA and MMP-9 expression in CCA tissues. In the current study we observed a very strong correlation between the presence of high levels of MAC387 positive cells in CCA regions that were also high level expressions of uPA and MMP-9. Double immunofluorescence studies confirmed that for most cases, uPA and MMP-9 immunoreactivities co-localized with MAC387 positive cells, indicating that MAC387 expression macrophages were the major site of tumor associated uPA and MMP-9 expression. In our study, CCA tumor cells were rarely expressed these

tissue invasion molecules comparing to the MAC387 positive cells. These data favor a model wherein MAC387 positive recently blood derived macrophages promote tumor invasion by degrading extracellular matrix via uPA and MMP-9 activities. This assumption is supported by the observation in this study that a high density of MMP-9 positive cells (macrophages) was highly associated with tumor metastasis/invasion in advanced stage-CCA.

Studies using the macrophage cell line THP-1 suggested a mechanism by which uPA and MMP-9 promote extracellular matrix degradation (Menshikov et al., 2002). Binding of uPA to its membrane receptor activated cell signaling which upregulated MMP-9 expression the release of MMP-9 protein. Therefore, uPA plays a role in activation of macrophage MMP-9. In the current study, coexpression of high level tissue MAC387, or uPA or MMP-9 positive cells at tumor front area was a sign of unfavorable outcome with a 3-5 fold shorter survival in patients co-expressing all three markers. The survival of patients was worst in the MAC387 positive group with combination of uPA and MMP-9 positive cells comparing to those with MAC387 positive and either uPA or MMP-9 positive. Notably, patients whose tissues were negative for all three markers had the best survival. However, multivariate analyses showed that only the level of MMP-9 positive cells (a molecule with direct activity promoting tissue invasion), age more than 56 years and nonpapillary type CCA were shown to be independent prognostic markers in Cox proportional hazard analyses for post-resectional survival of CCA patients.

Metastasis is a multistep process in which the cancer cells spread within local organs and subsequently throughout the body. This tissue invasive process requires tumor cells to have associated tissue degradative enzymes to facilitate this process. In the present study, we have identified the predominant pro-tumorigenic cell with CCA to be recent blood derived macrophages co-expressing the macrophage marker MAC387 as well as the tissue invasion associated molecules uPA and 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 all three markers. This is the first study to directly implicate recent blood derived macrophages expression 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. As a final point, tracking the level of tissue MAC387 expressing cells and their protease products (uPA and MMP-9 expression) may be a useful predictive factor for metastatic stage prognostic marker of CCA patients and for monitoring novel approaches addressing this important tumor invasion associated cell population.