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Prognostic Impact of CD163+ Macrophages in Tumor Stroma and CD8+ T-Cells in Cancer Cell Nests in Invasive Extrahepatic Bile Duct Cancer

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Abstract. *Aim: The aim of this study was to examine the clinicopathological influence of tumor-infiltrating cluster of differentiation (CD) 163+ macrophages and CD8+ T-cells, and to clarify the prognostic effects of these cells in patients with invasive extrahepatic bile duct cancer (EHBC). Materials and Methods: The numbers of CD8+ T-cells in cancer cell nests and CD163+ macrophages in tumor stroma were evaluated using immunohistochemistry in 101 resected EHBC specimens. Correlations with clinicopathological variables and overall survival were analyzed. Results: Perihilar EHBC and perineural invasion were significantly associated with a low number of tumor-infiltrating CD8+ T-cells. Poorly- differentiated histology and nodal metastasis were significantly associated with a high number of tumor-infiltrating CD163+ macrophages. A combination of high number of CD8+ T-cells and low number of CD163+ macrophages was independently related to better overall survival in the whole patient cohort (hazard ratio=0.127, p<0.001) and in patients treated with adjuvant chemotherapy (hazard ratio=0.139, p=0.021). Conclusion: Infiltrating CD163+ macrophages in tumor stroma and CD8+ T-cells in cancer cell nests have a prognostic impact in patients with EHBC following resection and also after adjuvant chemotherapy.*

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The incidence of extrahepatic bile duct cancer (EHBC) is rising in all racial groups (1). Survival in EHBC is extremely low, with many cases of recurrence and death after surgical resection (2, 3), while chemotherapy for biliary tract cancer has not given satisfactory results (4). In these circumstances, new therapeutic options are required. Immunotherapy for activation of natural elimination of cancer cells is one promising approach that has been shown to have potent effects in other cancer types (5).

Tumors contain cancer cells and recruit apparently normal cells, creating the tumor microenvironment (6). This microenvironment has many immune cells, among which macrophages are the most abundant (7). Macrophages are prominent in stromal compartments of tumors, and interactions between tumor cells and the associated stroma strongly influence cancer progression and prognosis (8, 9). Macrophages have two distinct patterns of polarized activation, which are recognized as M1 and M2, corresponding to T-helper type 1 and 2 immune response (10). Plasticity is also present between M1-like tumoricidal macrophages and M2-like pro-tumoral macrophages (10). Cluster of differentiation (CD) 163 was shown to be useful marker for M2-like macrophages in patients with intrahepatic cholangiocarcinoma (11). In pancreatic cancer, high numbers of infiltrating CD163+ macrophages in tumor stroma are a poor prognostic indicator (12, 13).

Infiltrating CD8+ T-cells in cancer cell nests are related to the effector function of activated cytotoxic T-cells and are predictors of better survival (14). This finding has also been related to favorable survival in EHBC (15, 16). However, the prognostic impact of tumor-infiltrating M2-like macrophages and the relationship of prognosis with CD8+ T-cells are unclear in patients with EHBC. Therefore, the aim of this study was to examine the clinicopathological influences of tumor-infiltrating M2-like CD163+ macrophages and CD8+ T-cells and clarify the prognostic importance of those cells in patients with invasive EHBC.

Materials and Methods

Patients and samples. A total of 115 patients underwent macroscopic curative resection at Hirosaki University Hospital between 2005 and 2011. Eight patients were excluded due to unavailability of samples for immunohistochemical evaluation or use of chemotherapy or immunotherapy preoperatively, and six with mucosal cancer were excluded due to the goal of evaluating an invasive tumor microenvironment. Therefore, 101 cases were included in the study. Clinicopathological variables that are well-known prognostic factors in perihilar or distal bile duct cancer were obtained from a review of medical records (17). The median age at the time of surgery was 69 years (range=36-83 years). Preoperative biliary drainage was placed to reduce jaundice (n=80) or to evaluate the extent of disease (n=5). Surgical procedures included resection of the extrahepatic bile duct plus right or left hepatectomy with caudate lobectomy (n=31), resection of the extrahepatic bile duct alone (n=4), pancreatoduo-denectomy (n=56), and hepatopancreato-duodenectomy (n=10). All patients underwent *en-bloc* dissection of regional lymph nodes.

Tumors were re-staged and classified according to the seventh TNM classification of the International Union Against Cancer (18). Metastatic lesions included microscopic nodal metastasis around the abdominal aorta or hepatic metastasis in the resected lobe. The degree of lymphatic, vessel and perineural invasion was scored as: 0, no invasion; 1, mild invasion; 2, moderate invasion; and 3, severe invasion, based on the General Rules for Surgical and Pathological Studies in Cancer of the Biliary Tract (19). Normal epithelium or dysplastic lesions including carcinoma *in situ* (CIS) at the ductal margin were defined as R0 resection because the presence of CIS at the ductal margin reportedly has no impact on survival compared to no CIS in extrahepatic bile duct cancer (20).

Since 2009, patients with invasive components who agreed to adjuvant therapy received S-1 chemotherapy at a dose of 80 mg/m^2 on days 1-14 every 3 weeks with the intention to continue for 1 year. One patient received uracil-tegafur in 2005 and one patient received gemcitabine plus S-1 in 2011. The survival period was censored at the last follow-up date for surviving patients or at the date of death for those who died for non-cancer-related reasons. The median follow-up period was 38 months (range=2-111 months). This study was approved by our Institutional Review Board (approval number: 2016-1003).

Immunohistochemistry. Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections of 4 μm thickness using standard techniques with a Ventana Benchmark HX Autostainer (Ventana Medical Systems, Tucson, AZ, USA). Antigen retrieval was carried out using Cell Conditioning Solution (CC1, Tris-based EDTA buffer, pH 8.0; Ventana). Following antigen retrieval, tissue samples were incubated for 32 min at 37˚C with mouse monoclonal antibodies against CD8 (C8/144B; 1:50 dilution; Dako, Glostrup, Denmark) or CD163 (MRQ-26; 1:100 dilution; Cell Marque, Austin, TX, USA). An iVIEW Universal DAB Detection Kit (Ventana) was used for detecting primary antibodies.

Evaluation of immunohistochemistry. Three areas with abundant CD8+ T-cells in cancer cell nests and three areas with many CD163+ macrophages in tumor stroma around cancer cells were selected at a magnification of ×100 (13, 14). Stroma areas where CD163+ macrophages were recruited as a result of necrosis, bleeding and destruction of tubular glands were not selected. Similarly, stroma areas around the liver were not included because it was difficult to distinguish tumor-associated macrophages from tissue-resident Kupffer cells in these areas. Photographs of selected areas at ×400 magnification were captured with an Olympus DP70 digital microscope camera (Olympus Optical, Tokyo, Japan). The number of immunopositive cells in the respective areas were counted using ImageJ software (National Institutes of Health, Bethesda, MD, USA) and the mean count from the three areas was calculated.

Statistical analysis. Comparisons of variables were performed by Fisher exact test. Overall survival (OS) was determined using the Kaplan–Meier method. Univariate analysis was performed for prognostic factors using a log-rank test. Significant factors in univariate analysis were included in multivariate analysis using a Cox proportional hazards model. All tests were two-sided and $p<0.05$ was considered significant. All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria) (21). More precisely, it is a modified version of R commander designed to add statistical functions frequently used in biostatistics.

Results

Relationships between clinicopathological variables and tumor-infiltrating immune cells. The median numbers of CD8+ T-cells in cancer cell nests and CD163+ macrophages in tumor stroma were 5.6 (range=0-130) and 71.3 (range=20.3-176.3), respectively. Cases were divided into high and low groups based on respective median numbers (Figure 1). Relationships of clinicopathological variables and the extent of tumor-infiltrating CD8+ T-cells or CD163+ macrophages were evaluated by the Fisher's exact test (Table I). Perihilar EHBC and perineural invasion were significantly associated with a low count of infiltrating CD8+ T-cells. Poorly-differentiated histology and nodal metastasis were significantly associated with high count of infiltrating CD163+ macrophages, but the extent of infiltration of CD163+ macrophages was not significantly correlated with that of CD8+ T-cells.

Relationship of clinicopathological variables with OS. In univariate analysis, age >70 years, carcinoembryonic antigen (CEA) >5 ng/ml, perihilar location, poor histology, high T stage, nodal involvement, distant metastasis, lymphatic invasion, venous invasion, perineural invasion, R1 status, no adjuvant chemotherapy were significantly associated with shorter OS (Table II). Counts of CD8+ T-cells in cancer cell nests alone and CD163+ macrophages in tumor stroma alone were not significant prognostic factors ($p=0.365$ and 0.178, respectively). We examined the prognostic influence of a combination of the counts of CD8+ T-cells in cancer cell

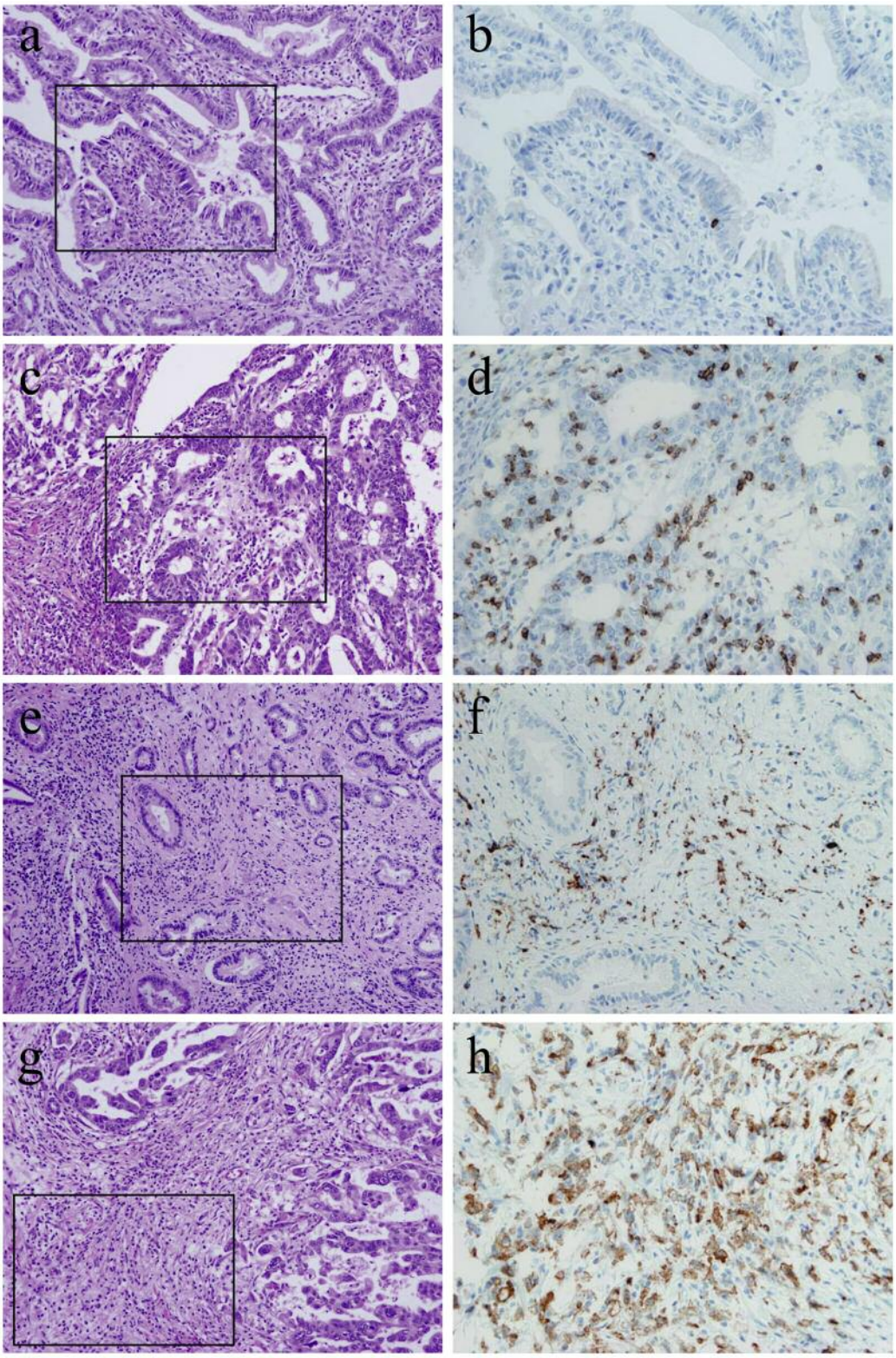

Figure 1. *Immunohistochemical (IHC) staining of tissues with low and high counts of tumor-infiltrating immune cells. a, c, e, g: Low-power view (×200) of hematoxylin and eosin-stained tissues; b, d, f, h: high-power view (×400) of areas shown in a, c, e, g stained by IHC. Low (b) and high (d) numbers of infiltrating cluster of differentiation (CD) 8^{+} T-cells in cancer cell nests, and low (f) and high (h) numbers of infiltrating CD163^{+} macrophages in tumor stroma are shown.*

Table I. *Relationships between clinicopathological variables and infiltrating immune cells.*

Variable		n	$CD8^+$ count, n (%)		*p*-Value	$CD163^+$ count, n (%)		*p*-Value
			Low	High		Low	High	
All cases		101	53 (52.5)	48 (47.5)		51 (50.5)	50 (49.5)	
Age	≤70 Years	54	30 (55.6)	24 (44.4)		27 (50.0)	27 (35.0)	
	>70 Years	47	23 (48.9)	24 (51.1)	0.553	24 (51.1)	23 (48.9)	1.000
Gender	Male	71	34 (47.9)	37 (52.1)		37 (52.1)	34 (47.9)	
	Female	30	19 (63.3)	11 (36.7)	0.193	14 (46.7)	16 (53.3)	0.667
Albumin	>3.5 g/dl	82	46 (56.1)	36 (43.9)		42 (51.2)	40 (48.8)	
	≤3.5 g/dl	19	7 (36.8)	12 (63.2)	0.202	9 (47.4)	10 (52.6)	0.804
Jaundice	No	18	10 (55.6)	8 (44.4)		12 (66.7)	6 (33.3)	
	Yes	83	43 (51.8)	40 (48.2)	0.801	39 (47.0)	44 (53.0)	0.193
CEA	≤5 ng/ml	91	50 (54.9)	41 (45.1)		46 (50.5)	45 (49.5)	
	>5 ng/ml	10	3 (30.0)	7 (70.0)	0.186	5 (50.0)	5 (50.0)	1.000
CA19-9	<100 U/ml	69	35 (50.7)	34 (49.3)		35 (50.7)	34 (49.3)	
	≥100 U/ml	32	18 (56.2)	14 (43.8)	0.671	16 (50.0)	16 (50.0)	0.986
Location	Distal	60	26 (43.3)	34 (56.7)		27 (45.0)	33 (55.0)	
	Perihilar	41	27 (65.9)	14 (34.1)	0.042	24 (58.5)	17 (41.5)	0.225
Histology	Pap/Well	27	12 (44.4)	15 (55.6)		21 (77.8)	6 (22.2)	
	Other	74	41 (55.4)	33 (44.6)	0.373	30 (40.5)	44 (59.5)	0.001
T	T1, T2	54	28 (51.9)	26 (48.1)		29 (53.7)	25 (46.3)	
	T3, T4	47	25 (53.2)	22 (46.8)	1.000	22 (46.8)	25 (53.2)	0.552
N	N0	65	39 (60.0)	26 (40.0)		39 (60.0)	26 (40.0)	
	N1	36	14 (38.9)	22 (61.1)	0.061	12 (33.3)	24 (66.7)	0.013
M	M0	94	50 (53.2)	44 (46.8)		50 (53.2)	44 (46.8)	
	M1	7	3 (42.9)	4 (57.1)	0.706	1 (14.3)	6 (85.7)	0.060
Lymphatic invasion	ly0, ly1	55	28 (50.9)	27 (49.1)		30 (54.5)	25 (45.5)	
	ly2, ly3	46	25 (54.3)	21 (45.7)	0.842	21 (45.7)	25 (54.3)	0.427
Venous invasion	v0, v1	55	29 (52.7)	26 (47.3)		30 (54.5)	25 (45.5)	
	v2, v3	46	24 (52.2)	22 (47.8)	1.000	21 (45.7)	25 (54.3)	0.427
Perineural invasion	pn0, pn1	33	11 (33.3)	22 (66.7)		18 (54.5)	15 (45.5)	
	pn2, pn3	68	42 (61.8)	26 (38.2)	0.011	33 (48.5)	35 (51.5)	0.672
Resection margin status	R0	86	44 (51.2)	42 (48.8)		42 (61.6)	44 (38.4)	
	R1	15	9 (60.0)	6 (40.0)	0.585	9 (60.0)	6 (40.0)	0.577
Adjuvant chemotherapy	No	56	31 (55.4)	25 (44.6)		27 (48.2)	29 (51.8)	
	Yes	45	22 (48.9)	23 (51.1)	0.553	24 (53.3)	21 (46.7)	0.690
$CD163^+$ count	Low	51	30 (58.8)	21 (41.2)				
	High	50	20 (46.0)	18 (54.0)	0.234			

Pap/Well: Papillary/well-differentiated; CEA: carcinoembryonic antigen; CA19-9: carbohydrate antigen 19-9; CD163: cluster of differentiation 163.

nests and $CD163^+$ macrophages in tumor stroma. Patients with high $CD8^+$ T-cells and low $CD163^+$ macrophage counts ($CD8^{Hi}CD163^{Lo}$) had significantly better survival than other patients (Figure 2). In multivariate analysis, $CD8^{Hi}CD163^{Lo}$ status was independently associated with better OS (Table II). Adjuvant therapy was also independently associated with better OS in multivariate analysis (Table II). In order to examine the impact of $CD8^{Hi}CD163^{Lo}$ status in patients after adjuvant chemotherapy, univariate and multivariate analyses were performed in such cases. $CD8^{Hi}CD163^{Lo}$ status was independently associated with better OS in these patients (Table III, Figure 2b).

Discussion

This study shows that the combination of high counts of $CD8^+$ T-cells in cancer cell nests with low counts of $CD163^+$ macrophages in tumor stroma has an independent effect on better survival. A low count of infiltrating $CD8^+$ T-cells was significantly associated with perineural invasion, as also shown previously (15). In addition, patients with perihilar EHBC had a low number of infiltrating $CD8^+$ T-cells and significantly poorer survival. The study of patients with intrahepatic cholangiocarcinoma found that tumor progression including perineural invasion depended on the immune

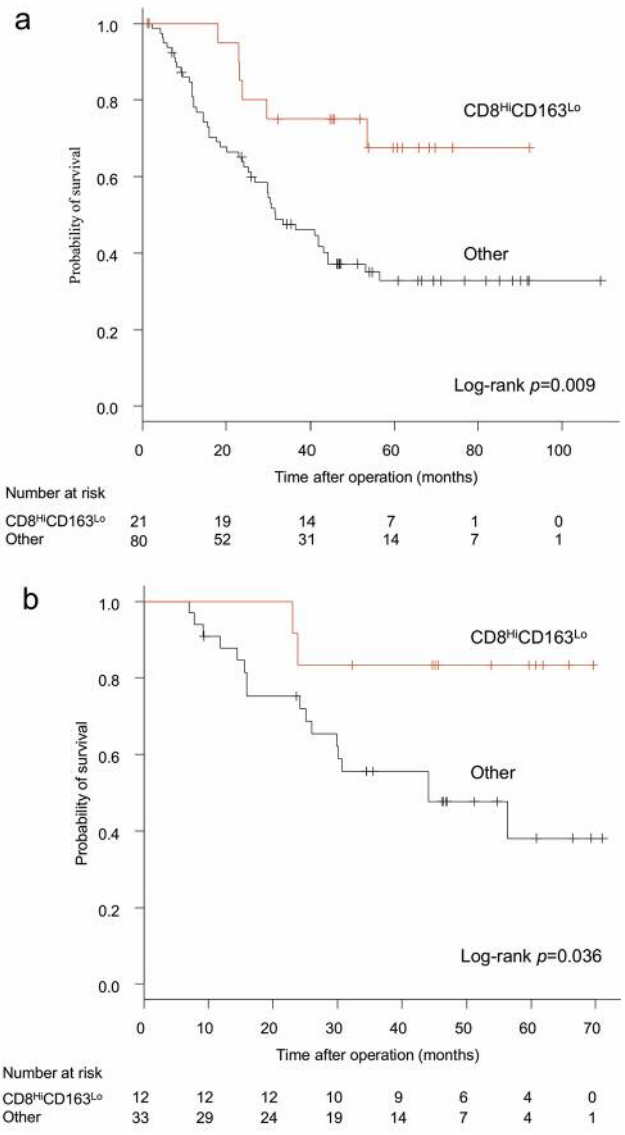

Figure 2. *Kaplan–Meier analyses of overall survival based on combined cell counts of cluster of differentiation (CD) 8+ T-cells and CD163+ macrophages. a: Patients with a combination of high CD8+ T-cell count and low CD163+ macrophage count ($CD8^{Hi}CD163^{Lo}$) had significantly better overall survival than other patients. b: $CD8^{Hi}CD163^{Lo}$ status was significantly associated with better overall survival among patients treated with adjuvant chemotherapy.*

evasion using tumor necrosis factor superfamily 6 (FAS/FASL) mechanisms (22). Those findings suggest that immune evasion might allow perineural invasion and low infiltration of CD8+ T-cells, leading to poor survival in patients with perihilar EHBC. In this study, the extent of CD8+ T-cells within cancer cell nests alone was not found to be a significant prognostic indicator as shown by previous studies (15, 16). The reason for this is thought to be that we excluded mucosal cancer, which is associated with high frequency of infiltrating CD8+ T-cells and favorable survival (14).

A high count of infiltrating CD163+ macrophages correlates with the grade of histological malignancy in breast cancer (23), and is associated with lymph node metastasis in pancreatic cancer (12). M2-like CD163+ macrophages induced an epithelial-to-mesenchymal transition represented by N-cadherin expression in cholangiocarcinoma cells (24). Intrahepatic cholangiocarcinoma cells altered peripheral blood mononuclear cells-derived macrophages to M2-like CD163+ macrophages with high expression of matrix metalloprotease 2 and vascular endothelial growth factor, leading to tumor growth (11). Consistent with these findings, the results of this study suggest that the presence of tumor-infiltrating CD163+ macrophages in EHBC is associated with poor differentiation of cancer cells and promotes nodal metastasis.

In patients with breast cancer, the extent of infiltration of macrophages and that of CD8+ T-cells are inversely correlated, which suggests that macrophages suppress infiltration of CD8+ T-cells in tumors, and combined counts of infiltrating macrophages and T-cells predict survival (25). In contrast, an inverse correlation of the extent of infiltration of CD163+ macrophages and CD8+ T-cells was not found in the current study, which does not support suppression of infiltration of CD8+ T-cells by CD163+ macrophages. However, M2-like macrophages are also involved in the functional suppression of CD8+ T-cells through inducing regulatory T-cells and inhibitory molecules such as programmed cell death ligand 1 (26, 27). Therefore, our results suggest that M2-like CD163+ macrophages might inactivate tumor-infiltrating CD8+ T-cells to promote tumor progression in patients with EHBC.

The levels of tumor-infiltrating CD8+ T-cells and tumor-associated macrophages can also predict the response to chemotherapy (25, 28). In the current study, adjuvant chemotherapy improved OS in patients with EHBC and this effect was promoted in patients with $CD8^{Hi}CD163^{Lo}$ status. A recent study discovered that the inhibition of macrophage phosphoinositide 3 kinase gamma reprogrammed M2-like protumoral macrophages to stimulate CD8+ T cell-mediated suppression of pancreatic cancer and improve the responsiveness to chemotherapy (29). Clinical trials of standard chemotherapy including adjuvant therapy in patients with EHBC are ongoing, but additional therapy to modulate these immune responses could also be really promising.

There are several limitations to this study. Firstly, the accuracy of discrimination between inflammation-associated macrophages and tumor-associated macrophages is pointed out. In fact, only a few CD163+ macrophages were found on the walls of extrahepatic bile ducts from surgical specimens obtained by a diagnosis of benign inflammatory biliary stricture (data not shown). Although patients with

Table II. *Univariate and multivariate analyses of association of clinicopathological variables with overall survival.*

			Univariate analysis		Multivariate analysis	
Variable		n	MST (months)	*p*-Value	Hazard ratio (95% CI)	*p*-Value
Age	≤70 Years	54	56.4			
	>70 Years	47	29.9	0.027		0.057
Gender	Male	71	31.5			
	Female	30	53.2	0.130		
Albumin	>3.5 g/dl	82	41.9			
	≤3.5 g/dl	19	53.5	0.756		
Jaundice	No	18	NA			
	Yes	83	31.7	0.084		
CEA	≤5 ng/ml	91	43.1			
	>5 ng/ml	10	17.0	0.049	5.580 (1.745-17.840)	0.004
CA19-9	<100 U/ml	69	53.5			
	≥100 U/ml	32	31.7	0.077		
Location	Distal	60	53.2			
	Perihilar	41	29.8	0.045	2.637 (1.322-5.262)	0.006
Histology	Pap/Well	27	NA			
	Other	74	30.7	0.005		0.891
T	T1, T2	53	NA			
	T3, T4	48	29.8	0.002	3.603 (1.768-7.342)	<0.001
N	N0	65	NA			
	N1	36	22.9	<0.001	2.558 (1.309-4.997)	0.006
M	M0	94	43.1			
	M1	7	12.1	0.003		0.318
Lymphatic invasion	ly0, ly1	55	NA			
	ly2, ly3	46	25.9	0.003		0.708
Venous invasion	v0, v1	55	NA			
	v2, v3	46	29.8	0.001		0.406
Perineural invasion	pn0, pn1	33	NA			
	pn2, pn3	68	31.7	0.044		0.715
Resection margin status	R0	86	53.2			
	R1	15	17.7	<0.001	3.040 (1.377-6.712)	0.006
Adjuvant chemotherapy	No	56	31.5			
	Yes	45	NA	0.023	0.375 (0.194-0.725)	0.004
$CD8^+$ count	Low	53	33.5			
	High	48	53.1	0.365		
$CD163^+$ count	Low	51	44.1			
	High	50	30.6	0.178		
$CD8^{Hi}CD163^{Lo}$	No	80	31.7			
	Yes	21	NA	0.009	0.127 (0.039-0.413)	<0.001

MST: Median survival time; CI: confidence interval; Pap/Well: papillary/well-differentiated; CEA: carcinoembryonic antigen; CA19-9: carbohydrate antigen 19-9; CD8/CD163: cluster of differentiation 8/163; Hi: high count; Lo: low count; NA: not available.

jaundice would have biliary inflammation resulting from preoperative biliary stenting, there was no significant association between the presence of jaundice and high counts of $CD163^+$ macrophages. Therefore, $CD163^+$ macrophages in this study were thought to be generally representative of M2-like tumor-associated macrophages. Secondly, we did not perform cytokine analyses based on the interaction among $CD163^+$ macrophages, $CD8^+$ T-cells and cholangiocarcinoma cells. It was shown that the up-regulation of macrophage expression of CD163 induced by cholangiocarcinoma cells accompanied the production of interleukin 10 and transforming growth factor-beta, which was associated with immunosuppression and tumor promotion (10, 11). However, further investigation will be necessary to define whether M2-like $CD163^+$ macrophages influenced $CD8^+$ T cell-dependent effects on cancer cells in patients with invasive EHBC.

Conclusion

In summary, our data suggest that infiltrating $CD163^+$ macrophages in tumor stroma and $CD8^+$ T-cells in cancer cell nests have a prognostic impact in patients with EHBC following resection and adjuvant chemotherapy. This suggests

Table III. *Univariate and multivariate analyses of clinicopathological variables on overall survival among patients with adjuvant chemotherapy.*

			Univariate analysis		Multivariate analysis	
Variable		n	MST (months)	*p*-Value	Hazard ratio (95% CI)	*p*-Value
Age	≤70 Years	25	NA			
	>70 Years	20	30.1	0.102		
Gender	Male	31	56.4			
	Female	14	NA	0.548		
Albumin	>3.5 g/dl	37	NA			
	≤3.5 g/dl	8	56.4	0.896		
Jaundice	No	12	NA			
	Yes	33	56.4	0.292		
CEA	≤5 ng/ml	40	NA			
	>5 ng/ml	5	15.9	0.213		
CA19-9	<100 U/ml	30	NA			
	≥100 U/ml	15	44.1	0.092		
Location	Distal	28	NA			
	Perihilar	17	44.1	0.257		
Histology	Pap/Well	12	NA			
	Other	33	44.1	0.038		0.730
T	T1, T2	23	NA			
	T3, T4	22	56.4	0.252		
N	N0	29	NA			
	N1	16	29.9	0.029		0.216
M	M0	42	NA			
	M1	3	20.8	0.042		0.102
Lymphatic invasion	ly0, ly1	27	NA			
	ly2, ly3	18	25.6	0.004		0.106
Venous invasion	v0, v1	21	NA			
	v2, v3	24	30.6	0.021		0.141
Perineural invasion	pn0, pn1	14	NA			
	pn2, pn3	31	56.4	0.226		
Resection margin status	R0	39	NA			
	R1	6	26.4	0.015	7.275 (1.631-32.440)	0.009
CD8^{+} count	Low	22	44.1			
	High	23	NA	0.052		
CD163^{+} count	Low	24	56.4			
	High	21	NA	0.933		
CD8^{Hi}CD163^{Lo}	No	33	44.1			
	Yes	12	NA	0.036	0.139 (0.026-0.739)	0.021

MST: Median survival time; CI: confidence interval; Pap/Well: papillary/well-differentiated; CEA: carcinoembryonic antigen; CA19-9: carbohydrate antigen 19-9; CD8/CD163: cluster of differentiation 8/163; Hi: high count; Lo: low count; NA: not available.

that immunotherapy to modulate this immune response might offer a new therapeutic approach in patients with EHBC.

Conflicts of Interest

The Authors declare that there are no conflicts of interest in regard to this study.

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