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Tumour-infiltrating inflammatory and immune cells in patients with extrahepatic cholangiocarcinoma

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Background: Inflammation and immune characteristics of the tumour microenvironment have therapeutic significance. The aim of this study was to investigate the clinical impact on disease progression in human extrahepatic cholangiocarcinoma (ECC).

Methods: A total of 114 consecutive ECC patients with curative resection between 2000 and 2014 were enrolled. Tumour infiltrating CD66b⁺ neutrophils (TANs; tumour associated neutrophils), CD163⁺ M2 macrophages (TAMs; tumour associated macrophages), CD8⁺ T cells, and FOXP3⁺ regulatory T cells (Tregs) were assayed by immunohistochemistry, and their relationships with patient clinicopathological characteristics and prognosis were evaluated.

Results: Tumour associated neutrophils were inversely correlated with CD8⁺ T cells ($P=0.0001$) and positively correlated with Tregs ($P=0.001$). High TANs ($P=0.01$), low CD8⁺ T cells ($P=0.02$), and high Tregs ($P=0.04$) were significantly associated with poor overall survival (OS). A high-risk signature, derived from integration of intratumoural inflammatory and immune cells, was significantly associated with poor recurrence-free survival ($P=0.01$) and OS ($P=0.0008$). A high-risk signature was correlated with postoperative distant metastases. Furthermore, a high-risk signature was related to the resistance to gemcitabine-based chemotherapy used after recurrence.

Conclusions: Our data showed that tumour infiltrating inflammatory and immune cells may play a pivotal role in ECC progression and a high-risk signature predicted poor prognosis in ECC patients.

In the tumour microenvironment, inflammatory reactions play key roles in carcinogenesis and cancer progression. The initiation of solid tumours is thought to be governed by genetic and epigenetic alterations of neoplastic cells during a multi-step tumour pathogenesis (Hanahan and Weinberg, 2011). In the past two decades, the tumour microenvironment has also emerged as an equally important determinant of tumour behaviour (Joyce and

Pollard, 2009; McAllister and Weinberg, 2014). Inflammatory reactions, which are important for wound repair, are also associated with stimulation of cancer cell proliferation, invasion, migration, metastasis, and tumour angiogenesis (Balkwill and Mantovani, 2001; Coussens and Werb, 2002; Grivennikov *et al*, 2010). In addition to the cancer cells and their surrounding stroma, the tumour microenvironment includes cells associated with both

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innate and adaptive immunity (de Visser *et al*, 2006). Intercellular communication controls and regulates tumour growth by direct contact or indirect interaction by autocrine and paracrine signalling of cytokines and chemokines (Grivennikov *et al*, 2010). Interest in targeting immune cells has increased not only in melanoma but also in gastrointestinal tumours including cholangiocarcinoma (Sabbatino *et al*, 2016; Fontugne *et al*, 2017).

Infiltration of inflammatory and immune cells such as neutrophils and macrophages that participate in defense against injury and infection can also induce tumour progression and metastasis, that is, as tumour associated neutrophils (TANs) and tumour associated macrophages (TAMs) (Qian and Pollard, 2010; Coffelt *et al*, 2016). Large numbers of tumour-infiltrating neutrophils indicate poor prognosis in patients with hepatocellular carcinoma (HCC) and renal cell carcinoma (Jensen *et al*, 2009; Li *et al*, 2011), as do large numbers of tumour-infiltrating macrophages in patients with HCC and Hodgkin's lymphoma (Zhu *et al*, 2008; Steidl *et al*, 2010). Moreover, previous studies revealed that TANs and TAMs are polarised toward an antitumour (N1 or M1) versus a protumour (N2 or M2) phenotype, depending on tumour microenvironment (Fridlender *et al*, 2009; Qian and Pollard, 2010). Regulatory T cells (Tregs) have also been shown to support tumour growth and invasion by suppressing host immune responses (Plitas and Rudensky, 2016; Tanaka and Sakaguchi, 2017). Large numbers of tumour-infiltrating Tregs have been associated with poor prognosis in patients with pancreatic and ovarian cancer (Curiel *et al*, 2004; Hiraoka *et al*, 2006). On the other hand, large numbers of tumour-infiltrating CD8⁺ T cells indicate a favourable prognosis in patients with colorectal and ovarian cancer (Naito *et al*, 1998; Zhang *et al*, 2003). Systematic analysis of tumour-infiltrating inflammatory and immune cells in cancer tissue would thus enable us to understand their role in tumour progression.

Extrahepatic cholangiocarcinoma, including perihilar and distal cholangiocarcinoma, is one of the most unfavourable cancer diagnoses because of its aggressive growth, early metastasis, and no effective treatment other than complete resection (Nakeeb *et al*, 1996; Takahashi *et al*, 2015). Although surgical techniques, perioperative management, and postoperative treatments have advanced, oncological outcomes remain unsatisfactory, even after curative resection, presumably because of lack of both effective additional treatment and predictive biomarkers of treatment response (Takahashi *et al*, 2015; Kobayashi *et al*, 2010). Pathological characteristics of human cholangiocarcinoma include infiltrating inflammatory and immune cells that participate in tumour development. Tumour-infiltrating cells do have a prognostic value in patients with biliary tract cancer (Oshikiri *et al*, 2003; Hasita *et al*, 2010; Gu *et al*, 2012; Goepfert *et al*, 2013), but it is challenging to link tumour biology and tumour inflammatory and immune cell status to treatment strategy.

The aim of this study was to investigate the role of tumour-infiltrating inflammatory and immune cells in patients with ECC. We found that a risk signature based on inflammatory and immune cell status was significantly relevant to disease progression.

MATERIALS AND METHODS

Patients. We retrospectively analysed 114 ECC patients with macroscopically complete resection (R0-1) at Kumamoto University Hospital between April 2005 and December 2014. All clinical data of patients were prospectively collected in the database of our department. Blood samples were obtained preoperatively. Patients were excluded if they died of postoperative complication within 30 days after surgery or R2 resection was performed. Ampullary carcinoma, gallbladder carcinoma, and intrahepatic

cholangiocarcinoma were excluded from this study. Paraffin-embedded sections were evaluated by immunohistochemistry. Pathological findings were prospectively evaluated following the Japanese classification of biliary tract cancers (Miyazaki *et al*, 2015). The TNM classifications were reclassified following the American Joint Committee on Cancer system, seventh edition (Edge *et al*, 2010). After initial surgery for the primary site, the patients were followed at 3- to 6-month intervals by clinical examinations and enhanced computed tomography (CT). Recurrence-free survival (RFS) was defined as the time between surgery and recurrence or death. Overall survival (OS) was defined as the time between surgery and death. Written informed consent was obtained from each patient, and the study procedures were approved by the Institutional Review Board.

Immunohistochemistry and evaluation. For CD66b (1:300 dilution; clone G10F5, BD Pharmigen, San Diego, CA, USA) and CD163 (1:300 dilution; clone 10D6; Novocastra, Newcastle, UK) staining, the protocol was previously shown (Komohara *et al*, 2008; Okabe *et al*, 2012). Paraffin-embedded tumour sections were dewaxed in xylene and ethanol, and autoclaved for 15 min in an antigen retrieval solution to retrieve their antigen epitopes; endogenous peroxidase activity was blocked by 3% hydrogen peroxide. Tissue sections were incubated overnight at 4 °C with primary antibodies, including rabbit polyclonal anti-CD8 (1:200 dilution; ab4055; Abcam, Cambridge, UK) and anti-FOXP3 (1:200 dilution; ab54501; Abcam, Cambridge, UK). The secondary antibody was incubated with horseradish peroxidase-labelled polymer (EnVision1kit; Dako, Carpinteria, CA, USA) for 30 min at 25 °C and incubated in 3,30-diaminobenzidine tetrahydrochloride (applied as a 0.02% solution containing 0.005% H₂O₂ in 0.05 M Tris-HCl; pH 7.6) at 25 °C for 5–15 min and counterstained with haematoxylin. Stained slides were evaluated by light microscopy at ×200 by two researchers (YK and YS) blinded to patients' clinicopathological data. For CD66b, CD163, CD8, and FOXP3 staining, serial sections from tumour blocks that contained the largest amount of tumour in each patient were evaluated and positive cells in each 1-mm-diameter field were counted at three fields, which contained a large amount of cancer cells and expressed as the mean (cells per field) of triplicate counts (Li *et al*, 2011).

Prognostic prediction. Prognostic prediction was estimated based on the cell count of TAN, TAM, CD8⁺T-cells, and Treg by immunohistochemistry. We determined optimal cut-off quartiles that fit the current outcome for prognostic analyses of single cells. The lowest quartiles of the continuous values of CD66b, CD163, and FOXP3, and the highest quartile of that of CD8 were determined as cut-offs (Supplementary Table S1). For instance, high CD66b stands for the number of cell superior or equal to the cut-off value of 9. Tumour associated neutrophil, TAM, and Treg were regarded as cells downregulating immune response to cancer cell, and CD8⁺ T cells as cells upregulating immune response. The risk signature model was estimated by combining expression pattern of these cells by using Nearest Template Prediction (NTP) algorithm in which a prediction of high- and low risk is made as implemented in the NTP module of the GenePattern (<http://software.broadinstitute.org/cancer/software/genepattern/>) analysis toolkit. Raw data shown in Supplementary Table S2 were put in the algorithm. Approach of the analysis with NTP was previously described (Hoshida *et al*, 2008; Nakagawa *et al*, 2016).

The predictive score was calculated by following scores of which cutoff values were already determined in single-cell analysis (Supplementary Table S1): (1) high CD66b, CD163, and FOXP3 defined as 1 point; (2) low CD66b, CD163, and FOXP3 as 0 point; (3) high CD8 as 0 point, and (4) low CD8 as 1 point. The final cut-off value was defined as three points in total because of first quartile of this continuous score.

Statistical analysis. Continuous variables were expressed as means \pm standard deviation; differences were assessed for significance using Student's *t*-test or the Mann–Whitney test. Categorical variables were evaluated using chi-square or Fisher exact tests, as appropriate. Cox proportional hazard regression analyses were performed to identify predictors of prognosis. The multivariate analysis was performed with clinicopathological factors with a *P*-value <0.05 in univariate analysis. RFS and OS rates were estimated by the Kaplan–Meier method, and survival curves were compared using the log-rank test. Pearson's correlation methods were performed to identify correlations for quantitative variables with normal distributions. *P* <0.05 was considered significant. All tests were performed on JMP software version 10.0.2 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Expression and correlations of TANs, TAMs, CD8⁺ T cells, and Tregs in ECC. Immune cells were found infiltrating more frequently around cancer cells than ducts of normal glands. One of those immune regulator, TAN expressing CD66b⁺, is shown in Figure 1A. Not only CD66b⁺ cells but also CD163⁺, CD8⁺, and FOXP3⁺ cells were detected in tumour stroma (Figure 1B). The distributions of positively labelled cells are shown in Figure 2A. The number of CD66b⁺ cells was negatively correlated with CD8⁺ cells ($r = -0.35$, $P = 0.0001$) (Figures 1C and 2B), positively correlated with FOXP3⁺ cells ($r = 0.30$, $P = 0.001$) (Figure 2C), and positively correlated with CD163⁺ cells ($r = 0.24$, $P = 0.009$) (Figure 2D). No significant correlation of CD163⁺ with either CD8⁺ ($r = 0.16$, $P = 0.08$) or FOXP3⁺ cells ($r = 0.16$, $P = 0.09$) was seen.

Prognostic value of tumour-infiltrating inflammatory and immune cells. The median patient follow-up was 62.6 months

(95% confidence limits: 51.4–94.1), measured by reversed Kaplan–Meier. There were 72 recurrences and 69 deaths among the 114 ECC patients. The cut-off values of cell count for positivity of each cell marker are shown in Supplementary Table S1. High TANs (HR, 2.20; 95% CI, 1.22–4.29; $P = 0.01$) (Figure 3A), low CD8⁺ T cells (HR, 2.03; 95% CI, 1.13–3.99; $P = 0.02$) (Figure 3C), and high Tregs (HR, 1.78; 95% CI, 1.03–3.26; $P = 0.04$) (Figure 3D) were significantly associated with worse OS; however, high TAMs were not (HR, 1.42; 95% CI, 0.84–2.53; $P = 0.21$) (Figure 3B).

Risk signature of tumour-infiltrating inflammatory and immune cells is correlated with poor prognosis. To investigate the relationship of tumour-infiltrating inflammatory and immune cells with patient characteristics and prognosis, we constructed a risk signature model relevant to poor overall survival based on expression profiles of inflammatory and immune cells (Figure 4A). Colour red stands for high number of tumour-infiltrating cells, and blue means low number. To investigate the prognostic impact of immune profile in ECC patients, we stratified the patients into high- and low-risk groups based on the expression pattern of four immune cells with an NTP algorithm as described above. Although there were no significant differences in the background clinicopathological features of the two groups (Table 1), the high-risk signature group had significantly worse RFS (HR = 1.76; 95% CI: 1.14–2.75; $P = 0.01$; Supplementary Figure S1) and OS (HR = 2.30; 95% CI: 1.40–3.82; $P = 0.0008$; Figure 4B) than the low-risk signature group. Moreover, to simplify this risk signature and use in further prospective study, we transposed the risk signature into a predictive score. Supplementary Figure S2a showed the survival curve of OS based on calculated score as described above. The high-score group determined by predictive score ≥ 3 had significantly worse OS than the low-score group with the score < 3 (HR = 2.81; 95% CI: 1.54–5.54; $P = 0.001$; Supplementary Figure S2b). Regarding RFS, high-score group also showed worse

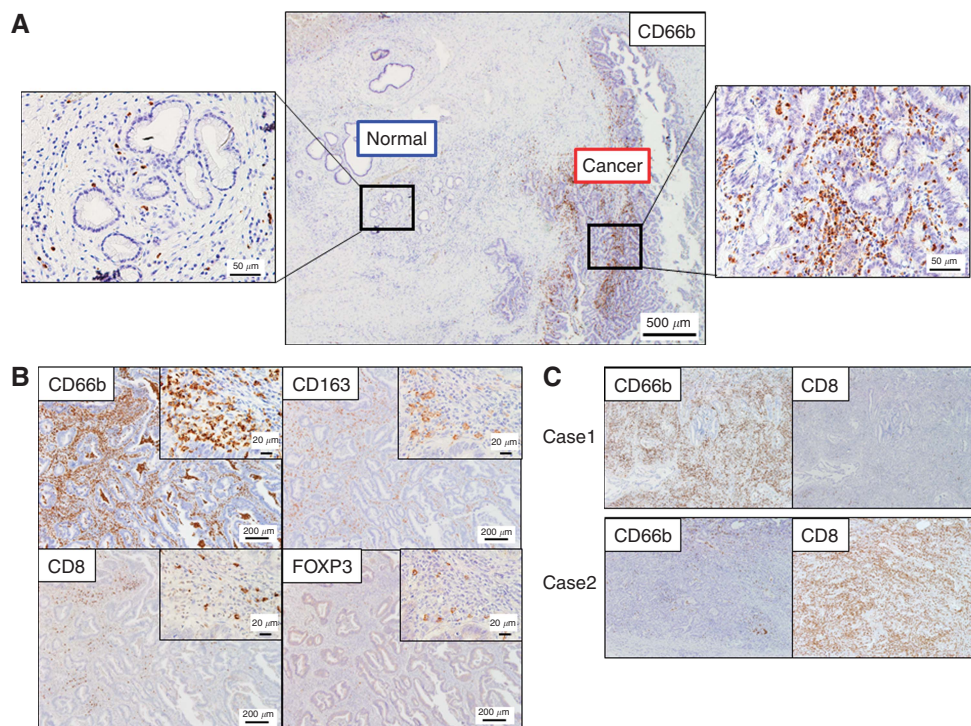


Figure 1. Immunohistochemistry on tumour-infiltrating CD66b⁺, CD163⁺, CD8⁺ and FOXP3⁺ cells. (A) Expression of CD66b⁺ cells in normal ducts and ductal cancer in patients with extrahepatic cholangiocarcinoma. (B) Representative pictures of immunohistochemical staining (CD66b, CD8, CD163 and FOXP3) with serial sections in extrahepatic cholangiocarcinoma are shown. (C) Representative cases with intratumoural exclusive expression patterns of CD66b⁺ and CD8⁺ cells.

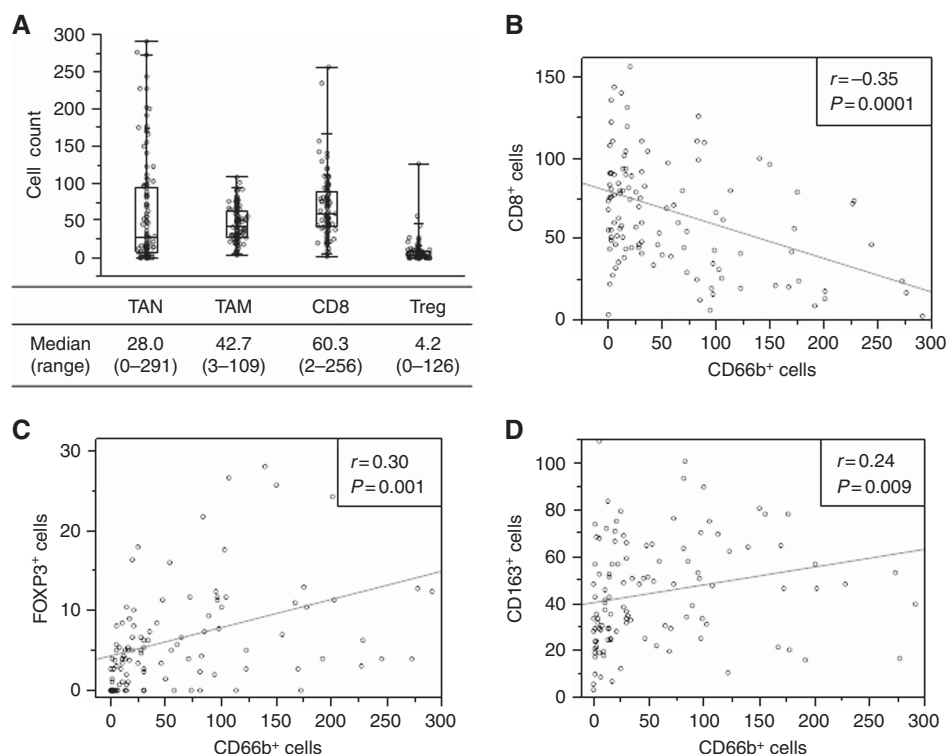


Figure 2. Distribution and relationships of tumour-infiltrating immune cells. (A) Distributions of tumour-infiltrating CD66b⁺, CD163⁺, CD8⁺ and FOXP3⁺ cells are shown in box plots. (B–D) The relationships of (B) CD66b⁺ and CD8⁺, (C) CD66b⁺ and FOXP3⁺, (D) CD66b⁺ and CD163⁺ expression are shown. One circle corresponds to one patient. TAM = tumour associated macrophage; TAN = tumour associated neutrophil; Treg = regulatory T cells.

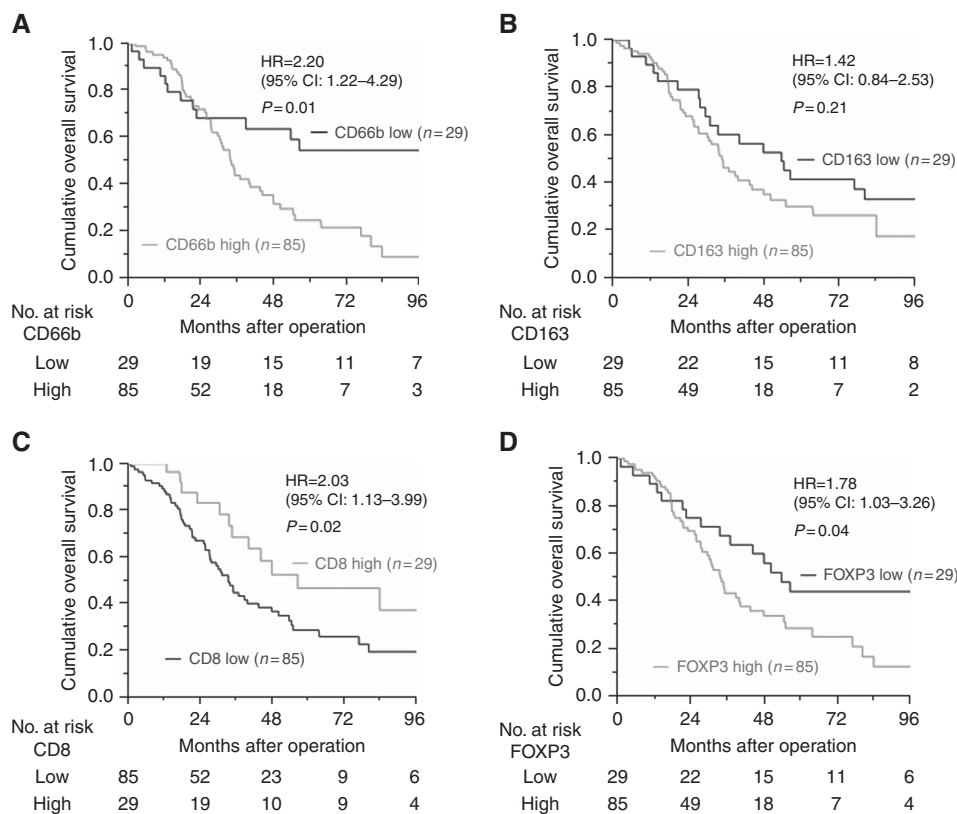


Figure 3. Overall survival and tumour-infiltrating immune cell status. Overall survival and (A) CD66b⁺ cell, (B) CD163⁺ cell, (C) CD8⁺ cell and (D) FOXP3⁺ cell status are shown. CI = confidence interval; HR = hazard ratio.

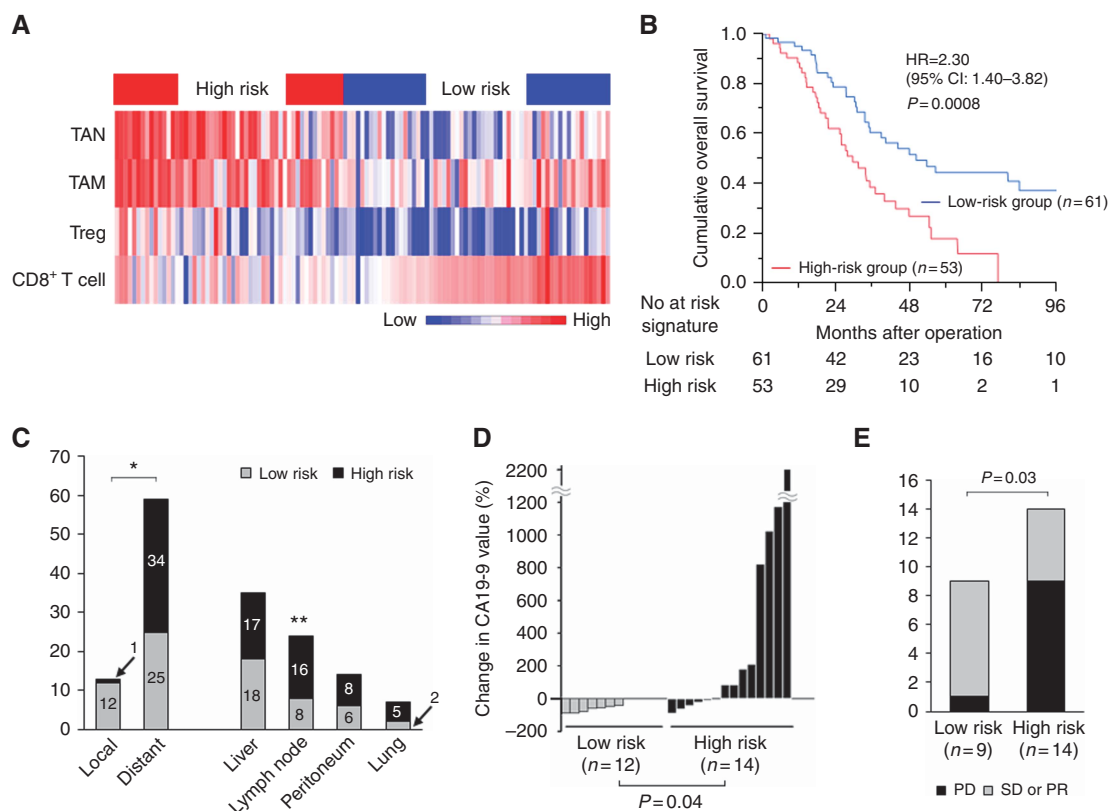


Figure 4. Risk signature model of tumour-infiltrating inflammatory and immune cells and postoperative outcome. (A) Risk signature is derived from the integration of tumour-infiltrating inflammatory and immune cell expression and shown as a heat map. (B) Overall survival based on the risk signature model of tumour-infiltrating inflammatory and immune cells is shown. (C) Comparison of recurrence sites between high- and low-risk signature. (D) Change in CA19-9 level between the initiation of gemcitabine-based chemotherapy and 3 months after the start in patients with recurrence. (E) The effect of chemotherapy on recurrent disease was assessed by RECIST criteria. Progressive disease (PD) was shown as 'not effective', whereas stable disease (SD) and partial response (PR) are shown as 'effective'. CI = confidence interval; HR = hazard ratio; TAN = tumour associated macrophage; TAM = tumour associated neutrophil; Treg = regulatory T cells. * $P = 0.005$; ** $P = 0.02$.

RFS than low-score group, although the difference did not reach statistical significance (HR = 1.61; $P = 0.07$; Supplementary Figure S2c and d).

Risk signature of tumour-infiltrating inflammatory and immune cells is independently correlated with both RFS and OS. Univariate and multivariate analyses for RFS and OS were performed (Supplementary Table S3 and Table 2). Univariate analysis for RFS revealed that low albumin ($< 3.8 \text{ g dl}^{-1}$), high CA19-9 ($> 74 \text{ U ml}^{-1}$), large tumour size ($> 25 \text{ mm}$), presence of lymph node metastasis, and high-risk signature were significantly associated with poor RFS. Multivariate analysis including those factors showed that the presence of lymph node metastasis (HR = 1.94, $P = 0.005$), high-risk signature (HR = 1.62, $P = 0.04$), and large tumour size (HR = 1.60, $P = 0.04$) were independent predictors of poor RFS. Univariate analysis found that high CA19-9, presence of lymph node metastasis, and high-risk signature were significantly associated with poor OS. High-risk signature (HR = 2.09, $P = 0.004$) and presence of lymph node metastasis (HR = 1.96, $P = 0.009$) were independent predictors of poor OS in multivariate analysis.

Risk signature of tumour-infiltrating inflammatory and immune cells is correlated with distant metastases and resistance to chemotherapy used after recurrence. To address why a high-risk signature was correlated with poor prognosis, recurrence patterns were carefully investigated. All patients with recurrence could be properly assessed for the analysis. Recurrences involving

the hepatic portal region were defined as local; recurrences involving organs such as the liver, lymph nodes, lungs, and peritoneum were defined as distant. As regional lymph nodes were routinely removed during curative surgery, recurrence of lymph node metastases was determined as distant metastases in the current study. A high-risk signature was correlated with distant recurrence, whereas low-risk signatures were correlated with local recurrence ($P = 0.0005$; Figure 4C). Since ECC tended to be resistant to chemotherapeutic reagents, resulting in the lack of effective standard chemotherapy, the impact of risk-signature on the activity of chemotherapy used after recurrence was also investigated. Carbohydrate antigen 19-9 values at the recurrence and 3 months after the beginning of treatment were included in the comparative analysis. Complete data were available in 26 patients receiving gemcitabine-based chemotherapy regimen and patients' background is shown in Supplementary Table S4. Intriguingly, a significantly greater decrease in CA19-9 was seen in the low-risk signature group than was in the high-risk signature group ($P = 0.04$; Figure 4D). Furthermore, chemotherapeutic effect evaluated by CT was confirmed by Response Evaluation Criteria in Solid Tumors v1.1 criteria. Recurrent tumour size was measurable in 23 patients, and we evaluated the chemotherapeutic effect at 3 months after an administration of chemotherapy. The number of progressive disease in the low-risk signature group ($n = 1$) was significantly less than in the high-risk signature group ($n = 9$) ($P = 0.03$, Figure 4E). Survival rates from recurrence of high-risk signature group was worse than low-risk group, although

Table 1. Baseline clinicopathological factors in patients with high- and low-risk signatures

	Inflammation risk signature			P-value
	Total (n = 114)	High risk (n = 53)	Low risk (n = 61)	
Age				0.11
Mean ± s.d.	67.5 ± 10.1	69.1 ± 8.1	66.1 ± 11.4	
Sex				0.26
Male	77 (67.5%)	33 (62.3%)	44 (72.1%)	
Female	37 (32.5%)	20 (37.7%)	17 (27.9%)	
Tumour location				0.57
Perihilar	57 (50.0%)	28 (52.8%)	29 (47.5%)	
Distal	57 (50.0%)	25 (47.2%)	32 (52.5%)	
DM				0.46
Present	27 (23.9%)	11 (20.8%)	16 (26.7%)	
Absent	87 (76.1%)	42 (79.2%)	45 (73.3%)	
BMI				0.56
Mean ± s.d.	23.4 ± 3.5	23.2 ± 3.8	23.6 ± 3.1	
Albumin (g dl⁻¹)				0.57
Mean ± s.d.	3.8 ± 0.4	3.7 ± 0.4	3.8 ± 0.5	
Bilirubin (mg dl⁻¹) (first visit)				0.71
Mean ± s.d.	6.8 ± 7.1	6.6 ± 7.5	7.1 ± 6.8	
CRP (mg dl⁻¹)				0.74
Mean ± s.d.	0.65 ± 1.0	0.69 ± 1.0	0.62 ± 1.0	
WBC (μl⁻¹)				0.38
Mean ± s.d.	5506 ± 1589	5647 ± 1714	5384 ± 1475	
Neutrophil (μl⁻¹)				0.29
Mean ± s.d.	3308 ± 1324	3424 ± 1368	3153 ± 1353	
Lymphocyte (μl⁻¹)				0.57
Mean ± s.d.	1598 ± 622	1623 ± 748	1555 ± 532	
CEA (ng ml⁻¹)				0.31
Mean ± s.d.	2.9 ± 7.8	3.7 ± 11.0	2.2 ± 2.4	
CA19-9 (U ml⁻¹)				0.95
Mean ± s.d.	328.1 ± 1166	320.0 ± 981	335.1 ± 1315	
Operation time (min)				0.07
Mean ± s.d.	669.6 ± 215.9	630.1 ± 153.8	703.9 ± 254.4	
Blood loss (ml)				0.61
Mean ± s.d.	1400 ± 1681	1315 ± 958	1478 ± 2139	
Operative procedures				0.60
Resection of extrahepatic bile duct	14 (12.2%)	7 (13.2%)	7 (11.5%)	
Hemihepatectomy	40 (35.1%)	19 (35.9%)	21 (34.4%)	
Trisectionectomy	4 (3.5%)	2 (3.8%)	2 (3.3%)	
Pancreaticoduodenectomy	54 (47.4%)	23 (43.4%)	31 (50.8%)	
Hepato-pancreaticoduodenectomy	2 (1.8%)	2 (3.8%)	0	
Postoperative complication (CD ≥ IIIa)				0.11
Present	65 (57.0%)	26 (49.1%)	39 (63.9%)	
Absent	49 (43.0%)	27 (50.9%)	22 (36.1%)	
Mortality within 30 days after surgery				0.48
Present	3 (2.6%)	2 (3.8%)	1 (1.6%)	
Absent	111 (97.4%)	51 (96.2%)	60 (98.4%)	
Tumour size (mm)				0.65
Mean ± s.d.	30 ± 15	31 ± 16	29 ± 15	
Histologic grade				0.59
Papillary	4 (3.5%)	2 (3.8%)	2 (3.3%)	
Well	51 (44.7%)	24 (45.3%)	27 (44.3%)	
Moderately	48 (42.2%)	24 (45.3%)	24 (39.3%)	
Poorly	11 (9.6%)	3 (5.7%)	8 (13.1%)	
pT				0.74
T1	8 (7.0%)	3 (5.7%)	5 (8.2%)	
T2	52 (45.6%)	24 (45.3%)	28 (45.9%)	
T3	50 (43.9%)	25 (47.2%)	25 (41.0%)	
T4	4 (3.5%)	1 (1.9%)	3 (4.9%)	
pN				0.87
N0	74 (64.9%)	34 (64.2%)	40 (65.6%)	
N1	40 (35.1%)	19 (35.8%)	21 (34.4%)	

Table 1. (Continued)

	Inflammation risk signature			P-value
	Total (n = 114)	High risk (n = 53)	Low risk (n = 61)	
Residual tumour				0.48
R0	79 (69.3%)	35 (66.0%)	44 (72.1%)	
R1	35 (30.7%)	18 (34.0%)	17 (27.9%)	
Adjuvant therapy				0.79
Present	51 (44.7%)	23 (43.4%)	28 (45.9%)	
Absent	63 (55.3%)	30 (56.6%)	33 (54.1%)	

Abbreviations: BMI = body mass index; CA19-9 = carbohydrate antigen 19-9; CD = Clavien-Dindo classification; CEA = carcinoembryonic antigen; CRP = C-reactive protein; DM = diabetes mellitus; pT = pathological tumour; pN = pathological node; s.d. = standard deviation; WBC = white blood cell.

Table 2. Univariate and multivariate analysis for overall survival

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	P-value	HR	95% CI	P-value
Age >70	1.02	0.68–1.79	0.69			
Female	1.42	0.86–2.30	0.17			
BMI >26.5	0.71	0.35–1.32	0.30			
Reduction of jaundice	1.15	0.65–2.21	0.64			
Bilirubin (first visit) >2.0 mg dl ⁻¹	1.16	0.70–2.01	0.57			
Albumin <3.8 g dl ⁻¹	1.57	0.97–2.55	0.07			
CEA >3.0 ng ml ⁻¹	0.95	0.50–1.69	0.87			
CA19-9 >74 U ml ⁻¹	1.87	1.14–3.03	0.01	1.56	0.95–2.55	0.08
Tumour size >25 mm	1.34	0.82–2.25	0.25			
Operation time >720 min	1.31	0.78–2.13	0.30			
Blood loss >1500 cc	1.45	0.86–2.37	0.16			
Postoperative complication	1.04	0.64–1.71	0.89			
Tumour location, perihilar	1.10	0.68–1.78	0.69			
Histologic grade, poorly	0.95	0.42–1.88	0.89			
T3, 4	1.19	0.74–1.02	0.47			
N1	2.07	1.26–3.39	0.005	1.96	1.19–3.21	0.009
R1	1.64	0.97–2.70	0.06			
Adjuvant therapy (-)	0.92	0.56–1.50	0.72			
High-risk signature	2.30	1.34–3.82	0.001	2.09	1.26–3.50	0.004

Abbreviations: BMI = body mass index; CA19-9 = carbohydrate antigen 19-9; CEA = carcinoembryonic antigen; CI = confidence interval; HR = hazard ratio. Bold values are statistically significant at P < 0.05.

difference was marginal (HR = 2.51, P = 0.051; Supplementary Figure S3).

DISCUSSION

As mentioned in hallmarks of cancer at the next generation, evading immune destruction and presence of tumour-promoting inflammation were additional hallmarks involving in the pathogenesis and progression of all cancers (Hanahan and Weinberg, 2011). We found that a high-risk signature defined by infiltration of inflammatory and immune cells was an independent predictor of poor prognosis in ECC patients with surgical resection. Although the high-risk signature was not associated with background characteristics relevant to tumour progression, it could predict disease recurrence and progression after surgery. Although the prognostic value of each cell type was substantial, we aimed to establish a predictor derived from tumour biological characteristics in the belief that multiple parameters would reduce current experimental limitations and increase the accuracy of patient classification. In the beginning, we had made a risk signature model with three types of cells (TANs, CD8⁺ T cells, and Tregs) which showed the significant correlation with OS in single-cell analysis (Supplementary Figure S4a), and this model showed nice correlation with patients' prognosis (RFS: HR = 1.38, P = 0.15; OS: HR = 1.72, P = 0.03 Supplementary Figure S4b). Interestingly,

however, four-cell type model including TAMs showed better correlation with patients' prognosis than three-cell type model. We believe that TAM somehow regulates immune cell status relevant to tumour progression, although TAM by itself is not associated with prognosis. The number of TANs was significantly correlated with that of TAMs, CD8⁺ T cells, and Tregs, suggesting that those cells collaborate to produce inflammation affecting tumour development, and in this patient series, the regulation of inflammatory circumstances was of great importance in ECC progression and has future implications for clinical therapeutics.

Large numbers of tumour-infiltrating neutrophils (Jensen *et al*, 2009; Li *et al*, 2011; Gu *et al*, 2012; Coffelt *et al*, 2016) and macrophages (Zhu *et al*, 2008; Hasita *et al*, 2010; Steidl *et al*, 2010; Zhu *et al*, 2008) are positively associated with disease progression in many types of cancer. In clinical practice, a small number of CD8⁺ T cells (Naito *et al*, 1998; Zhang *et al*, 2003) and a large number of Tregs (Curiel *et al*, 2004; Hiraoka *et al*, 2006; Tanaka and Sakaguchi (2017)) have also been associated with tumour development. High TAN, low CD8⁺ T cell, and high Treg populations were also significantly associated with worse OS in this series of ECC patients with surgical resection. Previous studies of tumour immune cells found that TAN depletion slowed tumour growth and resulted in an increase of activated CD8⁺ T cells (Chao *et al*, 2016; Coffelt *et al*, 2016; Fridlender *et al*, 2009). Tumour associated neutrophils have also been shown to

mediate the infiltration of macrophages and Tregs by secreting CCL2 and CCL17, which resulted in enhanced HCC growth and metastasis (Mishalian *et al*, 2014; Zhou *et al*, 2016). Our finding that the numbers of TANs, TAMs, and Tregs in ECC patients were positively correlated is in line with previous results. The collective evidence suggests that TAN may be a key regulator of inflammation and immune status. Further study of the interaction of tumour-infiltrating immune cells in cholangiocarcinoma is warranted (Elinav *et al*, 2013; Grivennikov *et al*, 2010).

The clinical relevance of the risk-signature to recurrence pattern depends on recent investigations showing that tumour-induced systemic accumulation of neutrophils and macrophages acts to produce premetastatic niches and promote distant metastasis. Inflammatory cells in those niches inhibit antitumour T-cell immunity and promote immunosuppressive cells, including Tregs (Coffelt *et al*, 2015, 2016; Qian and Pollard, 2010; Tuting and de Visser, 2016). As expected, high-risk signatures were correlated with postoperative distant recurrence. A high-risk signature may represent a more aggressive phenotype, which results in a higher rate of lymph node spread as well as a greater propensity to recur at a distant site. Patients with a high-risk signature may be candidates for intensified strategies. The high-risk signature should be validated prospectively and may be used to stratify patients, along with other prognostic factors (high CA19-9 value, lymph node metastasis), in further adjuvant studies.

To determine the clinical implications of high-risk signatures in addition to its prognostic value, we also carefully evaluated its impact on chemotherapeutic response. A high-risk signature was correlated with resistance to gemcitabine-based chemotherapy used after recurrence. Previous studies have suggested that inflammatory and immune cells are responsible for chemotherapy resistance or sensitivity. In HCC model, TANs, TAMs, and Tregs had the ability to promote neovascularisation and inhibit Sorafenib-induced anti-angiogenesis (Zhou *et al*, 2016). In ovarian cancer, CD8⁺ T cell-derived interferon γ abrogated resistance to platinum-based chemotherapy by altering glutathione metabolism of stromal fibroblasts in the tumour microenvironment (Wang *et al*, 2016). Increased intratumoural levels of the proinflammatory cytokines IL-6 and IL-8 levels have been associated with multidrug and apoptosis resistance in several cancers (He *et al*, 2011; Shi *et al*, 2012). Gemcitabine is a key drug for biliary tract cancer, and only effective drug therapy is cisplatin plus gemcitabine for locally advanced or metastatic biliary tract cancer (Valle *et al*, 2010). Activity of transcription factors promoting inflammation, including high mobility group A1 (HMGA1) and nuclear factor- κ B (NF- κ B), is associated with gemcitabine resistance in pancreatic cancer (Arlt *et al*, 2003; Kong *et al*, 2010). The genomic landscape of cholangiocarcinoma is slowly being unveiled with clinically significant results (Nakamura *et al*, 2015). For example, mutation-specific CD4⁺ T cell could be potential therapeutic targets (Tran *et al*, 2014). Immunotherapy targeting tumour-specific neoepitopes encourages personalised therapy in patients with metastatic cholangiocarcinoma (Loffler *et al*, 2016). The roles of tumour-infiltrating inflammatory and immune cells are becoming increasingly clear, and suggest that this risk signature focusing on these cells might be used to stratify patients by tumour microenvironment status and immune cell profile.

Since we acknowledge that there are several limitations in the current study, they are major issues to be addressed in the future work. (1) The number of patients was not enough for validation of the outcome. Multicentre analysis with different cohorts would confirm the impact of risk-signature in ECC. (2) Although the current study focused on the impact of the interaction of several major immune cells involved in tumour development by statistical approach with immunohistochemistry, the underlying mechanisms by which those major immune cells interact with each other remains unclear. *In situ* analysis by isolating those immune cells is

our next concern. (3) Risk-signature in primary tumour had a notable impact on the activity of chemotherapy on recurrent disease; however, the result is based on the concept that recurrent tumour is genetically similar to the primary tumour resected. Prognostic serological marker for risk-signature of the tumour is of great interest and under investigation.

In conclusion, tumour infiltrating inflammatory and immune cells might play a pivotal role in the ECC microenvironment. The risk signature derived from those cells had a clinical significance and independently indicated prognosis of ECC patients with curative resections.

ETHICAL STANDARDS

All procedures complied with the ethical standards of the relevant local and national committees on human experimentation and with the latest version of the Helsinki Declaration of 1964. Informed consent or acceptable substitute was obtained from all patients before study inclusion.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Conception and design: YKi, HO, YiY, SN and YKo; development of methodology: YKi, HO, SN, YS and YKo; acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.) and analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): YKi, HO and SN; writing, review and/or revision of the manuscript: YKi, HO, YiY, YKo and HB; administrative, technical, or material support (i.e., reporting or organising data, constructing databases): YKi, HO, YiY, SN, YS, YKo and HB; study supervision: YiY, KM, KI, DH, YKo, AC, TI and HB.

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