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ORIGINAL ARTICLE

Lymphocyte–C-reactive protein ratio as a prognostic marker associated with the tumor immune microenvironment in intrahepatic cholangiocarcinoma

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Abstract

Background Changes in immune cell and inflammation-associated protein levels, either independently or in combination, are commonly used as prognostic factors for various cancers. The ratio of lymphocyte count to C-reactive protein concentration (lymphocyte–CRP ratio; LCR) is a recently identified prognostic marker for several cancers. Here, we examined the prognostic value of LCR and its relationship to various aspects of the tumor immune microenvironment in patients with intrahepatic cholangiocarcinoma (ICC).

Methods This was a single-center, retrospective study of patients who underwent surgical resection for ICC between 1998 and 2018. Patients were dichotomized into high- and low-LCR status groups, and the relationships between LCR status, prognosis, and other clinicopathological characteristics were analyzed. Tumor-infiltrating CD8+ and FOXP3s+ lymphocytes and tumor expression of CD34 and programmed death-ligand 1 were evaluated by immunohistochemical staining of resected tumors.

Results A total of 78 ICC patients were enrolled and assigned to the high ($n=44$)- and low ($n=34$)-LCR groups. Compared with the high-LCR group, patients in the low-LCR group had a significantly higher serum CA19-9 level (median 20.6 vs. 77.3 U/mL, $P=0.0017$) and larger tumor size (median 3.5 vs. 5.5 cm, $P=0.0018$). LCR correlated significantly with tumor microvessel density ($r=0.369$, $P=0.0009$) and CD8+ T lymphocyte infiltration ($r=0.377$, $P=0.0007$) but not with FOXP3+ T lymphocyte infiltration or tumor PD-L1 expression. Low-LCR status was significantly associated with worse overall survival by multivariate analysis ($P=0.0348$).

Conclusions Low-LCR status may reflect a poor anti-tumor immune response and predict worse outcomes in ICC patients.

Keywords Lymphocyte · C-reactive protein ratio · Tumor-infiltrating lymphocytes · Tumor immune microenvironment · Intrahepatic cholangiocarcinoma

Abbreviations

ALP	Alkaline phosphatase
CA19-9	Carbohydrate antigen 19-9
CAR	CRP–albumin ratio
CCA	Cholangiocarcinoma
CEA	Carcinoembryonic antigen
CONUT score	Controlling Nutritional Status score
CRP	C-reactive protein
FOXP3	Forkhead box protein P3
GPS	Glasgow Prognostic Score
γ-GTP	γ-Glutamyl transpeptidase
HBV	Hepatitis B virus
HCV	Hepatitis C virus
H&E	Hematoxylin and eosin
ICC	Intrahepatic cholangiocarcinoma
ICG15	Indocyanine green retention rate at 15 min
IG	Intraductal growth
LCR	Lymphocyte–CRP ratio
LMR	Lymphocyte–monocyte ratio
MF	Mass-forming

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MVD	Microvessel density
NLR	Neutrophil–lymphocyte ratio
OS	Overall survival
PD-L1	Programmed death-ligand 1
PI	Periductal infiltrating
PLR	Platelet–lymphocyte ratio
PNI	Prognostic Nutritional Index
RFS	Recurrence-free survival
TIL	Tumor-infiltrating lymphocyte
TIME	Tumor immune microenvironment

Introduction

Intrahepatic cholangiocarcinoma (ICC) is the second most common primary liver tumor following hepatocellular carcinoma and a major cause of cancer mortality and morbidity worldwide [1]. Although surgical resection is a potentially curative treatment and improves the outcomes, the prognosis of ICC patients remains poor because of tumor progression. The precise risk factors that predict poor outcomes among ICC patients have not been fully investigated.

Inflammation is recognized as a hallmark of tumor progression and a key component of the tumor microenvironment [2, 3]. An increasing number of studies have indicated that changes in systemic inflammatory factor levels can predict surgical and oncological outcomes in several cancers, including ICC [4–6]. In particular, ratios of various systemic molecular and cellular factors, such as C-reactive protein (CRP), albumin, neutrophils, lymphocytes, monocytes, and platelets, have been revealed as prognostic markers. For ICC, the most commonly measured markers are the CRP–albumin ratio (CAR) [7], neutrophil–lymphocyte ratio (NLR) [8, 9], lymphocyte–monocyte ratio (LMR) [9], and platelet–lymphocyte ratio (PLR) [8]. Despite accumulating evidence that many of these markers correlate with tumor-related outcomes in various cancers, the best predictors of survival remain unclear. Recently, another biomarker, the lymphocyte–CRP ratio (LCR), was reported to be a useful marker for predicting surgical and/or oncological outcomes in patients with colorectal cancer [10] and suggested as a promising marker for predicting outcomes in patients with ICC [11, 12].

The tumor immune microenvironment (TIME), which describes the interplay among immune, tumor, and stromal factors within tumor tissues, plays an important role in the progression of many cancers, including ICC [13, 14]. Tumor-infiltrating lymphocytes (TILs) play a particularly pivotal role in cancer progression; indeed, alterations of specific TIL subtypes [e.g., cluster of differentiation 8 (CD8) or forkhead box protein P3 (FOXP3) positive TILs] have been shown to predict outcomes in several cancers, including lung adenocarcinoma and colorectal cancer [15–17]. We previously investigated the relationships among the component factors of the TIME, including tumor microvessels and TILs. The tumor microvessels can regulate anti-tumor immunity through the attenuation of CD8+ lymphocytes and activation of FOXP3+ T lymphocytes, which are associated with poor prognosis in ICC patients [18, 19]. Moreover, tumor expression of programmed cell death-ligand 1 (PD-L1) also plays a crucial role in tumor immunobiology in ICC [18–20].

Although the LCR has been identified as a predictive biomarker in some cancers, the relationship between LCR and various other aspects of the TIME has not been fully elucidated. Because several types of inflammatory cells are known to migrate from peripheral blood to tumor tissues, blood-based inflammatory markers can reflect local tumor immune status. The goal of the present study was to investigate the relationships among the outcomes of ICC patients and the blood-based LCR and components of the TIME.

Materials and methods

Patients and ethics

All patients who underwent hepatic resection for ICC at Kyushu University Hospital, Japan, between April 1998 and February 2018 were enrolled. Patients underwent resection for primary ICC without preoperative chemotherapy or radiation and were selected retrospectively. Preoperative and postoperative de-identified clinical data were obtained from electronic and paper records and were available for all patients. This study was approved by the ethics committee of our hospital according to the ethical guidelines of the Japanese government (approval number: 2020-628) and all patients provided consent for the research use of their resected tissue.

Histological evaluation of TILs

Sections were stained with hematoxylin and eosin (H&E) using standard protocols. The number of TILs was assessed using a standardized method for TIL analysis in solid tumors [21]. All sections obtained from each patient were reviewed using light microscopy (200 × magnification, 20 × objective lens and 10 × ocular lens; 0.950 mm^2 per field) by three independent observers (K.Y., S.I., and K.K.) who were blinded to the clinical data. If the TIL count obtained by the three observers differed by more than 10%, the sections were re-evaluated. The average count from the three observers was taken as the final TIL number.

Immunohistochemical staining and evaluation

Tissue sections were deparaffinized in xylene and rehydrated in a graded series of ethanol. Antigen retrieval was performed by incubation under the following conditions. For CD34, slides were incubated at 37 °C for 30 min in 0.2% trypsin in 10 mM phosphate-buffered saline; for CD8 and FOXP3, slides were microwaved at 98 °C for 20 min in Tris–EDTA buffer (pH 9.0); and for PD-L1, slides were autoclaved at 120 °C for 10 min in Tris–EDTA buffer (pH 9.0). Slides were treated with 0.3% H_2O_2 for 5 min to inhibit endogenous peroxidase activity and then incubated overnight at 4 °C with the primary antibodies: mouse monoclonal anti-CD34 (clone QBEnd 10; Agilent Technologies, Santa Clara, CA, USA), mouse monoclonal anti-CD8 (clone C8/144B; Agilent Technologies), mouse monoclonal anti-FOXP3 (236A/E7; Abcam, Cambridge, UK), and rabbit monoclonal anti-PD-L1 (E1L3N; Cell Signaling Technology, Danvers, MA, USA), all at a dilution of 1:100. The specimens were then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Color development was performed by the addition of 3,3′-diaminobenzidine followed by counterstaining with Mayer's hematoxylin.

After immunohistochemical staining, CD34 + microvessels were counted in five areas of the tumor having the highest vascular density by light microscopy (200 × magnification, 20 × objective lens, and 10 × ocular lens; 0.950 mm^2 per field). The average CD8+ and FOXP3+ TIL counts were calculated from five areas with the highest staining density in the intratumoral area by light microscopy (400 × magnification, 40 × objective lens, and 10 × ocular lens; 0.237 mm^2 per field). PD-L1 expression was evaluated on the cytoplasmic membrane of tumor cells (200 × magnification, 20 × objective lens, and 10 × ocular lens; 0.950 mm^2 per field). Immunohistochemical evaluations were performed independently by three observers (K.Y., S.I., and K.K.) as described above.

Statistical analysis

Data are presented as means, medians, frequencies, and percentages. Continuous variables were compared using the Mann–Whitney *U* test and Kruskal–Wallis test. Categorical variables were compared using the χ^2 test or Fisher's exact test. Univariate and multivariate survival analyses were performed using Cox proportional hazard models. Cumulative overall survival (OS) and recurrence-free survival (RFS) rates were calculated using the Kaplan–Meier method, and differences between curves were evaluated using the log-rank test. OS was calculated as the time from the date of surgery to the date of the last follow-up or death. Receiver operating characteristic (ROC) curve analysis of LCR was performed. The area under the ROC curve (AUC) was used to determine the optimal cut-off value for analyzing OS. The ROC analysis was performed with respect to the endpoint of death at 5 years after hepatic resection. To identify postoperative prognostic factors, variables found to be significant in univariate analyses were included in the overall multivariate Cox proportional model. All statistical tests were two sided, and a value of $P < 0.05$ was considered significant. All analyses were performed using JMP14 software (SAS Institute, Cary, NC, USA).

Results

The 78 enrolled patients included 55 men (70.5%) and 23 women (29.5%). The median age was 66 years (range 39–87), and the median OS and RFS times were 4.3 and 1.4 years, respectively. Regarding the etiology of ICC, 7 patients (9.0%) were infected with hepatitis B virus, 7 patients (9.0%) were infected with hepatitis C virus, and 10 patients (1.3%) had liver cirrhosis according to the pathological features.

Of the 78 patients, 63 (80.8%) were treated with complete resection (R0) and 15 (19.2%) with near-complete resection (R1). In our institution, lymph node dissection was performed according to whether lymph node metastasis was suspected on the preoperative abdominal computed tomography scan [22]. Pathological examinations revealed that 15 patients (19.2%) had lymph node metastasis.

The histopathological definition of cholangiocarcinoma (CCA) was based on the classification proposed by the World Health Organization. CCA is classified into intrahepatic (peripheral), perihilar, and distal types, on the basis of biliary tree location. Peripheral and perihilar CCAs were diagnosed in 59 (75.6%) and 19 patients (24.4%), respectively. Among peripheral CCAs, 56 patients (94.9%) had mass-forming and 3 patients (5.1%) had periductal infiltrating subtypes. No patients had an intraductal growth subtype of peripheral CCA. Recently, peripheral and perihilar CCAs have been reported to show similar pathologic characteristics and outcomes [23]. The large-duct type of ICC may share molecular features with perihilar CCA [24], and it is difficult to discriminate between perihilar and peripheral types solely by the tumor location. The peripheral CCA type can develop in the hepatic hilar area, which resembles the perihilar type. Therefore, this study included 19 perihilar CCAs as ICC.

Comparison of clinicopathological characteristics between patients with high and low LCR

The LCR was calculated as the lymphocyte count (number/μL) divided by the serum CRP concentration (mg/dL). The median LCR was 8981.1 (range 371.2–225,593), and a cut-off value of 7873.1 was calculated by ROC curve

analysis (AUC, 0.680; sensitivity, 64.7%; specificity, 70.5%, $P=0.0049$, Fig. 1). Using this cut-off value, 44 patients (56.4%) and 34 patients (43.6%) were assigned to the high- and low-LCR groups, respectively. The clinicopathological characteristics of the patients in the LCR groups are shown in Table 1. Overall, compared with the high-LCR group, the low-LCR group had significantly lower median levels of CRP (0.08 and 0.44, respectively, $P<0.001$), higher median levels of serum alkaline phosphatase (267.0 and 373.5 U/L, respectively, $P=0.0051$), gamma-glutamyl transferase (61.8 and 118.0 IU/L, respectively, $P=0.0031$), and CA19-9 (2.4 and 77.3 U/mL, respectively, $P=0.0017$), and had significantly larger tumor size (median 3.5 and 5.5 cm, respectively, $P=0.0018$). Moreover, all of the inflammation- or inflammation nutrition-based markers, except PLR, were significantly worse in patients with low LCR status than in those with high LCR status (NLR, $P=0.0123$; LMR, $P=0.0202$; Prognostic Nutritional Index (PNI), $P=0.0348$; CAR, $P<0.001$; modified Glasgow Prognostic Score (GPS), $P<0.001$; Controlling Nutritional Status (CONUT) score, $P=0.0151$). No significant difference was observed for preoperative cholangitis between the two groups. The other clinicopathological characteristics analyzed were not significantly different between the two groups.

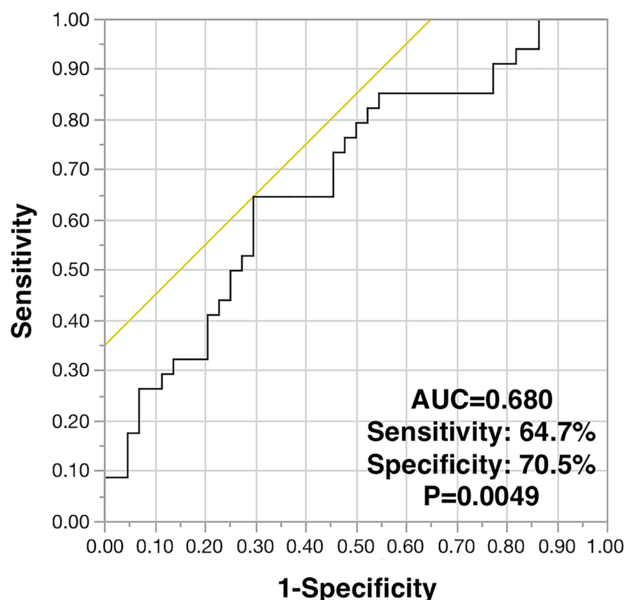


Fig. 1 Receiver operating characteristic curve analysis to determine the optimal cut-off value of the LCR. Area under the curve, sensitivity, and specificity were 0.680, 64.7%, and 70.5%, respectively

Prognosis of patients according to the LCR

Kaplan–Meier OS and RFS curves for the patients with high and low LCR are shown in Fig. 2. Patients with low LCR status had significantly worse prognosis than those with high LCR status for both OS (log-rank test, $P=0.0032$) and RFS (log-rank test, $P=0.0048$) (Fig. 2). The 1, 3, and 5 year OS rates for the high- and low-LCR groups were 97.4% and 79.5%, 66.3% and 39.2%, and 66.3% and 21.5%, respectively. The 1, 3, and 5 year RFS rates in the high- and low-LCR groups were 67.6% and 53.1%, 51.9% and 24.0%, and 51.9% and 18.0%, respectively.

In this study, CA19-9 ≥ 100 U/mL was used as the cut-off value because this level has been reported to be a significant predictor of poor prognosis for ICC [25]. Univariate analyses showed that the significant prognostic factors for OS were low LCR status, NLR, CAR, high modified GPS, CRP, high serum CA19-9 levels (≥ 100 U/mL), large tumor size (≥ 5 cm), positivity for microvascular invasion, and intrahepatic and lymph node metastases. For RFS, the significant prognostic factors were low LCR status, CAR, high modified GPS, CRP, high serum CA19-9 levels (≥ 100 U/mL), large tumor size (≥ 5 cm), positivity for microvascular invasion, and intrahepatic and lymph node metastases. In multivariate analysis, low LCR status and positivity for lymph node metastasis were identified as significant independent prognostic factors for OS; and high serum CA19-9 levels (≥ 100 U/mL), large tumor size (≥ 5 cm), and positivity for microvascular invasion were identified as significant independent prognostic factors for RFS (Table 2).

Relationships between the LCR and TIME features

Next, we evaluated sections of ICC tissues by histological and immunohistochemical staining to investigate the relationships between LCR and four key aspects of the TIME, namely the abundance of intratumoral CD34 + microvessels, CD8+ TILS, and FOXP3+ TILs, and the level of PD-L1 expression in cancer cells. The extent of intratumoral inflammatory cell infiltration was variable in ICC tissues, as evaluated by H&E staining [21] (Fig. 3A and B). We detected a highly significant relationship between the LCR and the density of TILs ($r=0.252$, $P=0.0263$; Fig. 4A). Interestingly, however, when we independently evaluated the density of CD8+ and FOXP3+ TILs by immunohistochemical staining (Fig. 3D and E and Fig. 4B and C, respectively), we detected a significant correlation between the LCR and CD8+ TIL density ($r=0.377$, $P=0.0007$; Fig. 4B), but not between the LCR and FOXP3+ TIL density ($r=-0.139$, $P=0.2249$; Fig. 4C). With

Table 1 Clinicopathological features of patients with high and low LCR following hepatic resection for intrahepatic cholangiocarcinoma

Factors	High LCR ($n=44$)	Low LCR ($n=34$)	P value
Age (year)	65 (41–87)	69 (39–87)	0.3914
Sex (male/female)	28/16	27/7	0.1297
HBV (+, %)	3 (6.8%)	4 (11.8%)	0.4485
HCV (+, %)	5 (11.4%)	2 (5.9%)	0.0649
Preoperative cholangitis (presence, %)	3 (6.8%)	3 (8.8%)	0.7417
Albumin (g/dL)	4.2 (3.3–4.9)	4.1 (3.3–5.3)	0.7076
Total bilirubin (mg/dL)	0.7 (0.2–1.7)	0.7 (0.3–8.7)	0.7076
ALP (U/L)	267.0 (141–1337)	373.5 (127–1344)	0.0051*
γ-GTP (IU/L)	61.8 (21–574)	118.0 (21–1071)	0.0031*
CRP (mg/dL)	0.08 (0.01–0.20)	0.44 (0.07–4.01)	< 0.001**
Lymphocytes (/μL)	1544.0 (526.3–3950.2)	1411.0 (363.0–2789.8)	0.1919
Platelets ($\times 10^4$/μL)	19.8 (7.4–40.2)	17.8 (5.2–44.0)	0.8285
ICG15 (%)	9.3 (1.9–28.5)	12.1 (2.6–31.0)	0.1524
NLR	1.96 (0.61–4.91)	2.59 (1.33–15.0)	0.0123*
PLR	128.0 (50.4–381.9)	130.3 (54.8–647.2)	0.5355
LMR	4.74 (1.40–11.3)	3.76 (0.53–13.2)	0.0202*
PNI	49.8 (39.5–59.8)	46.7 (36.1–58.8)	0.0348*
CAR	0.018 (0.002–0.054)	0.100 (0.016–1.146)	< 0.001**
Modified GPS: 0/1–2	43/1	21/13	< 0.001**
CONUT score: 0–1/≥ 2	29/15	13/21	0.0151*
CEA (ng/mL)	2.4 (0.4–41.8)	3.4 (0.6–30.7)	0.2005
CA19-9 (U/mL)	20.6 (0.6–21,100)	77.3 (0.6–40,795)	0.0017*
Tumor size (cm)	3.5 (0.5–8.0)	5.5 (1.2–12.0)	0.0018*
Tumor localization (peripheral type/perihilar type)	35/9	24/10	0.3608
ICC subtype ($n=59$) MF/PI/IG	32/3/0	24/0/0	0.1410
Poor differentiation (%)	26 (59.1%)	21 (61.8%)	0.8109
Microvascular invasion (%)	21 (47.7%)	18 (52.9%)	0.6479
Bile duct invasion (%)	18 (40.9%)	14 (41.2%)	0.9810
Intrahepatic metastasis (%)	13 (29.6%)	14 (41.2%)	0.2843
Lymph node metastasis (%)	7 (15.9%)	8 (23.5%)	0.3971
Histological liver cirrhosis (%)	5 (11.4%)	5 (14.7%)	0.6615

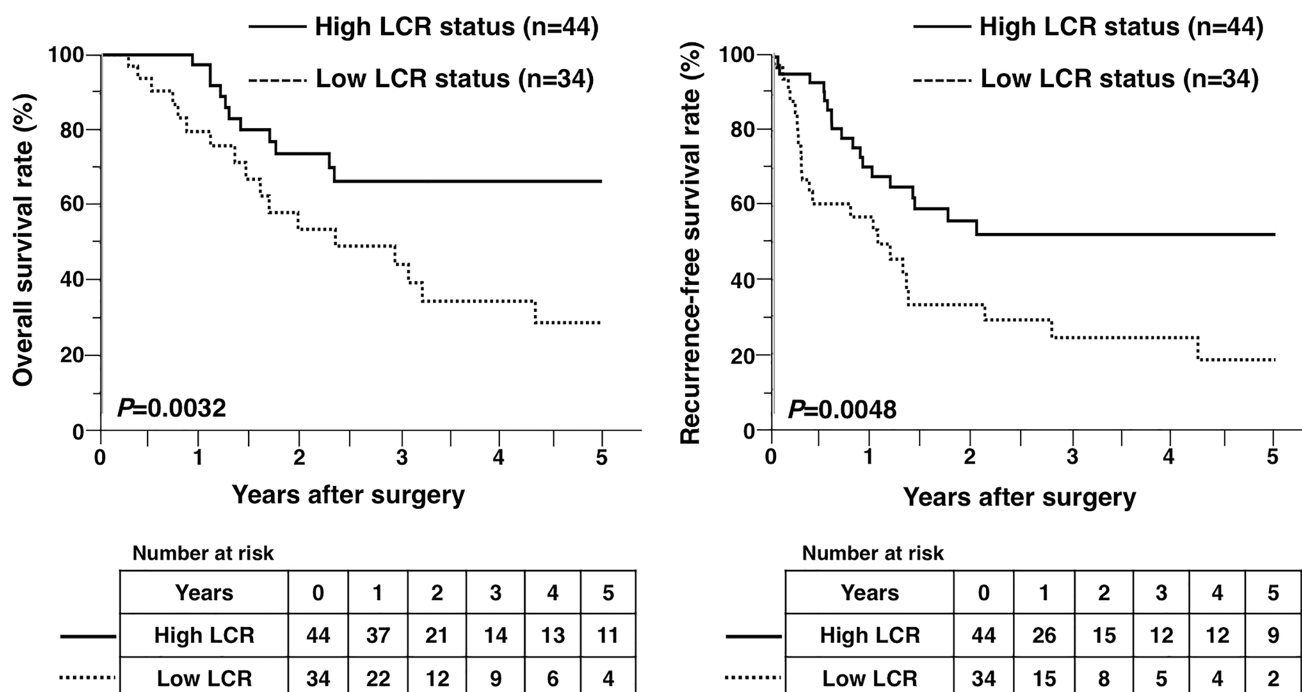
Data are presented as *n* (%) or median (range)

ALP alkaline phosphatase, *CA19-9* carbohydrate antigen 19-9, *CAR* CRP–albumin ratio, *CEA* carcinoembryonic antigen, *CONUT score* Controlling Nutritional Status score, *CRP* C-reactive protein, *γ-GTP* γ-glutamyl transpeptidase, *GPS* Glasgow Prognostic Score, *HBV* hepatitis B virus, *HCV* hepatitis C virus, *ICG15* indocyanine green retention rate at 15 min, *ICC* intrahepatic cholangiocarcinoma, *IG* intraductal growth, *LCR* lymphocyte–CRP ratio, *LMR* lymphocyte–monocyte ratio, *MF* mass-forming, *MVD* microvessel density, *NLR* neutrophil–lymphocyte ratio, *PI* periductal infiltrating, *PLR* platelet–lymphocyte ratio, *PNI* Prognostic Nutritional Index

*$P < 0.05$

**$P < 0.001$

respect to tumor characteristics, we observed a significant correlation between the LCR and microvessel density (MVD) ($r=0.369$, $P=0.0009$; Fig. 4D), but not between the LCR and PD-L1 expression level in ICC cells. Using a cut-off point for positive PD-L1 expression of > 1% of total cancer cells, as previously described [26], the patients were divided into PD-L1-negative ($n=36$, 46.2%) and PD-L1-positive ($n=42$, 53.8%) groups. As shown in Fig. 4E, there was no significant difference in PD-L1 positivity among tumors in the high- and low-LCR groups (50.0% vs. 58.8%, respectively, $P=0.4383$).



Years	0	1	2	3	4	5
High LCR	44	37	21	14	13	11
Low LCR	34	22	12	9	6	4

Years	0	1	2	3	4	5
High LCR	44	26	15	12	12	9
Low LCR	34	15	8	5	4	2

Fig. 2 Overall survival (OS) and recurrence-free survival (RFS) of patients with high and low LCR status after hepatic resection for ICC. Patients were dichotomized into low- and high-LCR status groups using the cut-off value defined by Fig. 1

Discussion

In the current study, we demonstrated that low LCR status was a significant predictor of OS and RFS in patients with ICC who underwent surgical resection and the LCR correlated significantly with some features of the TIME. Thus, a low LCR correlated significantly with low CD8+ TIL density and low MVD but not with FOXP3+ TIL density or PD-L1 expression in ICC. Overall, our data revealed that low LCR status was significantly associated with ICC progression, possibly reflecting an attenuated anti-tumor response.

Increasing evidence suggests that certain systemic inflammation, nutrition, and immunity markers are predictors of poor prognosis in several cancers. These markers can be categorized as inflammation-based, such as the NLR, PLR, and LMR, and inflammation nutrition-based, such as the PNI, CAR, GPS, and CONUT score. By consensus, these markers are now accepted as prognostic indicators for ICC patients who undergo radical resection. High NLR [5, 27] and PLR [28] and low LMR [5, 29] have been shown to correlate with poor outcome in resected patients with ICC, and low PNI [30], high CAR [7], high GPS [31], and high CONUT score [32] have also been associated with poor survival of ICC patients. However, among these markers, it is unclear which is the superior predictor of poor survival in ICC patients.

Recently, Okugawa et al. evaluated the prognostic value of the LCR in patients with colorectal cancer [10]. The authors analyzed five key systemic inflammation markers (neutrophils, lymphocytes, platelets, albumin, and CRP) to calculate the various indexes and identified LCR as a promising new marker with the highest accuracy for predicting oncological outcomes in these patients. More recently, Noguchi et al. and Lu et al. also analyzed these inflammatory and nutritional markers and, similarly, they identified LCR as the best prognostic marker in patients with ICC who underwent resection [11, 12]. Our results are consistent with these studies and showed that low LCR status was the strongest independent prognostic factor among the other inflammation- and inflammation nutrition-based markers that we evaluated in resected ICC patients. In addition, our analysis of the relationships between the LCR and clinicopathological features of ICC patients showed significant associations between a low LCR and large tumor size and high CA19-9 levels, which revealed that a decrease in anti-tumor immune cells may lead to ICC

Table 2 Univariate and multivariate analyses of risk factors associated with overall and recurrence-free survival following hepatic resection for intrahepatic cholangiocarcinoma

Factors	Overall survival				Recurrence-free survival			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Low LCR status	2.80 (1.37–5.75)	0.0048*	2.79 (1.08–7.24)	0.0348*	2.33 (1.27–4.27)	0.0061*	1.80 (0.82–3.94)	0.1428
NLR	1.25 (1.01–1.47)	0.0436*	1.07 (0.78–1.37)	0.6000	1.21 (0.99–1.42)	0.0636		
PLR	1.00 (0.99–1.00)	0.7017			1.00 (0.99–1.00)	0.7602		
LMR	0.87 (0.71–1.71)	0.1354			0.92 (0.78–1.06)	0.2414		
PNI	0.96 (0.90–1.03)	0.2878			0.97 (0.91–1.02)	0.2538		
CAR	1.02 (1.00–1.03)	0.0249*	1.12 (0.93–1.33)	0.2241	1.01 (1.00–1.03)	0.0448*	1.16 (0.99–1.35)	0.0649
Modified GPS (1–2)	2.56 (1.14–5.74)	0.0228*	2.20 (0.42–11.4)	0.3484	2.36 (1.15–4.84)	0.0193*	2.39 (0.74–7.71)	0.1443
CONUT score (≥ 2)	1.34 (0.66–2.71)	0.4143			1.25 (0.68–1.46)	0.4669		
Lymphocytes (/μL)	1.00 (0.99–1.00)	0.7573			0.99 (0.99–1.00)	0.9642		
CRP (mg/dL)	1.52 (1.05–2.05)	0.0270*	0.04 (0.00–6.89)	0.2015	1.45 (1.01–1.96)	0.0460*	0.01 (0.00–1.11)	0.0548
Age (≥ 67, median)	1.85 (0.91–3.74)	0.0887			1.09 (0.59–2.00)	0.7776		
Male	1.19 (0.55–2.58)	0.6536			1.24 (0.63–2.41)	0.5328		
Albumin (< 3.5 g/dL)	2.18 (0.65–7.25)	0.2051			2.02 (0.72–5.69)	0.1844		
CA19-9 (≥ 100 U/mL)	2.64 (1.23–5.66)	0.0130*	2.19 (0.87–5.50)	0.0963	2.77 (1.46–5.24)	0.0017*	2.27 (1.09–4.69)	0.0278*
Tumor size (≥ 5 cm)	2.60 (1.29–5.24)	0.0073*	1.90 (0.85–4.26)	0.1172	2.89 (1.54–5.41)	0.0009**	2.21 (1.10–4.43)	0.0256*
Poor differentiation	1.14 (0.57–2.31)	0.7075			1.47 (0.79–2.75)	0.2237		
Microvascular invasion (+)	2.41 (1.10–5.28)	0.0282*	2.29 (0.94–5.57)	0.0676	2.90 (1.50–5.63)	0.0016*	2.79 (1.30–5.97)	0.0083*
Bile duct invasion (+)	1.88 (0.92–3.86)	0.0841			1.22 (0.67–2.23)	0.5109		
Intrahepatic metastasis (+)	2.48 (1.23–5.03)	0.0114*	1.60 (0.28–1.41)	0.2593	2.66 (1.44–4.91)	0.0018*	1.72 (0.82–3.60)	0.1489
Lymph node metastasis (+)	3.41 (1.65–7.04)	0.0009**	3.65 (1.53–8.72)	0.0036*	2.37 (1.23–4.58)	0.0102*	1.73 (0.83–3.59)	0.1415
Histological liver cirrhosis (+)	1.24 (0.43–3.57)	0.6870			1.08 (0.42–2.76)	0.8740		

CA19-9 carbohydrate antigen 19-9, *CAR* CRP–albumin ratio, *CI* confidence interval, *CONUT score* Controlling Nutritional Status score, *CRP* C-reactive protein, *GPS* Glasgow Prognostic Score, *HR* hazard ratio, *LCR* lymphocyte–CRP ratio, *LMR* lymphocyte–monocyte ratio, *NLR* neutrophil–lymphocyte ratio, *PLR* platelet–lymphocyte ratio, *PNI* Prognostic Nutritional Index

*$P < 0.05$

**$P < 0.001$

proliferation. Thus, the LCR can be considered a likely systemic factor that reflects the host immune status. However, the mechanism underlying the association between LCR status and outcome in cancer has not been clarified. Based on our findings, we hypothesized that the LCR status might reflect the cytotoxicity of the intratumoral immune response, and we tested this hypothesis by examining several molecular and cellular features of the TIME.

Vigano et al. showed that high numbers of CD3+ and CD8+ TILs were associated with higher survival and lower recurrence rates in patients with ICC, and conversely, high numbers of regulatory FOXP3+ TILs were associated with worse prognosis [33]. We and others previously showed that a lower MVD correlated with tumor malignancy in ICC patients who underwent resection [18, 34], and we identified interactions between TILs and tumor angiogenesis as an important component of ICC progression [18]. In the present study, we identified a strong correlation between the LCR and the density of both CD8+ TILs and tumor microvessels. These findings suggest that the low LCR status in ICC patients, driven by the reduction in CD8+ TILs, is likely to reflect an attenuated anti-tumor response within the TIME. Although we detected a trend towards an inverse correlation between FOXP3+ TILs and LCR, this did not reach the level

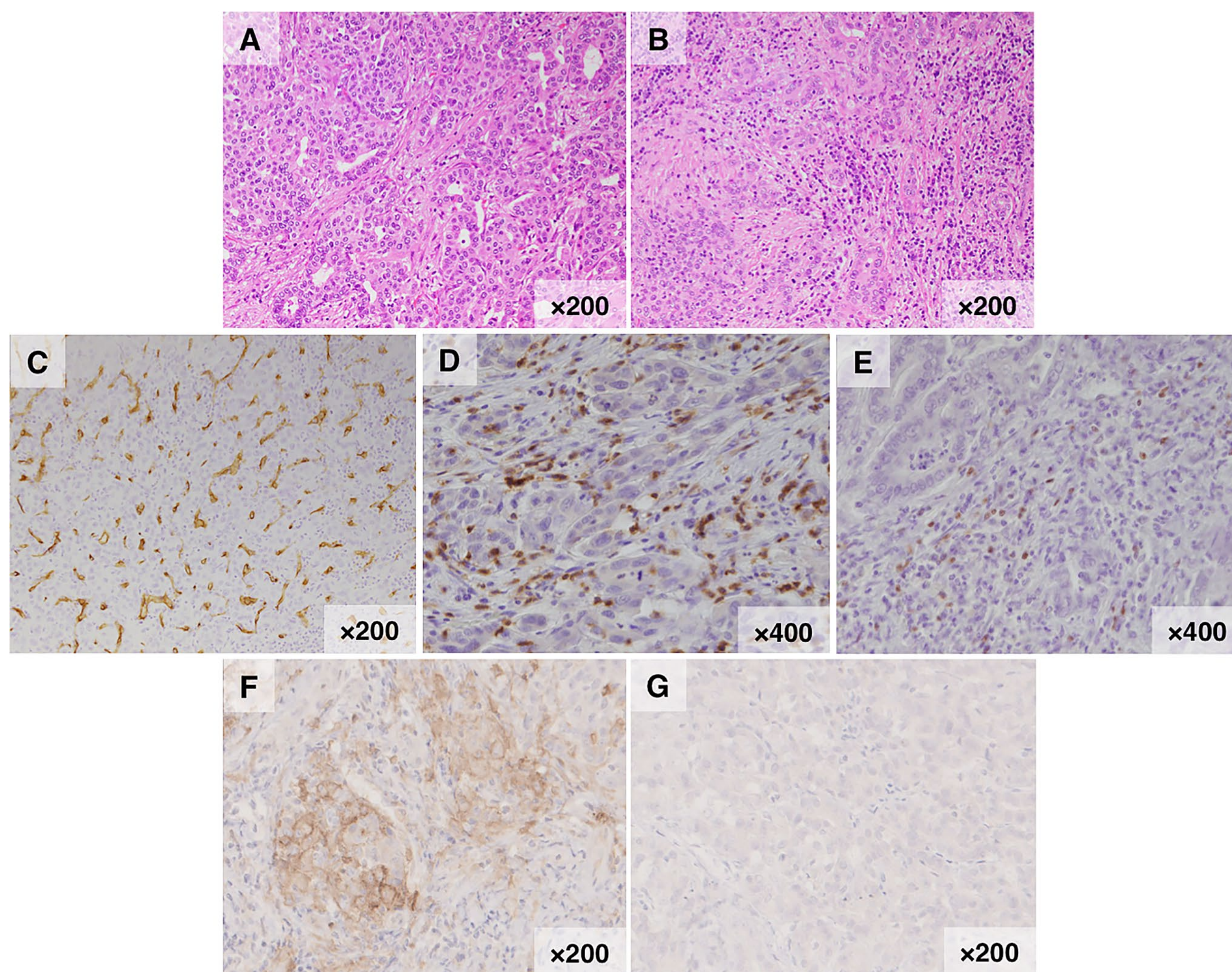


Fig. 3 Representative histochemical and immunohistochemical staining of tumors from ICC patients. Hematoxylin and eosin staining of **A** low-grade and **B** high-grade tumors. **C–E** Immunohistochemical staining of tumors for **C** CD34-positive microvessels, **D** CD8-positive TILs, and **E** FOXP3-positive TILs. Immunohistochemical staining of tumors showing **F** PD-L1-positive and **G** PD-L1-negative cancer cells. Magnification 200×(**A**, **B**, **F**, **G**) and 400×(**C–E**)

of statistical significance. The differentiation of regulatory T cells may be induced via cytokines, including transforming growth factor-β or interleukin-2 [35], that are not directly reflected by the LCR. Similarly, PD-L1 expression in ICC cells did not correlate with the LCR. The expression of PD-L1 may be regulated via metabolic [19] or endogenous [36] mechanisms that are unique to tumor cells. Therefore, PD-L1 expression may not be directly associated with a systemic inflammation-based marker under these conditions, which may be responsible for our negative results.

This study has some potential limitations. First, it was a single-center and long-term retrospective study designed to examine prognostic factors influencing OS and RFS. This case series included patients treated with postoperative adjuvant chemotherapy, which might have influenced their long-term outcomes. Second, we did not examine the detailed mechanisms underlying the associations between the TIME and LCR, such as molecular alterations. Finally, other tumor-infiltrating immune cells and factors that affect the outcomes of ICC patients were not investigated, and further work will be necessary to clarify their contributions.

In conclusion, our study demonstrated that low LCR status was an independent prognostic factor and reflected a low anti-tumor immune response in patients with ICC. Measurement of the LCR is routine and can easily be employed for risk stratification in the assessment of ICC patients. The LCR might also have utility as a reflection of the anti-tumor status of the TIME in ICC.

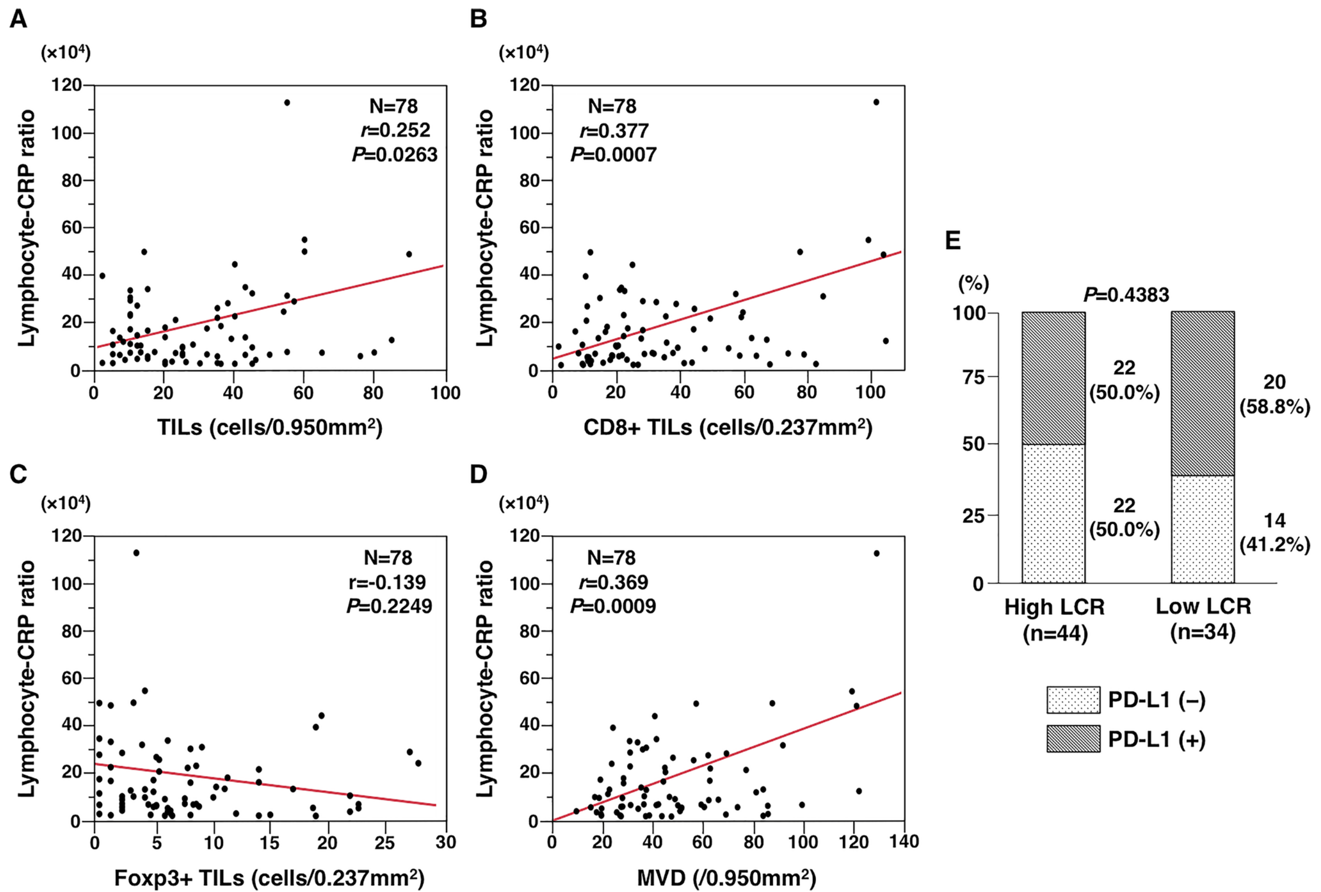


Fig. 4 Relationships between the LCR and TILs, MVD, and tumor PD-L1 expression in ICC patients. Relationship between the LCR and **A** total TILs, **B** CD8-positive TILs, **C** FOXP3-positive TILs, **D** tumor MVD, and **E** tumor PD-L1 expression

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Author contributions Study concepts: KY, SI, TY, and MM. Study design: KY, SI, TY, and MM. Data acquisition: KY, AM, and SI. Quality control of data and algorithms: KY, SI, TY, TT, NH, KK, and YO. Data analysis and interpretation: KY, SI, NI, TT, NH, KK, YO, and MM. Statistical analysis: KY, SI, and Y. Manuscript preparation: KY and SI. Manuscript editing: KY, SI, and TY. Manuscript review: TY, YO, and MM.

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Declarations

Conflict of interest The authors declare no conflicts of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was approved by the ethics committee of our hospital according to the ethical guidelines of the Japanese government (approval number: 2020-628). No animal studies were performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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