

Prognostic Implications of Lymphocyte/C-Reactive Protein Ratio With Tumor-Infiltrating Lymphocytes in Resected Intrahepatic Cholangiocarcinoma

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Research

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Abstract

Background

Lymphocyte/C-reactive protein (CRP) ratio (LCR), is a promising prognostic factor that can reflect tumor inflammation and systemic patient condition. The aim of this study was to investigate whether preoperative LCR can be a prognostic factor for post-surgical outcomes among patients with intrahepatic cholangiocarcinoma (IHCC). We also investigated the relationship between LCR and tumor infiltrating lymphocytes (TILs) to clarify whether systemic host immune parameters reflect local tumor immunity.

Methods

We enrolled 45 patients who had undergone hepatectomy for IHCC. Patients were divided into low LCR and high LCR groups, according to reactive operating characteristic curve (Cut-off value: 8836). We analyzed their overall survival (OS) and disease-free survival (DFS) with respect to LCR and other clinicopathological factors. We also investigated stromal TILs and numbers of CD8⁺ TILs in surgical specimens, and the relationship between LCR and TILs.

Results

Twenty-one patients (46.7%) were associated with low LCR. Low LCR was significantly correlated with older age, high CRP and advanced disease stage, and was a prognostic factor for OS and DFS. And multivariate analysis revealed that low LCR was an independent prognostic factor for worse OS (HR: 2.81 P<0.04). Regarding the relationship between LCR and local tumor immunity, while LCR and levels for stromal TILs were significantly related, LCR and levels for CD8⁺ TILs were significantly related.

Conclusions

Preoperative LCR levels could predict the post-surgical prognosis of patients with IHCC, and reflected numbers of intra-tumoral CD8⁺ TILs.

Trial registration

This study was approved by Tokushima University Hospital ethics committee and with the approval of corresponding regulatory agencies, and all the experiments were carried out in accordance with the approved guidelines (Tokushima Clinical Trial Management System Number; 3215). All the patients involved in this study signed informed consent forms and agreed to participate.

Background:

Although intrahepatic cholangiocarcinoma (IHCC) is the second-most common primary liver cancer after hepatocellular carcinoma (HCC), it is relatively rare, accounting for only 5% of primary liver cancers [1]. However, IHCC patients have been gradually increasing, whereas HCC patients have been decreasing [2].

It is a biliary tract cancer derived from the intrahepatic bile duct epithelium. Complete resection is the most effective treatment for IHCC, when possible. However, IHCC has a high recurrence rate after resection, and therefore has a poor prognosis [2].

Various combinations of blood test indicators have been studied in recent years as prognostic indicators for cancer patients, including the neutrophil/lymphocyte ratio (NLR) [3, 4], Modified Glasgow Prognostic Score (mGPS) [5, 6], and the Prognostic Nutrition Index (PNI) [7, 8], among others. These combinations can represent systemic inflammation, immune function, and/or nutritional status.

Reportedly, the preoperative platelet/lymphocyte ratio (PLR), systemic immune inflammation index (SII; platelets \times NLR), and lymphocyte/monocyte ratio (LMR) are significantly associated with outcomes in patients with IHCC who undergo curative resection [9–11]. We have already focused on the prognostic effects of some proteins in resected IHCC specimens or preoperative image findings of IHCC patients. High expression of Heat Shock Protein 1 (HSF-1), and loss of Secreted Frizzled-Related Protein-1 (SFRP-1) and F-box and WD repeat domain-contain 7 (FBXW7) in surgical specimens were associated with poor prognosis in IHCC patients [12–14]; as were low apparent diffusion coefficient of diffusion-weighted image magnetic resonance imaging, and central hypo-enhancement in hepatic arterial phase of dynamic computed tomography in preoperative images [15, 16].

After Okugawa et al. reported lymphocyte/C-reactive protein (CRP) ratio (LCR), a new immune-nutritional index, to be a strong prognostic factor for patients with colorectal cancer [17], LCR was also found to indicate prognosis of gastric cancer, pancreatic cancer, rectal cancer treated with chemoradiotherapy, and breast cancer. [18–21].

Whereas LCR and other immune-nutritional parameters represent the patient's systemic condition, tumor-infiltrating lymphocytes (TILs) may reflect local tumor immunity. Lymphocyte-predominant breast cancer (LPBC), which has high TIL levels, has a good prognosis [22]. Lee et al. reported a significant correlation between peripheral lymphocyte count and TILs in patients with cervical carcinoma [23], whereas Yoon et al. reported a significant relationship between TILs and peripheral neutrophil count in breast cancer [24]. CD8⁺ lymphocytes are considered to be especially crucial among TILs. Zhu et al. reported a correlation between tumoral programmed death ligand 1 (PD-L1) expression and CD8⁺ T-cell infiltration in IHCC patients [25], and Asahi et al. reported that numbers of CD8⁺ T-cells in a tumor's outer border area were associated with postsurgical outcomes in patients with IHCC [26].

This study investigated the relationships between preoperative LCR and postsurgical outcomes in patients with IHCC, and between LCR levels and intra-tumoral TILs, to clarify whether systemic immune indicators reflect local tumor immunity.

Methods:

Patients

We enrolled 45 patients who underwent primary hepatectomy for IHCC at Tokushima University Hospital between July 2002 and May 2015 in this study. We excluded patients who had undergone chemotherapy or radiation prior to surgery, or whose blood tests including cell fractions, were not measured within 4 weeks before surgery. We obtained data on other clinicopathological parameters, such as age, gender, blood parameters, image findings, operative information, pathological diagnosis, and so on, from the medical database. The patients' median follow-up period was 2.5 years (range: 0.17–17.08). This study was approved by Tokushima University Hospital ethics committee and with the approval of corresponding regulatory agencies, and all the experiments were carried out in accordance with the approved guidelines (Tokushima Clinical Trial Management System Number; 3215). All the patients involved in this study signed informed consent forms and agreed to participate.

Treatment strategy for IHCC

Patients were treated according to a previously described strategy for resectable IHCC [15, 27]. Briefly, limited hepatectomy was performed without typical lymph node dissection or extrahepatic bile duct resection for peripheral tumors. For tumors in the perihilar region, or in the peripheral region with hilar infiltration, anatomical hepatectomy and regional lymph node dissection were performed. Extrahepatic bile duct resection and reconstruction were performed if needed for surgical margins.

Preoperative immune parameters

Blood samples were taken prior to hepatectomy. LCR was calculated by dividing total lymphocyte count (TLC) (/ μ L) by serum CRP (mg/dL). The optimal cutoff value for LCR was determined by using receiver operating characteristics (ROC) curves on mortality five years after surgery. 5-year survival was 39.1% (Fig. 1a). The cutoff value of LCR determined by ROC curve was 8836 (1-Specificity, Sensitivity: 0.100, 0.667; area under curve: 0.815; Fig. 1b).

Assessment of TILs

TILs were evaluated according to the standardized methodology proposed by the International TIL Working Group [28]. In summary, rather than counting the number of lymphocytes, we assessed stromal TILs by calculating the ratio of TILs to stromal areas, excluding tumor cells, in hematoxylin and eosin (HE)-stained samples (Fig. 2a). We excluded the tumor cell areas (Fig. 2b) and calculated stromal and TILs areas (Fig. 2c) by using the image analysis software ImageJ (National Institute of Health). Assessments were performed in areas within the borders of the invasive tumor, and tumor zones with crush artifacts, necrosis, regressive hyalinization were excluded. We scored all mononuclear cells, including lymphocytes and plasma cells, but polymorphonuclear leukocytes or fibroblasts were excluded. After a full assessment of average TILs in the tumor border area, we assessed the stromal TILs averaged among three selected high-power fields (400 \times magnification), not focused on hotspots. Thus, we evaluated TILs for 38 patients whose HE slides were available, under the guidance of two expert pathologists who were blinded to clinical features.

Assessment of CD8⁺ TILs

Anti-CD8 antibody (dilution 1:100, M7103; Dako) was used as the primary antibody. We used immunohistochemistry procedures in our department that were previously reported by Ishikawa et al [29]. Selecting average regions within the tumor borders, we manually counted CD8⁺ TILs in three high-power fields (400 × magnification) per patient and calculated the mean number of CD8⁺ TILs, for the 31 patients whose samples were available. Figure 3 showed representative cases of high (Fig. 3a) and low (Fig. 3b) CD8⁺ TILs. These procedures were conducted under the guidance of two expert pathologists.

Statistical analysis

We used the unpaired Mann–Whitney U-test or the χ^2 test to compare clinicopathological variables between two groups. Overall survival (OS) and disease-free survival (DFS) curves were created using Kaplan–Meier method; differences were analyzed by log-rank test. Multivariate analysis was performed on factors which had significant difference in univariate analysis. The Cox proportional hazard regression model was used for multivariate analysis. $P < 0.05$ was considered significant.

Results:

Preoperative LCR levels can accurately reflect post-surgical prognosis

Patients were divided into the high LCR (LCR^{High}) group and the low LCR (LCR^{Low}) group. Median values indicated that the LCR^{Low} group was significantly older, and had higher CRP levels, longer operation time, and more advanced disease than the LCR^{High} group (Table 1). Other host, surgical, and tumor factors did not significantly differ between the two groups.

Table 1
Clinicopathological factors in the LCR high and low group

Variables	LCR > 8836 (n = 24)	LCR ≤ 8836 (n = 21)	P-value
Host factors			
Age (y)	67.1 ± 7.5	73.9 ± 7.1	< 0,01
Gender (M/F)	17 / 7	15 / 6	0.96
TLC (/μL)	1586 ± 578	1414 ± 455	0.42
ANC (/μL)	3480 ± 1005	4157 ± 1525	0.26
CRP (mg/dL)	0.10 ± 0.06	0.93 ± 1.60	< 0.01
NLR (< 2.24 / ≥2.24)	14 / 10	7 / 14	0.09
Frail (- / +)	18 / 6	11 / 10	0.11
HBV (- / +)	18 / 6	18 / 3	0.37
HCV (- / +)	21 / 3	20 / 1	0.35
Surgical factors			
Operation time (min)	346 ± 110	387 ± 75	0.02
Blood loss (mL)	259 ± 186	372 ± 289	0.22
Postoperative complication (- / +*)	21 / 3	19 / 2	0.75
Operation (Hr 0, 1 / Hr 2, 3)	8 / 16	6 / 15	0.73
Curability (A, B / C)	22 / 2	12 / 9	< 0.01
Tumor factors			
Size (< 3 cm / ≥3 cm)	7 / 17	6 / 15	0.68
Number (1 / 2 / 3)	24 / 0	16 / 3 / 2	0.02
LNM (- / +)	18 / 6	14 / 7	0.54
vp (- / +)	16 / 8	13 / 8	0.74
vv (- / +)	22 / 2	18 / 3	0.53
im (- / +)	24 / 0	16 / 5	< 0.01
Stage (I, II / III, IV)	11 / 13	2 / 19	< 0.01
CEA (< 10 / ≥ 10 ng/mL)	20 / 4	16 / 5	0.55
CA19-9 (< 100 / ≥100 U/mL)	15 / 9	10 / 11	0.32

ANC, absolute neutrophil count; CA19-9, carbohydrate antigen 19 – 9; CEA, carcinoembryonic antigen; CRP, C-reactive protein; HBV, hepatitis B virus; HCV, hepatitis C virus; im, intrahepatic metastasis; LCR, lymphocyte-CRP ratio; LMN, lymph node metastasis; NLR, neutrophil-lymphocyte ratio; TLC, total lymphocyte count; vp, portal vein invasion; vv, hepatic vein invasion.

*Complications of Clavien-Dindo \geq IIIa were considered positive.

Five-year OS rates after hepatectomy were significantly worse in the LCR^{Low} group (15.2%) than in the LCR^{High} group (57.5%; $P < 0.01$; Fig. 4a). In univariate analysis, advanced disease stage (III/IV), lymph node metastasis (LNM), low LCR, and high CA19-9 were associated with worse OS. In multivariate analysis, LNM, low LCR, and high CA19-9 were independent prognostic factors (Table 2).

Table 2
Univariate and multivariate analysis for overall survival

Variables	5-year OS (%)	Univariate	Multivariate	
		P-value	HR (95% CI)	P-value
Host factors				
Age (< 75 / \geq 75y)	43.6 / 28.6	0.11		
Gender (M/F)	39.1 / 32.5	0.64		
NLR (< 2.24 / \geq 2.24)	59.5 / 24.1	0.09		
LCR (> 8836 / \leq 8836)	57.5 / 15.2	< 0.01	2.81 (1.05–7.56)	0.04
Frail (- / +)	40.4 / 36.9	0.54		
Tumor factors				
Size (< 3 cm / \geq 3 cm)	47.6 / 37.6	0.95		
LNM (- / +)	40.8 / 18.3	0.02	2.67 (1.10–6.48)	0.03
vp (- / +)	46.6 / 16.9	0.05		
vv (- / +)	38.4 / 20.0	0.62		
im (- / +)	39.7 / 37.5	0.74		
Stage (I, II / III, IV)	72.2 / 25.8	0.01	1.71 (0.49–5.89)	0.39
CEA (< 10 / \geq 10 ng/mL)	40.4 / 33.3	0.73		
CA19-9 (< 100 / \geq 100 U/mL)	57.7 / 15.2	< 0.01	3.45 (1.33-9.00)	0.01

ANC, absolute neutrophil count; CA19-9, carbohydrate antigen 19 – 9; CEA, carcinoembryonic antigen; CRP, C-reactive protein; im, intrahepatic metastasis; LCR, lymphocyte-CRP ratio; LMN, lymph node metastasis;

NLR, neutrophil-lymphocyte ratio; TLC, total lymphocyte count; vp, portal vein invasion; vv, hepatic vein invasion.

Three-year DFS rates after hepatectomy were also significantly worse in the LCR^{Low} group (0%) than in the LCR^{High} group (31.8%; Fig. 4b). Univariate analysis associated age \geq 75 years old, advanced disease stage, LNM, portal vein invasion, and low LCR with poor prognosis, but none of these factors were found to be independent prognostic factors in multivariate analysis (Table 3).

Table 3
Univariate and multivariate analysis for disease-free survival

Variables	3-year DFS (%)	Univariate	Multivariate	
		P-value	HR (95% CI)	P-value
Host factors				
Age (< 75 / \geq 75y)	28.0 / 0	0.02	1.12 (0.38–3.34)	0.83
Gender (M/F)	20.8 / 20.0	0.84		
NLR (< 2.24 / \geq 2.24)	21.7 / 20.0	0.83		
LCR (> 8836 / \leq 8836)	31.8 / 0	< 0.01	1.87 (0.68–5.18)	0.23
Frail (- / +)	27.3 / 9.1	0.21		
Tumor factors				
Size (< 3 cm / \geq 3 cm)	30.0 / 16.7	0.10		
LNM (- / +)	27.0 / 0	0.02	1.65 (0.62–4.41)	0.32
vp (- / +)	26.1 / 9.1	0.04	1.36 (0.48–3.87)	0.57
vv (- / +)	20.7 / 25.0	0.48		
im (- / +)	22.6 / 0	0.28		
Stage (I, II / III, IV)	54.6 / 4.4	< 0.01	2.64 (0.70–9.92)	0.15
CEA (< 10 / \geq 10 ng/mL)	21.5 / 20.0	0.85		
CA19-9 (< 100 / \geq 100 U/mL)	28.6 / 8.3	0.22		

ANC, absolute neutrophil count; CA19-9, carbohydrate antigen 19 – 9; CEA, carcinoembryonic antigen; CRP, C-reactive protein; im, intrahepatic metastasis; LCR, lymphocyte-CRP ratio; LNM, lymph node metastasis; NLR, neutrophil-lymphocyte ratio; TLC, total lymphocyte count; vp, portal vein invasion; vv, hepatic vein invasion.

Preoperative LCR levels can reflect numbers of local tumor CD8⁺ TILs

The median percentages of TILs were 0.39% (interquartile range [IQR]: 0.23–0.97) in the LCR^{High} group, and 0.45% (IQR: 0.26–0.61) in the LCR^{Low} group. (Fig. 5a) LCR and stromal TILs were not significantly associated.

The median numbers of CD8⁺ TILs were 21.3/slide (IQR: 12.3–34.7) in the LCR^{High} group, and 12.8/slide (IQR: 6.7–15.9) in the LCR^{Low} group (Fig. 5b). The number of CD8⁺ TILs was related to LCR ($P = 0.0481$).

Discussion:

The present study suggests that preoperative LCR can indicate the postsurgical prognosis for patients who undergo resection for IHCC. Furthermore, as LCR was associated with CD8⁺ TILs, it indirectly reflects local tumor immune activity. This is the first report to find a correlation between a systemic immune marker and CD8⁺ TILs in patients with IHCC.

LCR is already reported to be a prognostic marker for colorectal, gastric, pancreatic, and breast cancers [17–21]. For patients with IHCC who undergo curative resection, NLR, LMR, PLR, and SII are reportedly significant prognostic factors [4, 9–11]. Many papers have especially suggested NLR as a strong prognostic marker for IHCC. In our result, NLR tended to be a prognostic factor in OS, but not significantly so, whereas LCR was found to be an independent prognostic factor. LCR is the combination of TLC and CRP. CRP is a well-established marker for inflammation. Elevated preoperative CRP level is widely reported to be associated with poor prognosis in various cancers, including IHCC [30–32]. CRP is synthesized by hepatocytes in response to IL-6, TNF and IL-1 β , which can activate cancer cell proliferation. CRP may reflect systemic inflammation, and cancer cell proliferation and protection from apoptosis. [33, 34]. TLC also reflect host immune status; several papers have associated low TLC with poor prognosis [35, 36]. Thus, LCR, which is the combination of CRP and TLC, may be stronger prognostic factor than NLR. Low LCR was also significantly associated with high age and longer operation time. TLC can reflect host's nutritional status. Elderly patients with malignancy easily lose their nutritional status. Since the low LCR group had many advanced stage patients, it was considered that the addition of bile duct reconstruction and lymph node dissection led to the extension of operation time.

However, the mechanism that links LCR to patients' long-term prognosis has been unclear. In the present study, we focused on the relationship between LCR and TILs, which are indicators of local tumor immunity. TILs have attracted recent attention with the development of immune checkpoint inhibitors. In addition, there were some reports TILs had the relationship between peripheral blood cells, such as TLC and absolute neutrophil count (ANC) [23, 24].

Interestingly, although this study found no significant relationship between LCR and stromal TILs, LCR and CD8⁺ TILs, specifically, were associated. TILs volume is reportedly correlated with tumor mutation burden (TMB) [37]. TMB is the number of gene mutations cancer cells have, and is associated with predicted immunotherapy response [38]. In breast cancer, which is reported to have relatively high TMB and TILs, patients who have more than 50% TILs are often defined as LPBC; <10% TILs are considered

minimal [28]. In the present study, the median TIL percentage in all patients was 0.43% (IQR: 0.26–0.77), which was much lower than for breast cancer. Biliary cancer is also reported to have relatively low TMB [39]. Thus, TILs in IHCC were expected to be low, and our result was consistent with that. Low TIL percentage in IHCC may be why LCR and TILs did not correlate. Furthermore, the TIL evaluation method included all mononuclear cells—not only lymphocyte. As IHCC apparently has low TILs, possibly cells other than lymphocytes were affected.

TILs include various types of lymphocytes, such as CD3⁺, CD4⁺, CD8⁺ cells; CD8⁺ T cells are considered especially important among them. Tumor infiltration by CD8⁺ T cells has been strongly associated with survival of cancer patients [26, 28]. Yoshida et al. also reported that CRP inhibited proliferation, activation, and function of CD8⁺ T cells in patients with melanoma [40]. Thus, CD8⁺ TILs may be more strongly associated with LCR, which is a robust prognostic factor. LCR can reflect not only the inflammatory response and immune-nutritional status, but also local tumor immunity via CD8⁺ TILs. Therefore, LCR can predict prognosis more accurately than the conventional indicators, such as NLR.

CD8⁺ TILs reported as the prediction factor of response to chemotherapy or immune check point therapy [26, 41]. Thus, preoperative LCR may predict the response to adjuvant therapy. IHCC has high recurrence rate, not only local recurrence but also intrahepatic recurrence or distant metastasis. Reduced surgery may be tolerated in patients with low LCR to maintain general condition. Furthermore, in patients with low LCR, nutrition and exercise therapy before and after surgery may contribute to improving prognosis.

This study had several limitations. First, it is a retrospective analysis from a single center, with a relatively small study cohort. Second, although lymphocytes come in various types, such as CD3⁺ or CD4⁺ lymphocytes, we assessed only CD8⁺ T cells as most representative type for tumor immunity. A larger study, and an assessment of non-CD8⁺ lymphocytes are issues for the future.

Conclusions:

In conclusion, LCR can predict prognosis of patients with IHCC via correlation with CD8⁺ TILs.

Abbreviations

absolute neutrophil count: ANC

apparent diffusion coefficient: ADC

computed tomography: CT

C-reactive protein: CRP

disease-free survival: DFS

F-box and WD repeat domain-contain 7: FBXW7

hematoxylin and eosin: H.E

hepatocellular carcinoma: HCC

intrahepatic cholangiocarcinoma: IHCC

intrahepatic metastasis: im

lymphocyte-C-reactive protein ratio: LCR

lymphocyte-monocyte ratio: LMR

lymph node metastasis: LNM

Lymphocyte-predominant breast cancer: LPBC

magnetic resonance imaging: MRI

modified Glasgow Prognostic Score: mGPS

neutrophil-lymphocyte ratio: NLR

overall survival: OS

platelet-lymphocyte ratio: PLR

prognostic nutrition index: PNI

programmed death ligand 1: PD-L1

receiver operating characteristics: ROC

Secreted Frizzled-Related Protein-1: SFRP1

systemic immune inflammation index: SII

total lymphocyte count: TLC

tumor-infiltrating lymphocytes: TILs

tumor mutation burden: TMB

portal vein invasion: vp

hepatic vein invasion: vv

Declarations

Ethics approval and consent to participate

This study was approved by Tokushima University Hospital ethics committee and with the approval of corresponding regulatory agencies, and all the experiments were carried out in accordance with the approved guidelines (Tokushima Clinical Trial Management System Number; 3215).

Consent for publication

All the patients involved in this study signed informed consent forms and agreed to participate.

Availability of data and material

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Not applicable.

Authors' contributions

1. KM participated in the research design, performance of the research, data analysis and writing manuscripts.
2. YM participated in the research design, performance of the research.
3. SI participated in performance of the research.
4. TI participated in performance of the research.
5. YS participated in performance of the research.
6. SY participated in performance of the research.
7. KT participated in performance of the research.
8. SO participated in performance of the research.
9. SY participated in performance of the research.
10. TO participated in performance of the research, and pathological assessment.
11. KT participated in performance of the research, and pathological assessment.
12. MS participated in the critical comments and administrative support.

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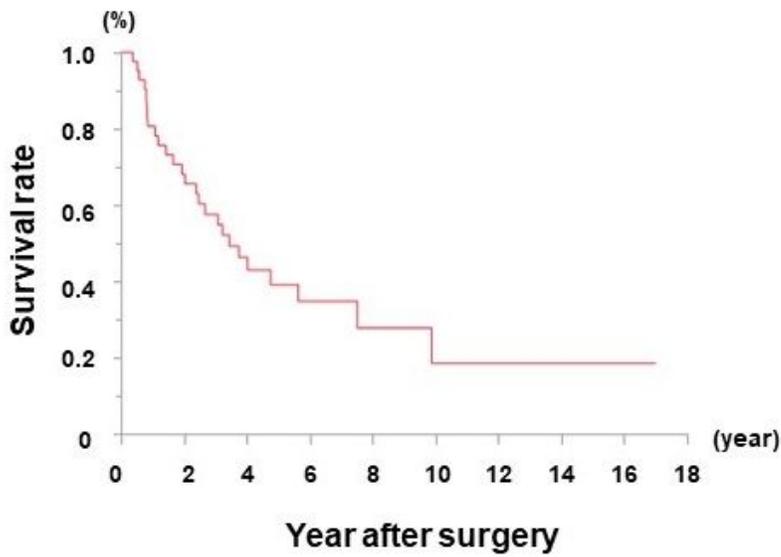
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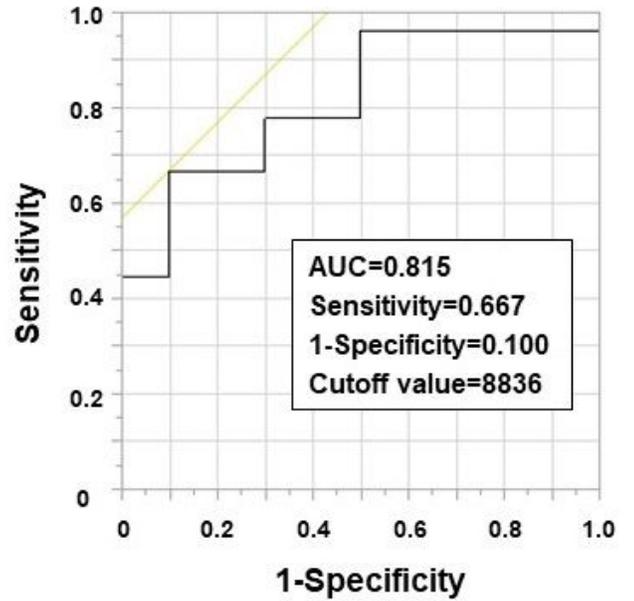
Figures

Overall survival



(a)

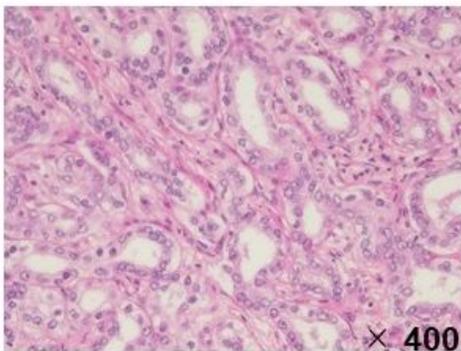
LCR cutoff value



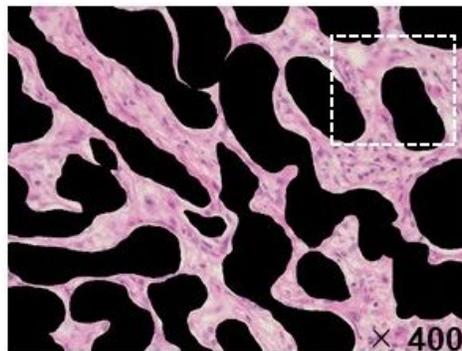
(b)

Figure 1

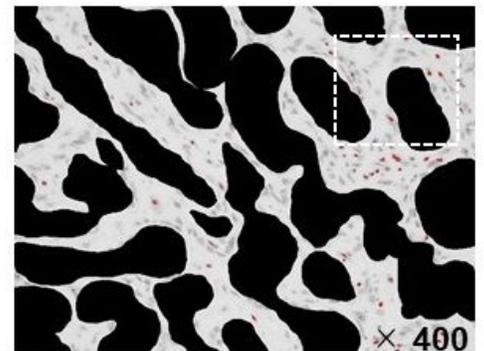
Survival curve and (ROC) curve for cutoff value to lymphocyte-C-reactive protein ratio. (a) Over all survival for all patients (5-year OS was 39.1%). (b) ROC curve for LCR (AUC=0.815, Sensitivity=0.667, 1-Specificity=0.100, Cutoff value=8836)



(a)



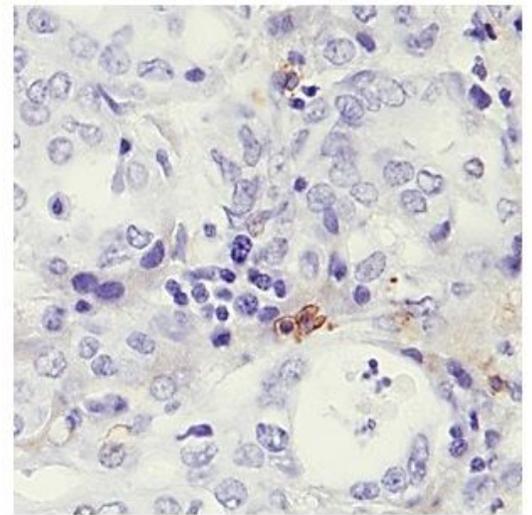
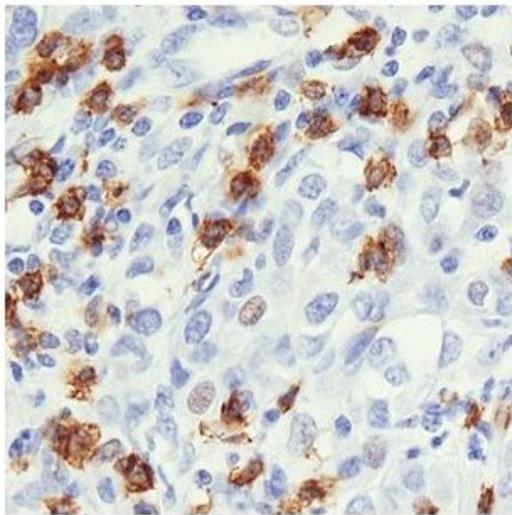
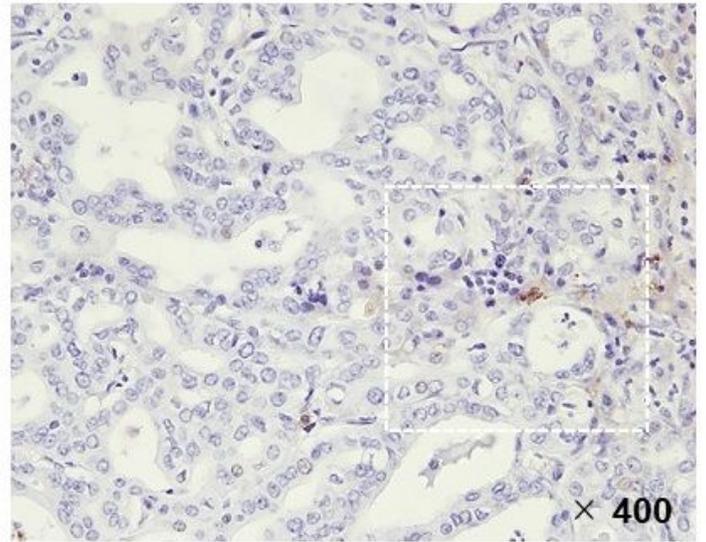
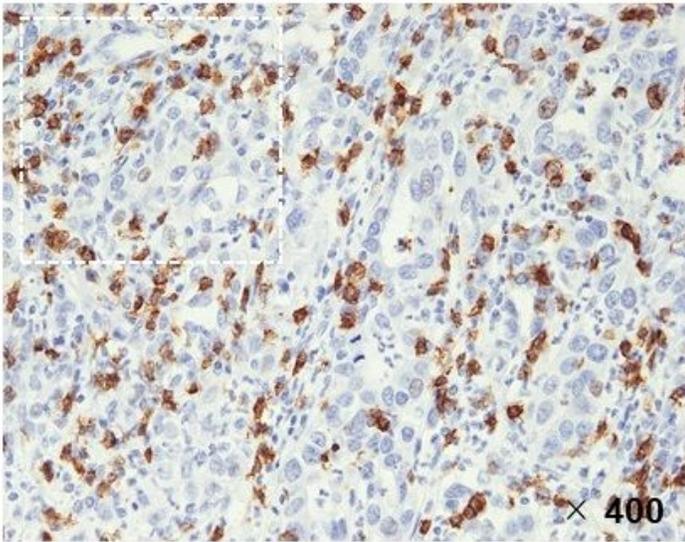
(b)



(c)

Figure 2

Method of assessing tumor-infiltrating leukocytes (TILs) using ImageJ. (a) For each patient, we selected 3 average regions in high-power fields (400× magnification); (b) excluded the tumor cells (black); and (c) calculated TILs as lymphocyte area (red) / stromal area (excluding black area), and averaged the score of TILs in the 3 fields.



(a)

(b)

Figure 3

Immunohistochemical staining for CD8+ tumor-infiltrating leukocytes (TILs). (a) Low CD8+ TILs; (b) high CD8+ TILs.

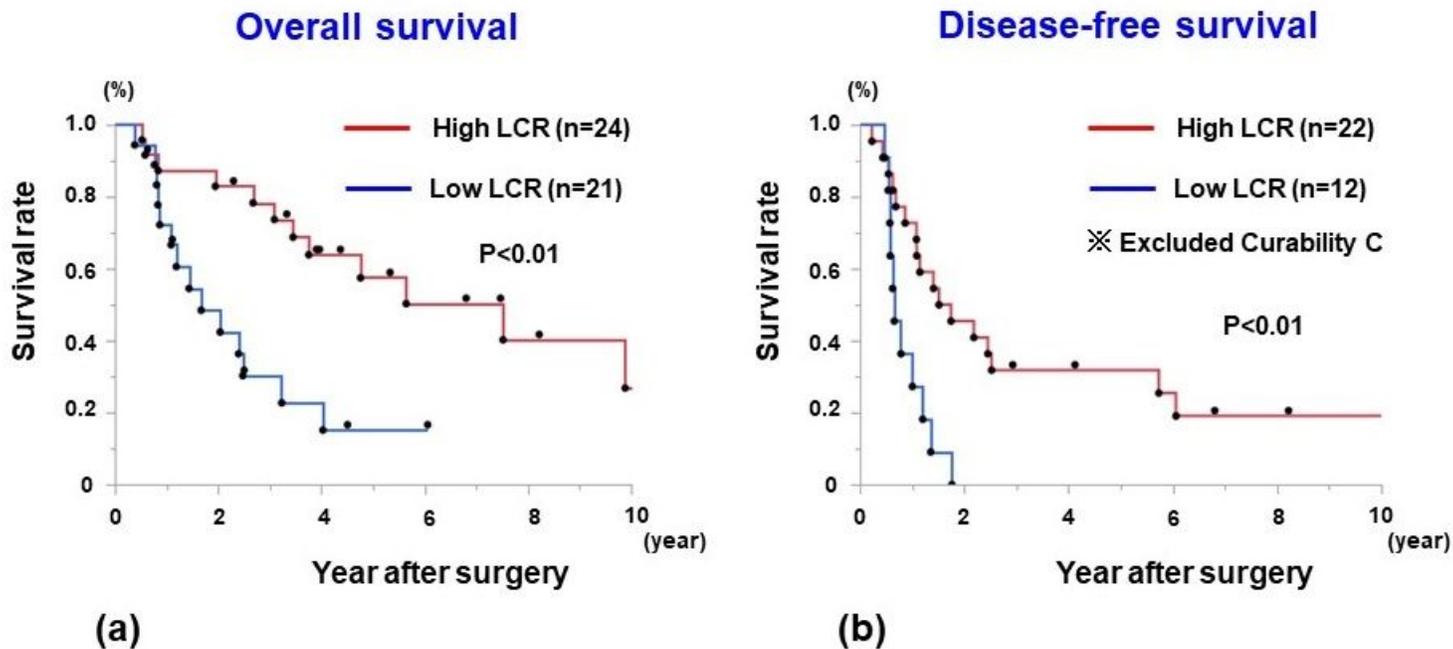


Figure 4

Survival curves. (a) Overall survival rates among patients with high or low lymphocyte/C-reactive protein ratio (LCR; P<0.01). (b) Disease-free survival rate among patients with high or low LCR (P<0.01)

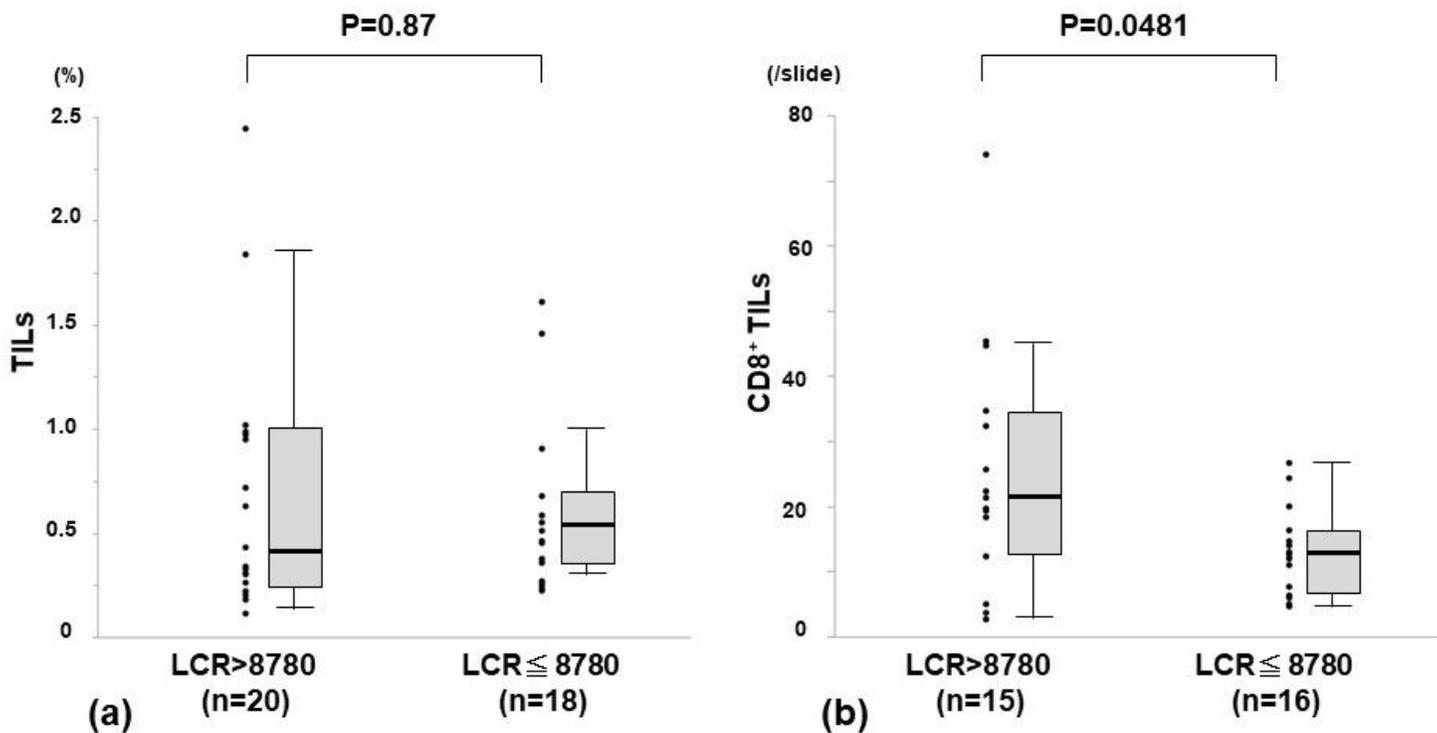


Figure 5

Association between LCR and TILs. (a) No significant association was found between lymphocyte/C-reactive protein ratio (LCR) and tumor-infiltrating leukocytes (TILs) of all types. (b) Lymphocyte/C-reactive

protein ratio (LCR) was significantly related to CD8+ TILs (P=0.0481).