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Immunohistochemical analysis of the aggregation of CD1a-positive dendritic cells in resected specimens and its association with surgical outcomes for patients with gallbladder cancer

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ABSTRACT

Gallbladder cancer (GBC) is an aggressive malignancy with a poor prognosis. Antigen-presenting dendritic cells (DCs) play a central role in antitumor immunity. DCs expressing CD1a (CD1a-DCs) are considered immature DCs. The aim of this study was to evaluate the clinical impact of CD1a-DC infiltration into GBC tissue. Seventy-five patients with GBC (excluding non-invasive and intramucosal cancer) were enrolled. Immunohistochemistry for CD1a, S100 and CD8 was performed using representative surgically resected specimens. The cases were divided into a high CD1a-DC group (27 cases, 36%) and low CD1a-DC group (48 cases, 64%) according to the degree of CD1a-DC infiltration/aggregation. The high CD1a-DC group contained fewer patients with distant metastasis ($P = 0.039$) and more patients given postoperative chemotherapy ($P = 0.038$). The high CD1a-DC group had significantly longer overall survival ($P = 0.001$) and disease-specific survival ($P = 0.002$) than the low CD1a-DC group. In contrast, S100-DC and CD8+ tumor-infiltrating lymphocyte statuses were without effect on OS or DSS. The results of multivariate analyses indicated that the degree of infiltration/aggregation of CD1a-DCs was an independent prognostic factor associated with a favorable prognosis after surgery.

Introduction

Gallbladder cancer (GBC) is the most common malignancy of the biliary tract and has an incidence that displays significant geographical variation [1,2]. GBC is an aggressive malignancy with a poor prognosis. Only 25% of patients with GBC undergo potential curative surgery, and just 16% of patients survive for more than 5 years [3]. Clinical diagnosis of GBC at an early stage is vitally important because the prognosis after surgery differs markedly according to the T stage [4]. It is often difficult, however, to make a definitive diagnosis based on radiological findings [5], especially in adenomyomatosis-accompanied cases [6]. Thus, GBC is often detected at an advanced stage, but an effective treatment for unresectable or recurrent GBC has not yet been unequivocally established.

Dendritic cells (DCs) are bone marrow-derived cells that seed in all tissues and are considered to be the most potent antigen-presenting cells that activate T and B lymphocytes [7–9]. Tumor antigen-loaded DCs can activate tumor-specific cytotoxic $CD8^+$ *T* lymphocytes by presenting the captured antigen as major histocompatibility complex (MHC) class I and MHC class II molecules [10]. Therefore, DCs are considered to be a potential novel target for cancer immunotherapy.

CD1a is a transmembrane glycoprotein associated with antigen presentation by DCs [11]. In contrast to S100 protein, which is usually expressed on both immature and mature DCs, CD1a is specifically expressed on immature DCs such as Langerhans cells in human skin [12]. Despite CD1a often being regarded as a marker for immature DCs, in many *in vitro* systems, CD1a is expressed as strongly on mature as on immature DCs. Additionally, co-expression of CD1a and CD83, a marker for mature DCs, has been demonstrated *in vivo*. Interestingly, antigen presentation by another group 1 CD1 molecule, CD1b, is as efficient on immature as on mature DCs. This might also prove true for CD1a-restricted antigen presentation. Taken together, these findings highlight

Abbreviations: CD1a-DCs, CD1a-positive dendritic cells; DC, dendritic cell; DSS, disease-specific survival; GBC, gallbladder cancer; GEM, gemcitabine; HPF, high-power field; IHC, immunohistochemistry; MHC, major histocompatibility complex; OS, overall survival; S100-DCs, S100-positive dendritic cells; SD, standard deviation; TIL, tumor-infiltrating lymphocyte; UFT, uracil/tegafur.

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that CD1a is not merely a marker of DCs or their maturation state but should be viewed in a different light [9].

Previous reports have indicated that tumor infiltration by CD1a-positive DCs (CD1a-DCs) is associated with favorable clinical outcomes in various types of malignancy including skin [12], oral [13], tongue [14], thyroid [15] and ovarian [16] cancer. However, the clinical impact of tumor infiltration by CD1a-DCs in patients with GBC remains unclear.

The aim of the present study was to clarify the status and clinical impact of CD1a-DC infiltration into GBC tissue.

Materials and methods

Patients

A total of 87 consecutive patients with GBC who underwent surgical resection of the primary lesion at Saga University Hospital between 1994 and 2012 were initially enrolled in the study. After the exclusion of patients with non-invasive or intramucosal cancer (pTis or pT1a), a total of 75 patients with GBC were included in the final analysis. All patients provided informed consent for the use of resected tissue, and the study protocol was approved by the Ethics Committee of the Faculty of Medicine at Saga University (No. 2020–04-R-20). Clinical and histopathological staging were based on the TNM Classification of Malignant Tumors (8th edition) provided by the Union for International Cancer Control [17].

Immunohistochemistry (IHC)

IHC for CD1a, S100 and CD8 was performed using a single representative block of formalin-fixed, paraffin-embedded GBC tissue specimen obtained from each patient. Sections (4 μm) were deparaffinized, and antigen retrieval was performed using Histofine® Heat Processor Solution pH 9 (Nichirei Biosciences, Tokyo, Japan) and an automatically controlled thermostat (Histofine® HEAT PROII; Nichirei Biosciences). The following primary antibodies were used: mouse monoclonal anti-CD1a antibody (clone 010; IS06930–2; prediluted; Dako, Glostrup, Denmark), mouse monoclonal anti-CD8 antibody (clone C8/144B; GA62361–2; prediluted; Dako) and rabbit polyclonal anti-S100 antibody (GA50461–2 J; prediluted; Dako). The Envision+® System (Dako) was used as the secondary antibody. Slides were visualized using diaminobenzidine tetrahydrochloride, and nuclei were counterstained with hematoxylin. An Autostainer Plus® (Dako) was used for staining of specimens.

Assessment of tumor-infiltrating DCs

The degree of tumor infiltration by DCs was assessed using a light microscope set to $a \times 200$ high-power field (HPF). Initially, we tried to count the number of DCs in a hot spot, but this was difficult in cases where DCs were highly aggregated, because the boundaries of individual DCs were unclear due to their complicated dendritic shapes. In most cases, DCs presented as focal aggregations in tumor tissue. Therefore, we defined a high level of CD1a-DC/S100-DC infiltration into the tumor tissue as the presence of at least one aggregation of > 10 DCs per × 200 HPF.

Assessment of CD8-positive tumor-infiltrating lymphocytes (TILs)

Three hot spots containing TILs were selected at × 200 magnification and digital images were captured. The numbers of $CD8^+$ TILs in the digital images were automatically counted using image-analysis software (Tissue Studio, Definiens, München, Germany). The mean number of $CD8^+$ TILs in the 3 hot spots was calculated for each case.

Statistical analysis

JMP Pro-version 13 software (SAS Institute, Cary, NC, US) was used for all statistical analyses. Normally distributed data are presented as the mean ± standard deviation (SD) and were compared between the two groups using Student's *t*-test (two-tailed). Count data are presented as *n* (%) and were compared between groups using a χ^2 or Fisher's exact test as appropriate (two-tailed). Overall survival (OS) was determined from the time of surgery to the time of death or most recent follow-up. Disease-specific survival (DSS) was determined from the time of surgery to the time of cancer-related death or the most recent follow-up. Survival curves were constructed using the Kaplan-Meier method and compared using the log-rank test. Cox proportional hazards modeling was used for univariate and multivariate analyses. Variables in the univariate analyses with a *P*-value < 0.2 were selected for the multivariate analysis. *P*-values < 0.05 were considered to be significant. All statistical analyses were supervised by a statistician (A.K.).

Results

Assessment of IHCs and distribution of CD1a-DCs

Representative images obtained from a patient in the high CD1a-DC group are shown in Fig. 1. DCs with a characteristic dendritic shape were clearly identified by IHC for CD1a. $CD8^+$ TILs were automatically detected by image analysis software. In most cases, CD1a-DCs were adjacent or very close to tumor cells. Fig. 2 shows representative images obtained from a patient in the high CD1a-DC group and a patient in the low CD1a-DC group. For patients in the high CD1a-DC group, CD1a-DCs were usually observed as focal aggregations at varying densities, and S100-DCs were also observed as focal aggregations. By contrast, sections from patients in the low CD1a-DC group usually exhibited zero or only a few CD1a-DCs, whereas aggregations of S100-DCs were clearly observed.

Clinicopathological features and degree of tumor infiltration by DCs and $CD8^+$ TILs

The clinicopathological features and degree of tumor infiltration by DCs and $CD8^+$ TILs for the 75 cases of GBC are summarized in Table 1. Twenty-three cases (30.7%) were male, 52 cases (69.3%) were female, and the mean age at the time of surgery was 68.9 ± 9.3 years. Twenty-seven cases (36.0%) were included in the high CD1a-DC group, and 42 cases (56.0%) were included in the high S100-DC group. The mean number of $CD8^+$ TILs per hot spot (× 200 magnification) was 219.7 ± 214.8. Twenty-two patients (29.3%) received adjuvant therapy or therapy for recurrent lesions after surgery, and the regimens used were as follows: TS-1 alone, 9 cases; TS-1 + gemcitabine (GEM); 3 cases; GEM alone, 2 cases; GEM followed by TS-1, 2 cases; GEM + radiotherapy, 1 case; GEM followed by uracil/tegafur (UFT), nimustine hydrochloride + cisplatin, 1 case; UFT alone, 1 case; UFT + radiotherapy, 1 case; UFT followed by cisplatin, 1 case; and radiotherapy alone, 1 case.

Clinicopathological features according to CD1a-DC status

The clinicopathological features of the patients in the high CD1a-DC group and low CD1a-DC groups are summarized in Table 2. The high CD1a-DC group contained a significantly lower proportion of patients with distant metastasis ($P = 0.039$) and a significantly higher proportion of patients who received adjuvant therapy ($P = 0.038$). There were no significant differences between the groups with regard to other clinicopathological characteristics. Although the mean number of $CD8^+$ TILs appeared to be numerically higher in the high CD1a-DC group than in the low CD1a-DC group, a statistically significant difference was not found.

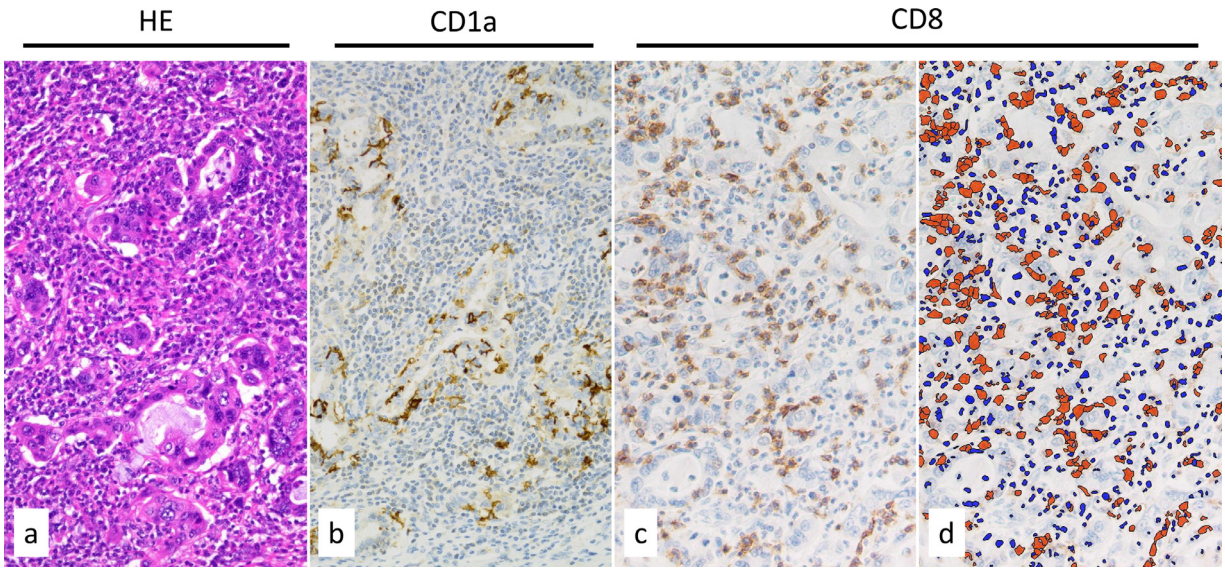

Fig. 1. Representative images obtained from a patient in the high CD1a-DC group. a: Hematoxylin/eosin (HE)-stained image (× 200 magnification). b: Immunohistochemistry for CD1a (× 200) revealed cells with a characteristic dendritic shape. c: Immunohistochemistry for CD8 (× 200). d: Automatic detection and counting of CD8-positive cells (orange) by Tissue Studio software.

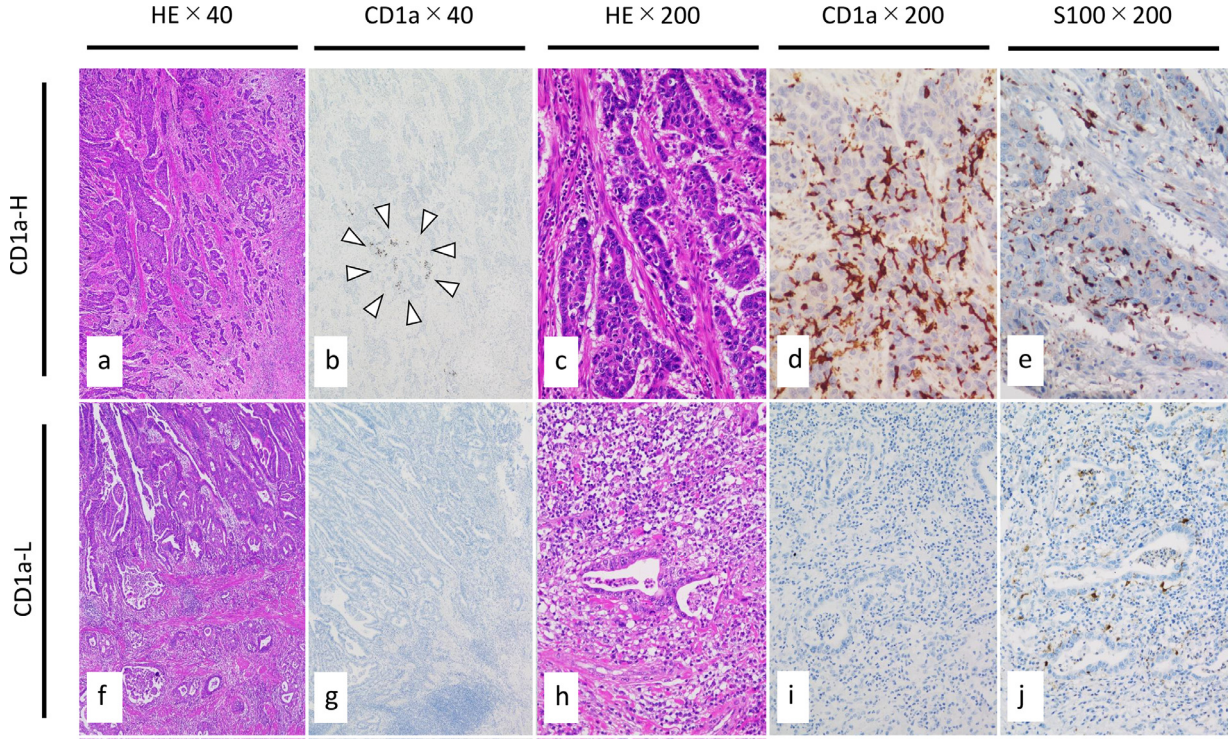

Fig. 2. Representative images obtained from a patient in the high CD1a-DC group (a-e) and a patient in the low CD1a-DC group (f-j). a, f: Hematoxylin/eosin (HE)-stained images (× 40). b, g: Immunohistochemistry for CD1a (× 40). Focal aggregations of CD1a-positive dendritic cells (CD1a-DCs; arrows) were observed for the patient in the high CD1a-DC group but not the patient in the low CD1a-DC group. c, h: HE-stained images (× 200). d, i: Immunohistochemistry for CD1a (× 200). e, j: Immunohistochemistry for S100 (× 200). Aggregations of S100-positive cells were observed in both groups.

Clinicopathological features according to S100-DC status

The clinicopathological characteristics of the patients in the high S100-DC and low S100-DC groups are presented in Table 3. There were no significant differences between the 2 groups with regard to age, gender, TNM stage or use of adjuvant therapy. Although the high S100-DC group appeared to have a greater number of $CD8^+$ lymphocytes than the low S100-DC group, the difference was not statistically significant.

OS and DSS according to CD1a-DC, S100-DC and $CD8^+$ TIL status

Survival curves for OS and DSS according to CD1a-DC, S100-DC and $CD8^+$ TIL status are shown in Fig. 3. Patients in the low CD1a-DC group exhibited significantly worse OS ($P = 0.001$) and DSS ($P = 0.002$) than patients in the high CD1a-DC group. By contrast, S100-DC and CD8+ TIL status did not have a significant impact on OS or DSS.

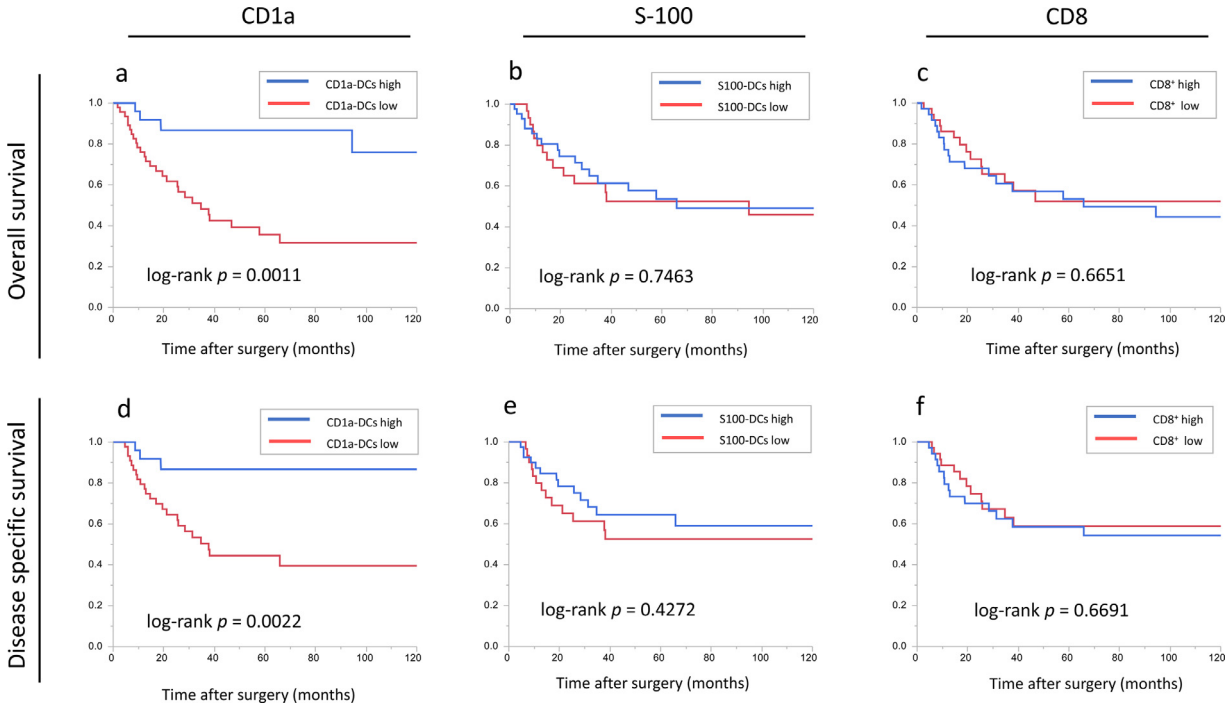

Fig. 3. Overall survival and disease-specific survival curves according to CD1a-positive dendritic cell status (a, d), S100-positive dendritic cell status (b, e) and $CD8^+$ tumor-infiltrating lymphocyte status (c, f).

Table 1
Clinicopathological features and the degree of tumor infiltration by DCs and $CD8^+$ TILs in patients with gallbladder cancer ($n = 75$).

Age (mean ± SD)	68.9 ± 9.3
Gender, *n* (%)	
Male	23 (30.7)
Female	52 (69.3)
T stage, *n* (%)	
1b	9 (12.0)
2a	11 (14.7)
2b	18 (24.0)
3	33 (44.0)
4	4 (5.3)
N stage, *n* (%)	
N0	36 (48.0)
N1	21 (28.0)
N2	18 (24.0)
M stage, *n* (%)	
M0	59 (78.7)
M1	16 (21.3)
Tumor infiltration by CD1a-DCs	
High	27 (36.0)
Low	48 (64.0)
Tumor infiltration by S100-DCs	
High	42 (56.0)
Low	33 (44.0)
Number of $CD8^+$ TILs (mean ± SD)	219.7 ± 214.8
Postoperative chemotherapy	
Yes	22 (29.3)
No	53 (70.7)

DCs, dendritic cells; TILs, tumor-infiltrating lymphocytes; SD, standard deviation.

Subset analyses for CD1a-DC according to TNM status in OS and DSS

We performed subset analyses according to T-stage (T1/T2, $n = 38$ *vs* T3/T4, $n = 37$), N-stage (N0, $n = 36$ *vs* N1/N2, $n = 39$), and M-stage (M0, $n = 59$ *vs* M1, $n = 16$) for OS and DSS. Survival curves for OS according to TNM status are shown in Fig. 4. Results of log-rank tests for OS are as follows: T1/T2: $P = 0.0192$, T3/T4: $P = 0.029$, N0: $P = 0.389$, N1/N2: $P = 0.002$, M0: $P = 0.006$, and M1: $P = 0.354$. Significant worse survival of low CD1a-DC group was shown in subsets of T1/T2, T3/T4, N1/N2, and M0 whereas no significant difference between high CD1a-DC and low CD1a-DC group was observed in the subsets of N0 and M1. Similar results were observed in the subset analyses for DSS. Results of the log-rank tests for DSS are as follows: T1/T2: $P = 0.020$, T3/T4: $P = 0.037$, N0: $P = 0.456$, N1/N2: $P = 0.002$, M0: $P = 0.018$, and M1: $P = 0.3534$. The results of DSS for the M1 subgroup was the same as for OS because all deaths in the M1 subgroup were cancer-related.

Univariate analyses of factors associated with os and dss

The results of univariate analyses of factors associated with OS and DSS are summarized in Table 4. As age and the number of TILs were continuous variables, these factors were analyzed using the median value as the cut-off. The factors significantly associated with OS were age (≥ 70 years *vs* < 70 years; $P = 0.046$), T stage (T1 or T2 *vs* T3 or T4; $P = 0.007$), N stage (N0 *vs* N1 or N2; $P = 0.007$), M stage (M0 *vs* M1; $P < 0.001$), CD1a-DC status (low group *vs* high group; $P < 0.001$) and use of adjuvant therapy (yes *vs* no; $P = 0.046$). The factors significantly associated with DSS were T stage (T1 or T2 *vs* T3 or T4; $P = 0.001$), N stage (N0 *vs* N1 or N2; $P < 0.001$), M stage (M0 *vs* M1; $P < 0.001$) and CD1a-DC status (low group *vs* high group; $P = 0.001$).

Multivariate analyses of factors associated with OS and DSS

The results of the multivariate analyses are listed in Table 5. Factors that were significantly associated with OS were CD1a-DC status (low group *vs* high group; $P = 0.012$) and use of adjuvant therapy (yes *vs* no; $P = 0.018$). The only factor significantly associated with DSS was CD1a-DC status (low group *vs* high group; $P = 0.033$).

Table 2
Comparison of the clinicopathological features of patients with gallbladder cancer between the high CD1a-DC and low CD1a-DC groups ($n = 75$).

Characteristic	High CD1a-DC ($n = 27$)	Low CD1a-DC ($n = 48$)	*P*-value
Age (mean ± SD)	69.7 ± 10.9	68.4 ± 8.3	0.585
Male / female	8 / 19 (29.6 / 70.4)	15 / 33 (31.3 / 68.7)	1
T1b or T2 / T3 or T4	15 / 12 (55.6 / 44.4)	23 / 25 (47.9 / 52.1)	0.632
N0 / N1 or N2	16 / 11 (59.3 / 40.7)	20 / 28 (41.7 / 58.3)	0.143
M0 / M1	25 / 2 (92.6 / 7.4)	34 / 14 (70.8 / 29.2)	0.039
$CD8^+$ TILs (mean ± SD)	254.6 ± 246.4	200.1 ± 194.9	0.329
Postoperative chemotherapy (yes / no)	12 / 15 (44.4 / 55.6)	10 / 38 (20.8 / 79.2)	0.038

Data are presented as *n* / *n* (% /%) unless stated otherwise. DCs, dendritic cells; SD, standard deviation; TILs, tumor-infiltrating lymphocytes.

Table 3
Comparison of the clinicopathological features of patients with gallbladder cancer between the high S100-DC and low S100-DC groups ($n = 75$).

Characteristic	High S100-DC ($n = 42$)	Low S100-DC ($n = 33$)	*P*-value
Age (mean ± SD)	69.6 ± 8.1	67.9 ± 10.6	0.425
Male / female	15 / 27 (35.7 / 64.3)	8 / 25 (24.2 / 75.8)	0.323
T1b or T2 / T3 or T4	22 / 20 (52.4 / 47.6)	16 / 17 (48.5 / 51.5)	0.818
N0 / N1 or N2	21 / 21 (50.0 / 50.0)	15 / 18 (45.5 / 54.5)	0.817
M0 / M1	35 / 7 (59.3 / 43.8)	24 / 9 (72.7 / 27.3)	0.395
$CD8^+$ TILs (mean ± SD)	244.8 ± 243.1	187.8 ± 170.6	0.257
Postoperative chemotherapy (yes / no)	12 / 30 (28.6 / 71.4)	10 / 23 (30.3 / 69.7)	1

Data are presented as *n* / *n* (% /%) unless stated otherwise. DCs, dendritic cells; SD, standard deviation; TILs, tumor-infiltrating lymphocytes.

Table 4
Univariate analyses of factors associated with overall survival and disease-specific survival.

Factor	OS		DSS	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age < 70 years	2.077 (1.012–4.492)	0.046	2.163 (0.986–5.081)	0.054
Female gender	1.270 (0.603–2.913)	0.541	1.741 (0.740–4.769)	0.213
T3 or T4	2.711 (1.315–5.815)	0.007	3.753 (1.665–9.247)	0.001
N1 or N2	3.629 (1.730–8.136)	0.001	4.576 (1.988–11.818)	< 0.001
M1	4.477 (2.081–9.279)	< 0.001	6.080 (2.718–13.405)	< 0.001
Low CD1a-DCs	4.884 (1.903–16.557)	< 0.001	5.347 (1.857–22.571)	0.001
Low S100-DCs	1.124 (0.545–2.281)	0.747	1.364 (0.626–2.971)	0.430
Low $CD8^+$ TILs (< 139)	0.855 (0.413–1.741)	0.665	0.845 (0.383–1.839)	0.669
No postoperative chemotherapy	2.434 (1.016–7.202)	0.046	1.954 (0.796–5.857)	0.152

CI, confidence interval; DCs, dendritic cells; DSS, disease-specific survival; HR, hazard ratio; OS, overall survival; TILs, tumor-infiltrating lymphocytes.

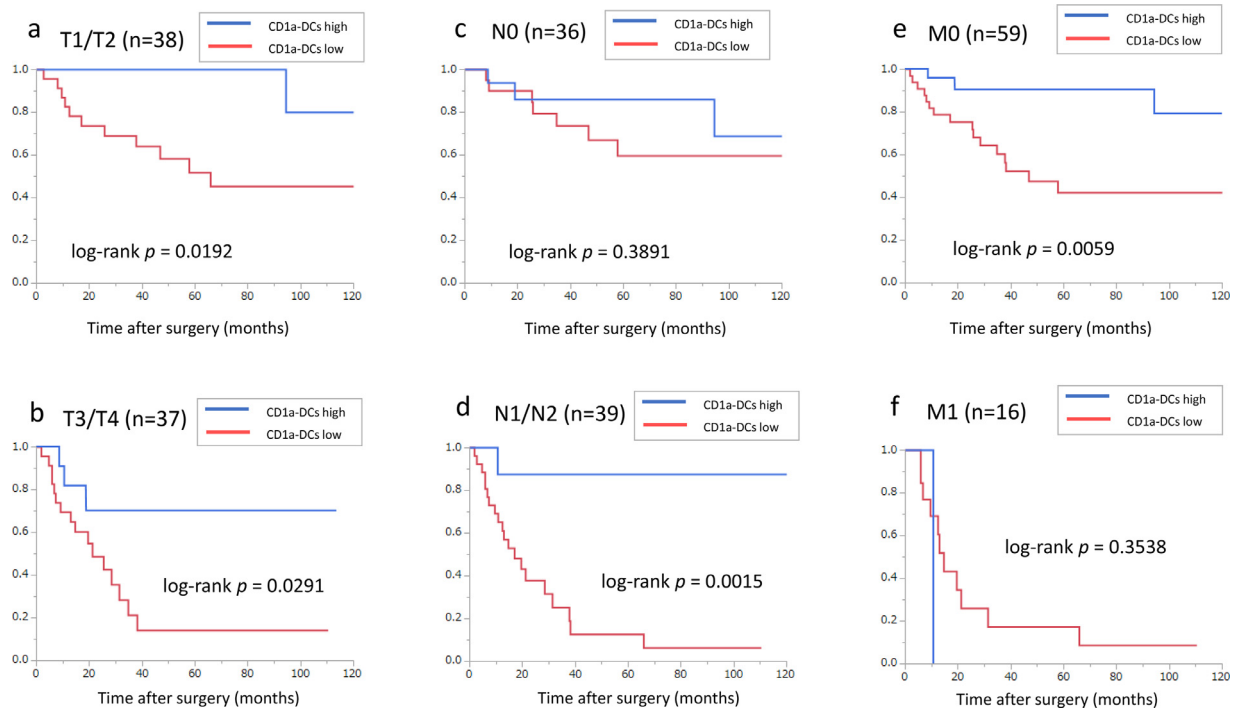

Fig. 4. Overall survival curves according to the CD1a-positive dendritic cell status in each subset of T1/T2 (a), T3/T4 (b), N0 (c), N1/N2 (d), M0 (e) and M1 (f).

Table 5
Multivariate analyses of factors associated with overall survival and disease-specific survival.

Factor	OS		DSS	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age < 70 years	1.883 (0.867–4.287)	0.111	1.827 (0.779–4.541)	0.167
T3 or T4	2.092 (0.852–5.386)	0.109	2.568 (0.940–7.571)	0.066
N1 or N2	1.964 (0.690–5.507)	0.201	1.756 (0.347–5.743)	0.340
M1	1.947 (0.783–4.986)	0.151	2.567 (0.978–7.209)	0.056
Low CD1a-DCs	3.635 (1.302–12.995)	0.012	3.452 (1.096–15.307)	0.033
No postoperative chemotherapy	3.040 (1.199–9.411)	0.018	2.451 (0.937–7.735)	0.069

CI, confidence interval; DCs, dendritic cells; DSS, disease-specific survival; HR, hazard ratio; OS, overall survival.

Discussion

Our study revealed that the degree of CD1a-DC infiltration into GBC tissue had a significant impact on the prognosis after surgery. Moreover, the degree of CD1a-DC infiltration was an independent prognostic factor for both OS and DSS. This finding suggests that the pathological assessment of CD1a-DC status in resected specimens might have important clinical implications with regard to the selection of the postoperative strategy for patients with GBC.

Various antibodies (detecting S100, CD1a, ATPase, CD83, CD207 and CD208) and measurement methods have been used to investigate the infiltration of DCs into cancer tissue, especially skin and oral carcinomas [12–14]. Infiltration of DCs has also been studied in carcinomas of the gastrointestinal tract such as colorectal [18] and gastric [19–21] cancers. To the best of our knowledge, only two studies have evaluated DC infiltration into GBC. Nakakubo et al. [22] investigated 45 cases of GBC using S100 protein as the DC marker and defined ≥ 20 DCs per × 200 HPF as the high DC infiltration group. Their results indicated that a high level of DC infiltration (observed in 22 cases, 48.9%) was significantly associated with longer OS. Furihata et al. [23] investigated DC infiltration in 29 cases of GBC using antibodies to both CD83 and CD1a. To our knowledge, their report is the only previous study that has evaluated CD1a-DCs in GBC. The investigators assessed the mean number of DCs per 10 HPFs (× 200) in both tumor and peritumoral areas and defined the CD83 index as: CD83 - DCs / (CD83 - DCs + CD1a-DCs). Their analysis indicated that the CD83 index was significantly associated with 5-year survival. However, the authors did not specifically investigate the association of CD1a-DC infiltration with clinicopathological factors. Thus, the present study is the first to report CD1a-DC infiltration as an independent prognostic factor for patients with GBC who were surgically treated. Although validation by future research is required, assessment of CD1a-DC status should be considered as an influence on decision-making regarding the initiation of adjuvant therapy after curative surgery for GBC or the need for additional surgery in cases of incidental GBC after cholecystectomy.

In contrast to the relevance of CD1a-DC infiltration, S100-DC infiltration had no significant impact on prognosis in the present study. As S100-DCs represent both mature and immature DCs, our results indicated that immature (CD1a-positive) DCs likely have more influence on the prognosis of GBC than mature DCs. Consistent with our findings, a previous study of 53 patients with oral squamous cell carcinoma found that peritumoral CD1a-DC infiltration but not CD83-positive DC infiltration was significantly associated with OS [13].

Theoretically, the number of TILs should correlate with the infiltration of CD1a-DCs. However, our analysis did not detect a significant impact of TILs on survival. Two possible reasons were considered for these results. First, as the mean number of TILs was numerically greater in the high CD1a-DC group compared to the low CD1a-DC group, it is possible that our study was underpowered to detect a real difference between the groups and that a significant difference may have been detected if a larger number of GBC cases had been included. Second, variations in the techniques used to evaluate TILs may have contributed to the differences between our findings and those of others. Previous studies of GBC that reported a significant impact of TILs utilized a variety of assessment methods including manual counting at 3 hot spots under a light microscope [22, 24], manual counting at 5 randomly selected locations [25], automated counting (by software) at 5 randomly selected locations [26] and automated counting in 1 region of interest [27]. It is noteworthy that the cut-off values of these previous studies varied widely.

The reason why CD1a-DC infiltration had a strong effect on the prognosis of patients with GBC remains to be elucidated. Based on our assessments of S100-DCs and TILs, the effects on prognosis cannot be explained simply by the processes of DC maturation and antigen presentation. A possible hypothesis to consider is that CD1a-DCs have some unknown functions that play important roles in the prevention of cancer progression. It is known that there are many subsets of DCs with unique and specific functions [28] in humoral and cellular immune responses [10] as well as immune tolerance [29]. The modulation of malignancy by the immune system is highly complex and although DCs play a pivotal role in the tumor microenvironment [30], the relevance of CD1a-DCs in cancer tissue remains unclear at the present time. Thus, further analysis of CD1a-DC functions in cancer tissue is required to establish whether CD1a-DCs has the potential to be a new therapeutic target for the treatment of GBC.

Our subset analyses revealed no significant association of CD1a-DCs on survival in the N0 and M1 group despite other subgroups showing significantly worse survival of the low CD1a-DC group. As the M1 subgroup ($n = 16$) contained only two CD1a-high cases, we consider that it is difficult to discuss the effects of CD1a-DCs in patients with distant metastases in the present study. In contrast, the N0 subgroup ($n = 36$) contained 16 cases of CD1a-high cases. It is an interesting to highlight that 20 CD1a-low cases in the N0 subgroup showed favorable prognosis. It has been reported that DCs initiate adaptive immune responses in lymph nodes and migrated into tumor tissues [31,32], the effects of CD1a-DC may be more significant in GBC patients with lymph node metastasis. However, we could not provide definite reasons for this result as we did not evaluate CD1a-DCs status in regional lymph nodes.

In the present study, the high CD1a-DC group contained a significantly smaller proportion of patients with distant metastasis than the low CD1a-DC group. The mechanism underlying this interesting observation is unknown because the functions of CD1a-DCs in cancer tissue have not yet been characterized. There are two possible explanations for this finding. First, it may be that patients with GBC and high levels of CD1a-DC infiltration rarely develop metastatic lesions. However, validation of this hypothesis is difficult because the presence or absence of metastasis at the time of surgery generally depends on the timing of the diagnosis. Second, infiltration of CD1a-DCs may depend on the host's immunocompetence, hence patients in the low CD1a-DC group may have followed an unfavorable clinical course. As no previous research has specifically focused on this issue, further studies are needed to test the above hypotheses.

The limitations of the present study include its retrospective design, the relatively small number of patients included, and the long

period required for enrollment. In addition, only one tissue block was used for IHC experiments despite the infiltration of CD1a-DCs being heterogenous. Finally, although DCs were clearly stained by anti-CD1a antibody, even in the older tissue specimens used for our analyses, it cannot be ruled out that age of the specimen or the duration of fixation with formalin may have affected the results of the IHC experiments.

In conclusion, the clinical impact of CD1a-DC infiltration into GBC tissue was investigated. The results indicate that the degree of infiltration/aggregation of CD1a-DCs was an independent factor associated with a favorable prognosis after surgery. DC-therapies using DC vaccines are already used for tumor immunotherapy and many results of clinical trials of DC-based tumor immunotherapy have been reported [33]. However, to the best of our knowledge, no immunotherapy has been developed to target specifically CD1a, and neither has the detailed role of CD1a-DCs been unequivocally clarified. Further research to clarify the role of CD1a-DCs in cancer tissue will almost certainly contribute to the development of a novel treatment for GBC or the improved selection of appropriate therapeutic strategies for patients with GBC.

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Author contributions

Conceptualization, project administration and acquisition of funding: Kai K; data curation and formal analysis: Kai K, Tanaka T, Ide T and Kawaguchi A; validation: Aishima S and Noshiro H; writing of the original draft: Kai K; writing - review & editing: Tanaka T, Ide T, Kawaguchi A, Noshiro H and Aishima S.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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