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ORIGINAL ARTICLE

Tumor-Infiltrating Lymphocytes and Macrophages in Intrahepatic Cholangiocellular Carcinoma. Impact on Prognosis after Complete Surgery

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Abstract

Background Immune infiltrate impacts prognosis of several tumors. To assess the prognostic impact of tumor-infiltrating lymphocytes and macrophages in patients undergoing resection for intrahepatic cholangiocellular carcinoma (ICC).

Methods All consecutive patients undergoing surgery for ICC between 2008 and 2016 were considered. Inclusion criteria were complete resection and follow-up > 12 months. Tissue sections were immunostained for CD3+, CD4+, CD8+, Foxp3+, and CD68+. The number of positive cells was quantified using a computer-aided image analysis system. Different cut-off values were tested as predictors of overall survival (OS).

Results Fifty-three patients were analyzed. ICC were T1 in 28 patients, multifocal in 11, and N+ in 13. After a median follow-up of 42 months, 5-year OS was 52.1%. The following immune infiltrate values were associated with better OS: CD3+ > 0.10% (5-year OS 63.3% vs. 13.6% if ≤ 0.10%, $p = 0.001$); CD8+ > 0.10% (56.2% vs. 28.6% if ≤ 0.10%, $p = 0.051$); Foxp3+ absent (59.4% vs. 16.0% if present, $p = 0.049$). CD4+ and CD68+ infiltrates were not associated with OS. Three-year OS rates in patients with 0, 1, and ≥ 2 negative prognostic factors were 73.6%, 47.3%, and 14.3%, respectively ($p < 0.001$). CD3+ infiltrate stratified prognosis in T1 tumors (3-year OS 71.7% if CD3+ > 0.10% vs. 14.3% if ≤ 0.10%, $p < 0.001$).

Conclusions Tumor-infiltrating lymphocytes are associated with prognosis of ICC patients after complete surgery. CD3+ and CD8+ infiltrate is associated with higher survival and lower recurrence risk, while Foxp3+ infiltrate is associated with worse prognosis. CD3+ infiltrate allows refining prediction of prognosis in early tumors.

Keywords Intrahepatic cholangiocellular carcinoma · Tumor-infiltrating lymphocytes and macrophages · Liver surgery · Prognosis and survival · Staging · Immunology

Introduction

Intrahepatic cholangiocellular carcinoma (ICC) is a disease at increasing incidence.[1,2] Liver resection (LR) is the standard treatment of ICC and achieves 5-year survival rates ranging from 20 to 35%.[3–9] Several postoperative prognostic factors have been depicted, including the completeness of the procedure (R0/R1), the presence of lymph node metastases, the number and the size of ICC, and the presence of vascular invasion.[5,6,8–12] The prediction of prognosis is crucial to select candidates for surgery and to modulate perioperative treatments, even if available chemotherapy regimens have a limited effectiveness.[3, 4] Precise criteria to assess the tumor biology are still lacking, but the analysis of the interaction between the immune system and the tumor could be the key for major progresses.[13–15] Since 2000, clinical studies have shown a

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prognostic value of the immune infiltrate in several tumors, the most relevant evidences concerning colorectal cancers.[16–21] The CD3+ and CD8+ infiltrate has been associated with better survival rates.[19–21] Galon et al. even proposed an immunoscore, based on the combination of $CD3^+$ and $CD8^+$ values, to integrate or even replace TNM staging.[21] Similar data have been reported for hepatocellular carcinoma,[22–24] while only few preliminary studies are available for ICC.[25–27]

The aims of the present study were to analyze the association between immune infiltrate and standard pathology data, to elucidate the impact of tumor-infiltrating lymphocytes and macrophages on prognosis after liver resection, and to evaluate if they refine present prognosis prediction based on TNM staging system.

Materials and Methods

Subject Enrollment

All consecutive 73 patients undergoing an LR for ICC between January 2008 and June 2016 were considered. The following inclusion criteria were used: ICC diagnosis confirmed at final pathology; de novo ICC; mass-forming ICC; complete resection (R0/R1); follow-up > 12 months; the possibility to review the pathology specimen. Patients with perihilar tumors were excluded as well as patients having operative mortality. According to these criteria, 53 consecutive patients were included in the present analysis. The Institutional Review Board of our hospital approved this retrospective study and the requirement of informed consent was waived.

Analysis of the Immune Infiltrate

Formalin-fixed, paraffin-embedded tissue sections from ICC were immunostained for CD3+ (T lymphocyte marker; 1:100, Dako/Agilent Technologies), CD4+ (helper/inducer T lymphocyte marker; 1:100, Origene), CD8+ (suppressor/cytotoxic T lymphocyte marker; 1:100, Biocare), Foxp3+ (Treg cells-lymphocyte marker; 1:100, AbCam), and CD68+ (macrophage marker; 1:200, Dako/Agilent Technologies). All the relevant tumor-infiltrating immune cells in ICC[27] were analyzed. All antibodies were mouse monoclonal antibodies. Intratumoral sections were considered. Only adequate sections were retained for the analysis. In patients with multiple tumors (20% of patients), the largest nodule was considered. Briefly, the 2-μm sections were deparaffinized and rehydrated following standard protocols. The antigen unmasking was performed using EDTA solution pH 9 at 98 °C for 30 min (Dako/Agilent Technologies) and, after washes, the sections were incubated in peroxidase blocking solution (Dako/Agilent Technologies). The tissue sections were incubated with primary antibodies, described above, overnight, at room temperature and then, after washes, with Dako EnVision+ System–HRP Labelled Polymer Anti-Mouse at room temperature for 30 min. The reaction was visualized with DAB (3, 3′-diaminobenzidine), counterstained with hematoxylin, dehydrated in ethanol, and mounted with media. As negative controls, the primary antibody was omitted.

The images were taken by an image analysis system consisting of a Leica DMLA microscope (Leica, Italy) equipped with an *x*–*y* translator table and a digital camera (Leica DC200), and the number of positive cells was quantified using a computer-aided image analysis system. The computer program automatically selected the surface covered by the immunopositive inflammatory cells (CD3+, CD8+, CD4+, Foxp3+, and CD68+). After analysis with the pathologist, we selected the tumor area and we excluded unfilled natural holes, vascular and biliary cavities, sinusoidal spaces, or artificial spaces due to histological manipulations. Representative results of immunohistochemistry for tumor-infiltrating immune cells are shown in Fig. 1. Images with less than 0.010% of stained cells were classified as negative (0 cells). Below the threshold of 0.010%, the stained cells are extremely rare and any stratification of patients on the basis of a numeric value would be largely inaccurate. Two experienced hepatic pathologists (LDT and MR) reviewed all the specimens and verified the results of analyses.

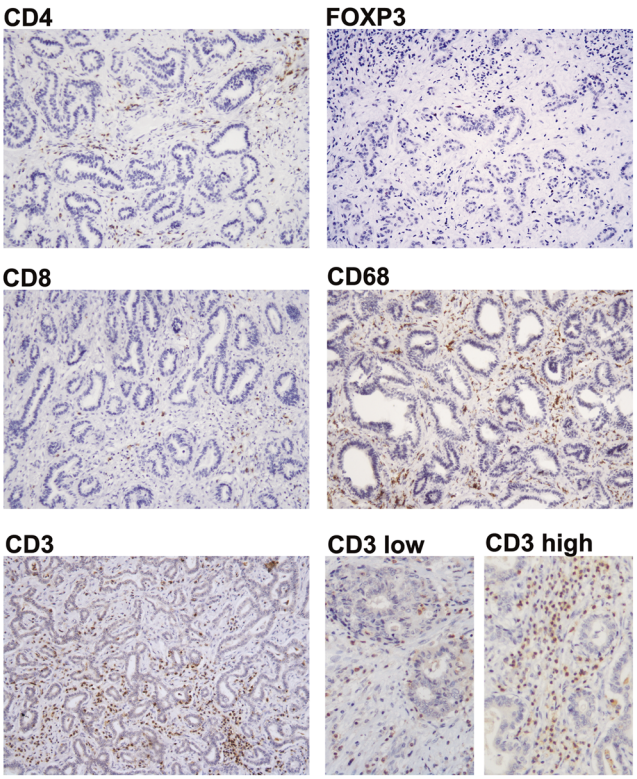

Fig. 1 Formalin-fixed, paraffin-embedded tissue sections from ICC were immunostained for CD3+, CD4+, CD8+, Foxp3+, and CD68+

Study Design

The following issues were analyzed: (1) the association between immune infiltrate and ICC pathology data; (2) the association between tumor-infiltrating lymphocytes and macrophages and survival rates after LR (overall survival, OS, and recurrence-free survival, RFS); (3) the prognostic impact of tumor-infiltrating immune cells in different tumor stages. Different cut-off values of CD3+, CD4+, CD8+, Foxp3+, and CD68+ (every 0.05% increase) were tested as predictors of OS, and the value with the lowest p value was considered (Supplementary Fig. 1).

In addition, the neutrophil to lymphocyte (Neu/Lymph) ratio and the platelet to lymphocyte (PLT/Lymph) ratio were computed from serum analyses for all the patients and their association with pathology data, immune infiltrate, and prognosis was analyzed.

Patient Management and Surgical Technique

Preoperative staging of ICC patients included thoracoabdominal computed tomography, hepatic magnetic resonance imaging with tissue-specific contrast agent, and positron emission tomography/computed tomography. A multidisciplinary team discussed the management of every patient. Surgery was scheduled whenever a complete resection was achievable. The authors' surgical technique has been previously reported.[28–31] The administration of adjuvant chemotherapy was evaluated on a case-by-case basis in a multidisciplinary setting. Follow-up was performed every 3 months and included ultrasonography, computed tomography, or magnetic resonance imaging.

Definitions and Statistical Analysis

Patients were identified from a prospectively maintained database and retrospectively analyzed. Major hepatectomy was defined as resection of ≥ 3 Couinaud's segments. Operative mortality was defined as death within 90 days after surgery or before discharge from the hospital (excluded patients). Morbidity included all the postoperative complications and was classified according to Dindo-Clavien classification.[32] The eighth edition of TNM staging was adopted for all patients.

Categorical variables were compared using the χ^2 or Fisher's exact test. Continuous variables were compared using the unpaired t test, or the Mann–Whitney U test. The Kaplan–Meier method was used to estimate survival probabilities, which were compared using the log-rank test. Follow-up was updated until July 2017, and no patient was lost to follow-up. Multivariable analysis was performed using a Cox proportional hazard model to identify independent prognostic factors of OS. Multivariate analysis was completed for factors with a p value ≤ 0.10 in the univariate analysis. A p value < 0.05 was considered significant for all tests.

Results

Overall, 53 patients undergoing LR for ICC were analyzed. Clinical and pathological patients' features are summarized in Table 1. ICC were multiple in 11 (21%) patients, > 50 mm in 26 (49%), and N+ in 13 (25%). Twenty-one patients (40%) had a T1a tumor, 7 (13%) a T1b, 17 (32%) a T2, and 8 (15%) a T3. Half of patients underwent major hepatectomy. Overall and severe morbidity rates were 38% and 17%, respectively.

Tumor-Infiltrating Immune Cells

The median value (range) of CD8+ was 0.41% (0.04–2.01%), of CD4+ was 0.28% (0.01–1.77%), and of CD68+ was 0.97% (0.10–2.96%). Forty-three (81%) patients had no evidence of Foxp3+ infiltrate; the median FoxP3+ value in positive patients was 0.05% (0.02–0.16%). The larger the tumor size, the lower the CD4+, CD8+, and CD68+ infiltrate ($p = 0.039$, $p = 0.042$, and $p = 0.031$, respectively). CD8+ infiltrate was inversely associated with T stages (median value 0.53% in T1 tumors, 0.37% in T2, and 0.27% in T3, $p = 0.034$) and N stages (0.18% in N+ patients vs. 0.51% in the others, $p = 0.016$). Data are summarized in Fig. 2 and in Supplementary Table 1.

Table 1 Patient characteristics and tumor details

	# (%)
Age > 70 years	25 (47.2%)
Sex (male)	31 (58.5%)
BMI > 30 kg/m^2	5 (9.4%)
Metabolic syndrome	7 (13.2%)
HCV infection	10 (18.9%)
Liver cirrhosis	8 (15.1%)
ICC data	
Single tumor	42 (79.2%)
Size >50 mm	26 (49.1%)
G1–2	40 (75.5%)
T1	28 (52.8%)
N+	13 (24.5%)
Microscopic vascular invasion	17 (32.1%)
Major hepatectomy	22 (41.5%)
Adjuvant chemotherapy	10 (18.9%)

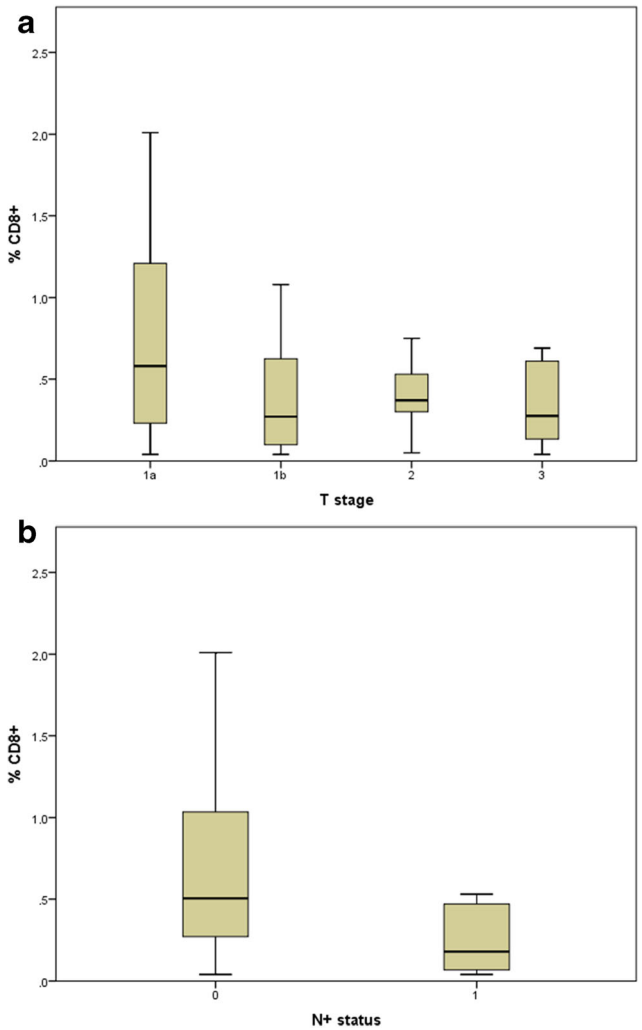

Fig. 2 Association between CD8+ infiltrate and T (**a**) and N (**b**) tumor stages ($p = 0.034$ and $p = 0.016$, respectively)

Survival Analysis

After a median follow-up of 42 months, 5-year OS was 52.1% and 3-year RFS was 40.5%. CD3+ > 0.10% and CD8+ > 0.10% were associated with a higher OS (at 5 years 63.3% if CD3+ > 0.10% vs. 13.6% if CD3+ ≤ 0.10%, $p = 0.001$, Fig. 3a; 56.2% if CD8+ > 0.10% vs. 28.6% if CD8+ ≤ 0.10%, $p = 0.051$, Fig. 3b); Foxp3+ infiltrate was associated with a lower OS (at 5 years 16.0% if Foxp3+ present vs. 59.4% if Foxp3+ absent, $p = 0.049$, Fig. 3c). CD4+ and CD68+ infiltrates were not associated with OS. Combining the CD3+, CD8+, and Foxp3+ data, median OS was not achieved in patients with no negative prognostic factor, 29.7 months in patients with one negative prognostic factor, and 16.3 months in patients with 2 or more negative prognostic factors (3-year OS, 73.6%, 47.3%, and 14.3%, respectively, $p < 0.001$, Fig. 4). At the multivariable analysis (Supplementary Table 2), CD3+ and Foxp3+ infiltrates were confirmed as independent prognostic factor of OS [CD3+ > 0.10%, hazard ratio (HR) = 0.272, $p = 0.012$; Foxp3+ present, HR = 3.255, $p = 0.028$].

CD3+ infiltrate stratified prognosis in T1 tumors (3-year OS 79.7% if CD3+ > 0.10% vs. 14.3% if CD3+ ≤ 0.10%, $p < 0.001$, Fig. 5). Of note, 4 patients with a T1 tumor had a CD8+ infiltrate ≤ 0.10% and none was alive 3 years after LR (vs. 73.8% if CD8+ > 0.10%). The small number of patients in different subgroups did not allow an adequate analysis for the other tumor stages.

Considering the RFS, the positive prognostic impact of CD3+ > 0.10% and of CD8+ > 0.10% was confirmed (3-year RFS 48.5% vs. 9.1% if CD3+ ≤ 0.10%, $p < 0.001$; 44.3% vs. 14.3% if CD8+ ≤ 0.10%, $p = 0.004$, respectively).

Neutrophil to Lymphocyte and Platelet to Lymphocyte Ratios

In the 53 analyzed patients, preoperative median values of Neu/Lymph and PLT/Lymph ratios were 2.5 (0.1–14.7) and 115.8 (11.7–289.2), respectively. Neu/Lymph ratio was not associated with the pathology data, while PLT/Lymph ratio was inversely associated with the tumor size ($p = 0.003$) and the presence of microscopic vascular invasion ($p = 0.003$). Neu/Lymph and PLT/Lymph ratios had no association with the immune infiltrate and had no impact on survival.

Discussion

Cholangiocarcinoma is a disease at increasing incidence and poor survival.[1,2] Surgery is the only curative option, but the resectability rate is low and post-surgical recurrence risk is high.[1–4] Prognosis prediction is paramount. To date, it is based on morphology (number and size of lesions, vascular invasion), lymph-nodal diffusion (N stage), and completeness of surgery.[5–12] Reliable molecular markers are still lacking as well as an adequate assessment of tumor biology. The analysis of the immune infiltrate works in this direction. The oncologic impact of tumor-infiltrating lymphocytes has been analyzed in several tumors. The largest evidences concern colorectal cancers,[16–21] but some consistent data are also available for HCC and colorectal liver metastases.[22–24, 33–38] Concerning ICC, only preliminary analyses are available,[25–27,39,40] in some cases, mixing intrahepatic and extrahepatic cholangiocarcinoma.[27,39]

The first studies about immune infiltrate explored its association with tumor pathology data and TNM stages. In colorectal cancers, Galon et al. demonstrated that CD3+, CD4+, and CD8+ infiltrates are inversely associated with microvascular, lymphatic, and perineural infiltration of the tumor.[18] This is not the case for primary liver tumors. No association between tumor-infiltrating lymphocytes and microscopic vascular infiltration was reported for HCC by Sun et al.[24] and for

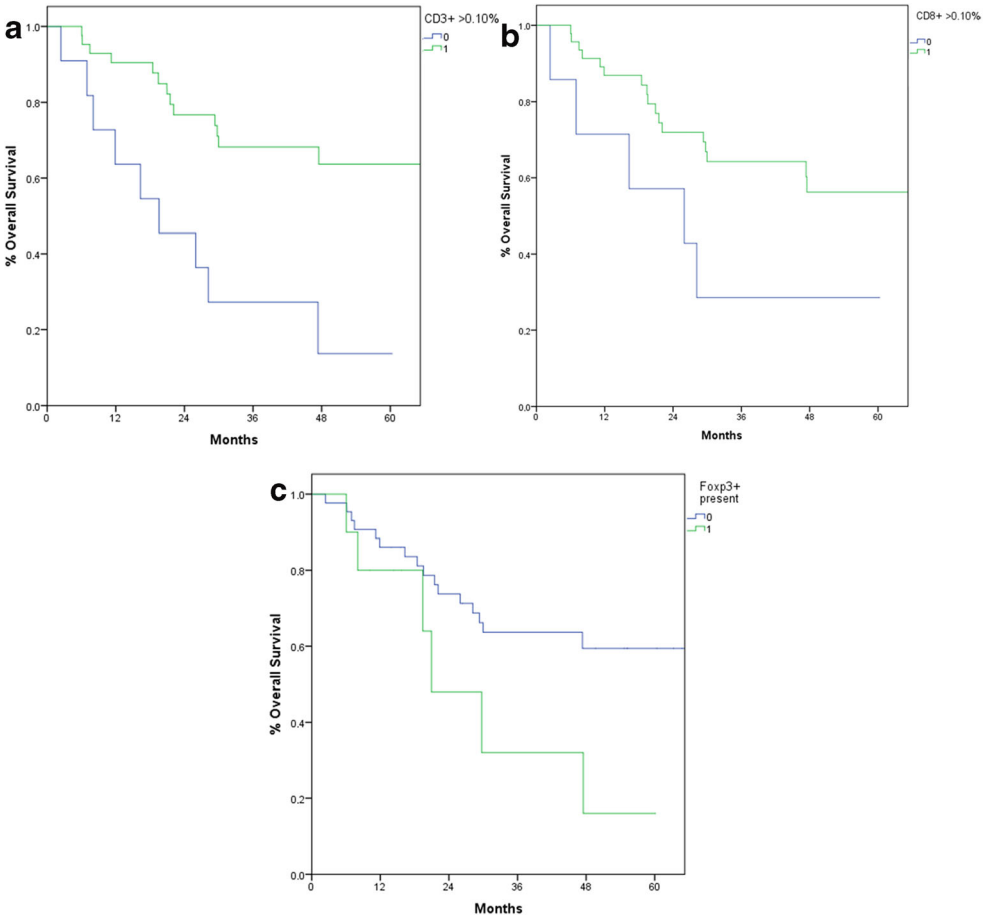

Fig. 3 Overall survival curves of the whole series according to CD3+ (**a**), CD8+ (**b**), and Foxp3+ (**c**) infiltrate

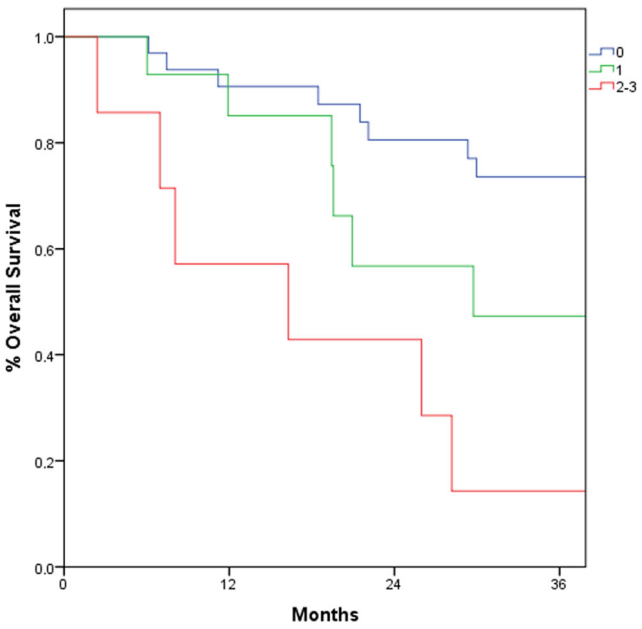

Fig. 4 Overall survival curves according to the combined presence of CD3+, CD8+, and Foxp3+ infiltrate. One point was assigned for each negative prognostic factor that is CD3+ ≤ 0.10%, CD8+ ≤ 0.10%, and Foxp3+ present

ICC by the present analysis. However, we observed an association between tumor-infiltrating lymphocytes and tumor size of ICC (the larger the tumor size, the lower the infiltrate). Even more interesting, CD8+ infiltrate was inversely associated with T and N stages. Similar data have been reported in colorectal cancers[18,41] and in biliary tract cancers (including intrahepatic and extrahepatic cholangiocarcinoma).[27]

The prognostic impact of the immune infiltrate is the most relevant issue. In 2011, a meta-analysis considering different tumors reported a prognostic value for CD3+ and CD8+ infiltrates.[42] In colorectal cancers, patients with high CD3+ and CD8+ infiltrates had higher survival and lower recurrence risk in comparison with patients with low infiltrates.[19–21] The survival benefit was reported in every tumor stage.[19] These data were the basis for the elaboration of an immunoscore[21] that is an immunological staging system based on the CD3+ and CD8+ presence. In the proposers' opinion, it could integrate or even replace the TNM staging. Considering liver tumors, few data are available. In 2003, Okano et al. firstly suggested an association between tumor-infiltrating lymphocytes and prognosis in 41 colorectal liver metastases patients.[33] Subsequent analyses about the same disease showed that CD3+ and CD8+ infiltrates have an inverse

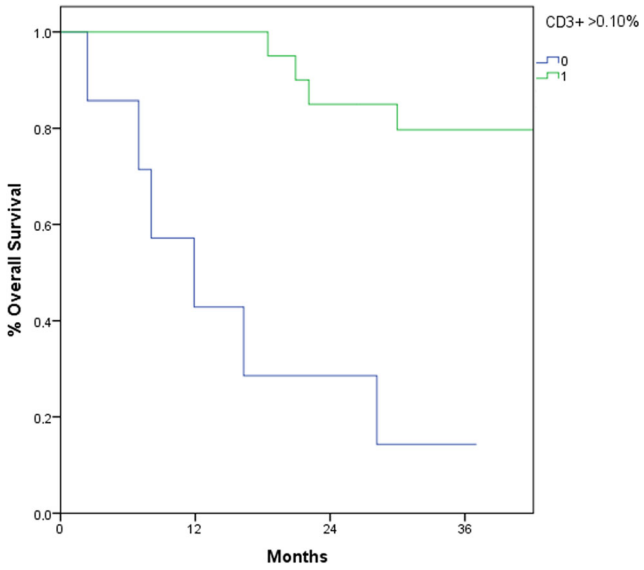

Fig. 5 Overall survival curves of patients with T1 tumor according to CD3+ infiltrate

association with tumor doubling time[43] and a positive association with survival.[35,37] The same positive prognostic value of CD3+ and CD8+ was described in HCC patients.[23,24] Due to the consistency of data with those reported for colorectal cancers, some authors even advanced the proposal of an immunoscore for HCC patients.[22,24] Considering ICC, two papers reported a prognostic impact of LI-17+ cell infiltrate,[25,26] while a large study performing a complete analysis of tumor-infiltrating lymphocytes and macrophages failed to demonstrate any prognostic impact of the immune infiltrate.[27] The present study offered some new insights. CD3+ and CD8+ infiltrates were associated with a better prognosis. A large survival advantage was observed when a relevant infiltrate was evident. Further, CD3+ and CD8+ refined prognosis prediction in T1 tumors. Again, the immune infiltrate could implement TNM staging. Even a preliminary attempt of immunoscore was advanced. A good stratification of prognosis was achieved combining the analysis of CD3+ and CD8+ infiltrates. The small sample size accounts for the need of validation by larger series, even if the concordance with the data observed in other tumors strengthens the present observation.

Some additional data deserve consideration. First, Foxp3+ T regulatory cells have been rarely analyzed. In colorectal primary tumors and liver metastases, data are controversial.[34,38,42–47] In the present series of ICC patients, a Foxp3+ infiltrate was evident in one-third of patients. It had a strong negative prognostic impact, being associated with a 16% 5-year survival rate (vs. 59% when Foxp3+ infiltrate is not evident). Second, we analyzed the macrophages (CD69+) infiltrate. Zhou et al. reported a positive association between macrophages and prognosis in colorectal metastases.[48] This was not confirmed in ICC, in which macrophages had no impact on survival. Finally, some recent papers investigated the prognostic impact of circulating neutrophils, lymphocytes and platelets, and their ratios. In ICC patients, Neu/lymph and PLT/Lymph ratios have been associated with survival.[49–52] The present series did not confirm those data. Neu/Lymph and the PLT/Lymph were associated neither with the immune infiltrate at pathology nor with prognosis. The tumor-infiltrating lymphocytes at pathology and not the ratios of circulating cells were prognosticators.

The impact of the present data on clinical practice is still to explore, but some hypotheses can be advanced. The analysis of tumor-infiltrating lymphocytes on the surgical specimen could refine the indication to adjuvant treatment after complete resection. A preoperative evaluation of the immune infiltrate could improve the selection of patients candidate for surgery and the patients' treatment allocation. Finally, immunotherapies have an emerging impact on several tumors, including ICC.[53,54] The tumor-infiltrating lymphocytes and macrophages could have a major role in this scenario. PD-L1 is an important biomarker predicting response to immunomodulators. In ICC, PD-L1 is expressed by neoplastic cells (rarely, about 9% of cases) and by inflammatory cell aggregates (more common, 53%), and the global PD-L1 expression is associated with CD3+ infiltrate.[55] ICC with dense intratumoral lymphocytic infiltration might represent good candidates for PD-L1/PD-1 blocking agents. In this sense, the analysis of the immune infiltrate assumes a crucial role to predict response to immunotherapy and to identify new treatment targets.

The present study may have some limitations. It is a retrospective study, but all the specimens were reviewed, and a completely automatized computer-aided specimen analysis was performed. Of note, 53 patients of a rare tumor with a complete adequate follow-up were included in the study. A detailed analysis of the immune infiltrate was performed, including not only the standard parameters (CD3+, CD4+, and CD8+) but also some additional data (Foxp3+ and macrophages). In colorectal cancer, the analysis of lymphocytes at the tumor margin together with the intratumoral ones contributed to prognosis prediction.[21] In the present series, the retrospective design did not allow a systematic evaluation of both the peritumoral and the intratumoral immune infiltrate, and only the latter was considered. Anyway, the available data about liver tumors did not confirm the prognostic impact of peritumoral lymphocytes.[22,23,36] Future studies have to analyze this issue as well as the intratumoral heterogeneity of the immune infiltrate. Subgroups analyses concerned a limited number of patients, but some preliminary evidences were achieved. They should be the starting point for further analyses, especially considering the coherence with previous studies in colorectal cancer and HCC. A large prospective cohort is needed to validate the present results.

Conclusion

ICC have a clinically relevant immune infiltrate. Even if preliminary, our data suggest that tumor-infiltrating lymphocytes impact prognosis of ICC after complete surgery. CD3+ and CD8+ infiltrates were associated with higher survival and lower recurrence rates, while Foxp3+ infiltrate was associated with a worse prognosis. CD3+ and CD8+ infiltrates implemented prognosis prediction of patients with early tumors.

Authors' Contribution Substantial contributions to the conception or design of the work: Viganò, Roncalli, Aghemo, Torzilli

Acquisition, analysis, or interpretation of data for the work: Viganò, Soldani, Franceschini, Cimino, Lleo, Donadon, Di Tommaso

Drafting the work: Viganò, Soldani, Franceschini, Cimino, Lleo, Donadon, Di Tommaso

Revising it critically for important intellectual content: Viganò, Roncalli, Aghemo, Torzilli

Final approval of the version to be published: all the authors

Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved: all the authors

Compliance with Ethical Standards

The Institutional Review Board of our hospital approved this retrospective study and the requirement of informed consent was waived.

Conflicts of Interest The authors declare that they have no conflict of interest.

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