The Characteristics of the Immune Cell Profiles in Peripheral Blood in Cholangiocarcinoma Patients

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Keywords: cholangiocarcinoma, immune cell profiles, CD3+ T cells, CD4+ T cells, helper T cells, regulatory T cells, naïve regulatory T cells, effector regulatory T cells, CD8+ T cells, myeloid-derived suppressor cells

Posted Date: February 25th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-238309/v1>

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Version of Record: A version of this preprint was published at Hepatology International on March 22nd, 2021. See the published version at <https://doi.org/10.1007/s12072-021-10177-8>.

Abstract

Background

Immune related cells are known to be closely related to the therapeutic effects and prognoses of cancer patients. In this study, we analyzed immune cell profiles (ICP) of cholangiocarcinoma patients (CCA).

Methods

To measure the frequency of immune cells, peripheral blood mononuclear cells of 41 CCA and 10 healthy volunteers (HV) were analyzed by FACS.

Results

There were significant differences between CCA and HV in ICP, and these differences were a consequence of tumor-bearing status because many items in ICP before surgery were restored to levels in HV after surgery. Therefore, these changes were specifically attributable to cholangiocarcinoma, and we examined if they can function as biomarkers for therapeutic effects and prognoses. A shorter overall survival was associated with a lower frequency of helper T cells (HT) ($p=0.001$), a higher frequency of effector regulatory T cells (eTregs) ($p=0.008$), and a lower frequency of CD80+ eTregs ($p=0.024$) in the best supportive care group, with a lower frequency of CD25+ naïve Tregs (nTregs) ($p=0.005$) in the chemotherapy group, and with a lower frequency of OX40+ HT ($p=0.022$), CD25+ CD8+ T cells ($p=0.017$), and OX40+ CD8+ T cells ($p=0.032$) in the surgery group. The recurrence factors were a higher frequency of CD4+ T cells ($p=0.009$), CCR6+ nTregs ($p=0.014$), and CXCR3+ nTregs ($p=0.012$), and a lower frequency of PD-1+ HT ($p=0.006$), OX40+ HT ($p=0.004$), CD8+ T cells ($p=0.001$), and CTLA-4+ CD8+ T cells ($p=0.036$).

Conclusions

The ICP in CCA are specifically attributable to cholangiocarcinoma, and may be biomarkers for therapeutic effects and prognoses.

Introduction

Cholangiocarcinoma is a malignant disease associated with a poor prognosis¹. Surgery at the early stage is the only curative option. However, recurrence after surgery is not uncommon¹. Patients with metastatic cholangiocarcinoma have a particularly high risk of mortality, with a median survival of 8-12 months even with combined modality therapy¹. A novel therapeutic approach is needed to improve the prognosis of cholangiocarcinoma patients (CCA).

Immunotherapy is an attractive new therapeutic approach. Cancer peptide vaccines can induce tumor-associated antigen-specific cytotoxic T lymphocytes (CTLs)² and are clinically useful, especially for hepatocellular carcinoma (HCC)². However, an enzyme-linked immunospot assay of peripheral blood

mononuclear cells (PBMCs) in HCC and cholangiocarcinoma revealed that the frequency of CTLs is not high³. The low frequency of induced CTLs may result in the failure of immunotherapy. The presence of cells controlling negative immune responses, represented by regulatory T cells (Tregs), and myeloid-derived suppressor cells (MDSCs) are believed to be one of the causes of the insufficient exertion of anti-tumor immunity against malignant diseases. In HCC, the mechanism of insufficient exertion of anti-tumor immunity has been gradually clarified⁴. In cholangiocarcinoma, however, the details regarding the insufficient exertion of anti-tumor immunity remain unclear. Characterization of immunosuppressor cells in cholangiocarcinoma may help to improve the insufficient exertion of anti-tumor immunity against cholangiocarcinoma, thereby improving the outcome of immunotherapy. In this study, we analyzed the immune cell profiles (ICP) in peripheral blood of CCA focusing on the anti-cluster of differentiation (CD)4+ T cells, Tregs, CD8+ T cells, and MDSCs.

Materials And Methods

Patients and clinical data

Forty-one CCA were enrolled. As a control, 10 healthy volunteers (HV) and 20 hepatitis C virus sustained virological response¹² (HCV SVR12) patients sex- and age-adjusted to CCA who were confirmed to be cancer-free were enrolled. Cholangiocarcinoma was categorized into the following types according to the anatomic location along the biliary tree: intrahepatic cholangiocarcinoma (iCCA), perihilar cholangiocarcinoma (pCCA), gallbladder carcinoma including cystic duct carcinoma (GBCA), and distal cholangiocarcinoma (dCCA). In this study, pCCA was included in iCCA. CCA were also categorized into the three following treatment groups: best supportive care (BSC) group, chemotherapy group, and surgery group.

Clinical data for patient characteristics, risk factors of cholangiocarcinoma (primary sclerosing cholangitis, hepatolithiasis, cirrhosis, hepatitis B virus infection (HBV), HCV infection (HCV), obesity (body mass index ≥ 25), diabetes mellitus (DM), chronic alcohol use (>80 g/day), tobacco, and occupation (1,2-dichloropropane, dichloromethane)), and outcomes were obtained from the medical records of 41CCA. For clinical data, laboratory test results before and after treatment were compared in the chemotherapy group and surgery group. For laboratory testing after chemotherapy, a sample before the second course of chemotherapy was used. For laboratory testing after surgery, a sample at PBMCs collection after surgery was used. We evaluated the tumor stage using the TNM staging of the Union Internationale Contre Le Cancer (UICC) system (8th version) (UICC stage). This study was approved by the ethics committee of Kanazawa University (No.1237), and all patients provided written informed consent to participate in accordance with the Declaration of Helsinki.

Analysis of ICP in peripheral blood

For the analysis of ICP in peripheral blood, PBMCs of 41CCA, 10 HV and 20 HCV SVR12 patients were used. CCA with cholangitis underwent biliary drainage to improve cholangitis. PBMCs before treatments,

such as surgery and chemotherapy, were used for 41 patients. For patients who underwent surgery, ICP of PBMCs after surgery were also investigated. PBMCs after discharge with improvement of postoperative complications were used as postoperative PBMCs. For patients who underwent chemotherapy, ICP of PBMCs after chemotherapy were not investigated. For HCV SVR12 patients, PBMCs at SVR12 were used. To measure the frequency of immune cells, FACS analysis using the FACS Aria II system (Becton Dickinson, Franklin Lakes, NJ, USA) was performed employing the following antibodies: CD3, CD4, CD8, CD14, CD15, CD25, CD80, CD45RA, human histocompatibility leukocyte antigen (HLA)-DR, forkhead box P3 (FoxP3), cytotoxic T-lymphocyte antigen-4 (CTLA-4), programmed cell death-1 (PD-1), PD-1 ligand (PD-L1), C-C chemokine receptor (CCR)4, CCR6, C-X-C motif chemokine receptor 3 (CXCR3), 4-1BB, and OX40 (Becton Dickinson).

Preparation of PBMCs and Statistical analysis

The details are provided in *Supplementary materials and methods*.

Results

Patient profile

The characteristics of 41 CCA, 10 HV, and 20 HCV SVR12 patients are shown in Table. 1 and Supplementary Table. 1. Of 41 CCA, the median age was 75 (48-90) years old, and 29 were male and 12 female. There were 23 patients with iCCA, 10 with GBCA, and 8 with dCCA. CCA were treated by surgery (n=18), chemotherapy (n=12), or BSC (n=11). As risk factors of cholangiocarcinoma, 2 had cirrhosis, 3 had HBV, 1 had HCV, 9 had obesity, 11 had DM, 3 had chronic alcohol use, and 22 had tobacco. Of 10 HV, the median age was 27 (23-32) years old, and 3 were male and 7 female. Of 20 HCV SVR12 patients, the median age was 75 (60-86) years old, and 10 were male and 10 female.

There were significant differences in sex ($p=0.028$) and age ($p<0.001$) between CCA and HV. There were no significant differences in sex ($p=0.157$) or age ($p=0.847$) between CCA and HCV SVR12 patients. There were significant differences in the values of aspartate transaminase (AST) ($p<0.001$), alanine transaminase (ALT) ($p<0.001$), and γ -glutamyltranspeptidase (γ GTP) ($p<0.001$) between CCA and HV. There were also significant differences in the values of white blood cell ($p<0.001$), AST ($p<0.001$), ALT ($p<0.001$), alkaline phosphatase ($p<0.001$), γ GTP ($p<0.001$), and total bilirubin ($p=0.001$) between CCA and HCV SVR12 patients.

The characteristics of each treatment group are shown in Table. 2. The surgery group consisted of 14 of 18 patients in whom ICP was able to be assessed before and after surgery. On comparison of laboratory test results before and after treatment in each treatment group, in the chemotherapy group, the values of carbohydrate antigen 19-9 (CA19-9) after chemotherapy was significantly higher than that before chemotherapy ($p=0.015$). In the surgery group, the values of ALT ($p=0.039$) and γ GTP ($p=0.022$) after surgery were significantly lower than those before surgery. Recurrence was observed in 9 patients after surgery.

ICP in peripheral blood between CCA and HV including HCV SVR12 patients

CD4⁺ T cells and CD8⁺ T cells were isolated, and CD4⁺ T cells were further separated into helper T cells (defined as CD3⁺CD4⁺CD45RA^{+/−}FoxP3[−] cells), naïve Tregs (nTregs) (defined as CD3⁺CD4⁺CD45RA⁺FoxP3^{low} cells), and effector Tregs (eTregs) (defined as CD3⁺CD4⁺CD45RA[−]FoxP3^{high} cells), as previously reported⁵ (Figure. 1a). In addition, PD-1, CD25, CTLA-4, CXCR3, CCR4, CCR6, CD80, OX40, and 4-1BB expression levels were measured in each T cell fraction (helper T cells, nTregs, eTregs (Supplementary Figures. S1a-S1c), and CD8⁺ T cells (Figure. 1b)). Peripheral blood MDSCs were separated into CD14⁺HLA-DR^{low} MDSCs and CD14[−]CD15⁺ MDSCs based on CD14, CD15, and HLA-DR expression levels. In addition, the PD-L1 expression level was measured in each fraction (Supplementary Figure. S1d).

There were significant differences between CCA and HV in the frequency of the following immune cells: OX40⁺ helper T cells, PD-1⁺ eTregs, CD80⁺ eTregs, OX40⁺ eTregs, nTregs, CD25⁺ nTregs, CCR6⁺ nTregs, CXCR3⁺ nTregs, CD25⁺ CD8⁺ T cells, (Figures. 1c, and 1d), lymphocytes, CD4⁺ T cells, CCR4⁺ helper T cells, CCR6⁺ helper T cells, CD80⁺ helper T cells, CTLA-4⁺ eTregs, PD-1⁺ nTregs, CCR4⁺ nTregs, CD80⁺ nTregs, OX40⁺ nTregs, CD80⁺ CD8⁺ T cells, CCR6⁺ CD8⁺ T cells, CCR4⁺ CD8⁺ T cells, PD-1⁺ CD8⁺ T cells, and monocytes. (Supplementary Figures. S2a-S2c).

There were significant differences between CCA and HCV SVR12 patients in the frequency of the following immune cells: lymphocytes, monocytes, nTregs, CD80⁺ nTregs, 4-1BB⁺ nTregs, eTregs, CTLA-4⁺ eTregs, PD-1⁺ eTregs, CCR4⁺ eTregs, CD80⁺ eTregs, OX40⁺ eTregs, 4-1BB⁺ eTregs, CTLA-4⁺ helper T cells, PD-1⁺ helper T cells, CCR4⁺ helper T cells, CD80⁺ helper T cells, OX40⁺ helper T cells, CXCR3⁺ helper T cells, CD25⁺ CD8⁺ T cells, CTLA-4⁺ CD8⁺ T cells, PD-1⁺ CD8⁺ T cells, CXCR3⁺ CD8⁺ T cells, CD80⁺ CD8⁺ T cells, and OX40⁺ CD8⁺ T cells (Supplementary Figures. S3a-S3d).

There were significant differences between HV and HCV SVR12 patients in the frequency of the following immune cells: CCR4⁺ eTregs, CXCR3⁺ eTregs, 4-1BB⁺ eTregs, nTregs, PD-1⁺ nTregs, CCR4⁺ nTregs, CCR6⁺ nTregs, CXCR3⁺ nTregs, 4-1BB⁺ nTregs, OX40⁺ nTregs, CTLA-4⁺ CD8⁺ T cells, CCR6⁺ CD8⁺ T cells, CXCR3⁺ CD8⁺ T cells, and OX40⁺ CD8⁺ T cells (Supplementary Figures. S4a-S4c).

The changes in ICP after biliary drainage in CCA compared with HV

The changes of patient characteristics of 3 CCA after biliary drainage are shown in Supplementary Table. 2. Cholangitis was improved by biliary drainage in these CCA. These patients were not included in the 41 CCA because they have not been followed for a long time. On comparison of ICP of 3 CCA before biliary drainage with those of HV, the frequencies of CD4⁺ T cells ($p=0.028$), PD-1⁺ eTregs ($p=0.014$), OX40⁺ eTregs ($p=0.014$), PD-1⁺ nTregs ($p=0.007$) and OX40⁺ helper T cells ($p=0.007$) in CCA were significantly higher than those in HV (Supplementary Figure. S5). After biliary drainage, the frequency of CD4⁺ T cells of CCA was comparable to that of HV ($p=0.287$), but the frequencies of PD-1⁺ eTregs ($p=0.007$), OX40⁺ eTregs ($p=0.007$), PD-1⁺ nTregs ($p=0.014$), and OX40⁺ helper T cells ($p=0.007$) in CCA remained higher than those in HV (Supplementary Figure. S5).

The differences in ICP based on the anatomic locations and risk factors of cholangiocarcinoma

ICP based on obesity, DM, and tobacco are shown in Supplementary Figures. S6a-S6d. In particular, CCA with DM exhibited significant differences in the frequencies of immune cells compared with CCA without DM. ICP for each location are shown in Supplementary Figure. S6e. The frequencies of CCR6+ eTregs, CD80+ nTregs, CXCR3+ CD8+ T cells, and OX40+ CD8+ T cells were significantly different among iCCA, GBCA, and dCCA.

The changes in ICP after surgery in CCA compared with HV

The immune cells that demonstrated a significant difference before surgery compared with HV but reached a level comparable to that in HV after surgery were lymphocytes, PD-1+ CD8+ T cells, CXCR3+ nTregs, CD80+ nTregs, eTregs, CTLA-4+ eTregs, PD-1+ eTregs, and CXCR3+ eTregs (Figures. 2a-2c).

In contrast, the frequencies of CD4+ T cells, helper T cells, CD25+ CD8+ T cells, CD80+ CD8+ T cells, PD-1+ nTregs, CCR4+ nTregs, CCR6+ nTregs, OX40+ nTregs, and OX40+ eTregs were significantly higher in CCA even after surgery (Figures. 2a-2c). These items were compared among three groups: non-recurrent CCA (n=5), recurrent CCA (n=9), and HV (n=10). The frequencies of CD4+ T cells, PD-1+ nTregs, CCR6+ nTregs, and OX40+ nTregs before surgery were higher in recurrent CCA than in HV, and comparable between non-recurrent CCA and HV (Figure. 2d).

The changes in ICP after surgery in CCA

The changes in ICP after surgery in CCA are shown in Supplementary Figure. S7. The frequencies of CTLA-4+ eTregs ($p=0.030$), CXCR3+ eTregs ($p=0.004$), CD80+ eTregs ($p=0.035$), CD80+ nTregs ($p=0.004$), CTLA-4+ helper T cells ($p=0.048$), CXCR3+ helper T cells ($p=0.041$), 4-1BB+ helper T cells ($p=0.017$), CD80+ helper T cells ($p=0.019$), CD25+ CD8+ T cells ($p=0.004$), PD-1+ CD8+ T cells ($p=0.002$), CXCR3+ CD8+ T cells ($p=0.002$), 4-1BB+ CD8+ T cells ($p=0.048$), and CD80+ CD8+ T cells ($p=0.003$) after surgery were significantly lower than those before surgery. The frequency of lymphocytes after surgery was significantly higher than that before surgery ($p=0.013$).

The relationship between ICP and overall survival (OS) in each treatment group

The relationships between OS and disease stage, tumor marker, or the frequency of immune cells before treatment were examined.

In the BSC group, there was no association between disease stage and OS ($p=0.249$). A higher carcinoembryonic antigen (CEA) level was weakly associated with a shorter OS ($p=0.053$). There was no association between CA19-9 levels and OS ($p=0.312$). Univariate analysis of ICP revealed that a lower frequency of helper T cells ($p=0.001$), higher frequency of eTregs ($p=0.008$), and lower frequency of CD80+ eTregs ($p=0.024$) was associated with a shorter OS (Figure. 3a).

In the chemotherapy group, no association between disease stage and OS was found ($p=0.515$). A higher CEA level was associated with a shorter OS ($p=0.025$). There was no association between CA19-9 levels and OS ($p=0.143$). Univariate analysis of ICP demonstrated that a lower frequency of CD25+ nTregs before chemotherapy was associated with a shorter OS ($p=0.005$) (Figure. 3b).

In the surgery group, no association between disease stage and OS was found ($p=0.240$). There was no association between CEA levels and OS ($p=0.599$), nor was there an association between CA19-9 levels and OS ($p=0.541$). Univariate analysis of ICP revealed that lower frequencies of OX40+ helper T cells ($p=0.022$), CD25+ CD8+ T cells ($p=0.017$), and OX40+ CD8+ T cells ($p=0.032$) before surgery were associated with a shorter OS (Figure. 3c).

The relationship between ICP and recurrence-free survival (RFS) in the surgery group

The relationships between RFS and disease stage, tumor marker, or the frequency of immune cells before surgery were examined.

A higher disease stage was weakly associated with a shorter RFS ($p=0.053$). There was no association between CEA levels and RFS ($p=0.800$), nor was there an association between CA19-9 levels and RFS ($p=0.860$). Univariate analysis of ICP revealed that higher frequencies of CD4+ T cells ($p=0.009$), CCR6+ nTregs ($p=0.014$), and CXCR3+ nTregs ($p=0.012$), and lower frequencies of PD-1+ helper T cells ($p=0.006$), OX40+ helper T cells ($p=0.004$), CD8+ T cells ($p=0.001$), and CTLA-4+ CD8+ T cells ($p=0.036$) before surgery were associated with a shorter RFS (Figure. 4).

Discussion

In this study, we investigated the differences in ICP between CCA and HV to clarify the characteristics of ICP in cholangiocarcinoma. The ICP of CCA differed significantly from those of HV. Major differences in ICP were observed for CD4+ T cells, CD8+ T cells, helper T cells, and Tregs, whereas no significant difference was found for MDSCs. Among the immune cells with major differences, the frequencies of OX40+ helper T cells, CCR6+ nTregs, CXCR3+ nTregs, CD80+ nTregs, OX40+ nTregs, PD-1+ eTregs, CD80+ eTregs, OX40+ eTregs, and CD25+ CD8+ T cells were higher in CCA, whereas those of CCR6+ helper T cells, CD25+ nTregs, and CCR6+ CD8+ T cells were higher in HV.

To confirm that these differences in ICP were due to tumor-bearing status, we examined whether the frequency of immune cells that differed between CCA before surgery and HV became similar to that in HV after surgery. This study demonstrated that many immune cells are restored to a level comparable to that in HV after surgery. Thus, the differences in ICP between CCA and HV may be directly attributable to cholangiocarcinoma. However, the HV we used as the control group had significant differences in sex, age, and laboratory test results from CCA. These differences, especially in laboratory test results and age, may have affected the ICP of CCA. Regarding the differences in laboratory testing, it was suggested that cholangitis is present in CCA. In order to exclude the possibility that ICP of CCA are different from those of HV due to cholangitis, the ICP before and after biliary drainage of CCA were compared with those of

HV. As a result, the ICP of CCA in this study were less affected by cholangitis and attributable to cholangiocarcinoma because the ICP of CCA did not reach a level comparable to that of HV only by performing biliary drainage. Regarding the difference in age, HCV SVR12 patients, who are not completely HV but equivalent in age to CCA, were designated as a control group equivalent to HV. On comparison of ICP of CCA with HCV SVR12 patients, significant differences were found in the frequencies of many immune cells. Comparing this result with ICP of CCA obtained by comparing with HV, the frequencies of eTregs, helper T cells, and CD8+ T cells were almost comparable, but the frequencies of CD4+ T cells and nTregs were different. Indeed, ICP between HV and HCV SVR12 patients were different, especially in nTregs. The differences in nTregs may have been due to HCV SVR12 patients having differences in liver fibrosis and age from HV. From the above, ICP of CCA obtained by comparing HV with CCA were considered to be almost specific to cholangiocarcinoma, but it should be noted that age may have slightly affected the ICP of CCA. Cholangiocarcinoma has different biological malignancy and clinical characteristics depending on risk factors and locations⁶. Furthermore, immunological characteristics differed depending on the risk factors and locations of cholangiocarcinoma in this study.

We also examined the possibility that ICP of CCA can function as biomarkers for the therapeutic effects and prognoses. Univariate analysis in each treatment group suggested that OS is influenced by numerous immune cells. Helper T cells, Tregs, and CD8+ T cells were found to be important immune cells, whereas CD4+ T cells had little influence on OS. Helper T cells support CTLs by promoting effector functions and have direct cytotoxicity against cancer cells via effector cytokines⁷. CD8+ T cells produce pro-inflammatory cytokines, such as tumor necrosis factor- α and interferon- γ , and exhibit tumor-specific cytotoxicity against cancer cells⁸. Tregs function in the inhibition of immune responses to cancer cells in malignant diseases⁹. Regarding immunosuppression by Tregs, a range of possible mechanisms have been proposed, including secretion of inhibitory cytokines, such as TGF- β and interleukin-10, direct contact inhibition via PD-1 and CTLA-4, and the inhibition of effector T cells such as CD4+ and CD8+ T cells¹⁰⁻¹³. Thus, Tregs have been reported to play an important role in tumor immunity and influence outcomes in cancer patients¹⁴.

The immune cells affecting OS were suggested to differ depending on treatment approach. In the BSC group, a lower frequency of helper T cells, higher frequency of eTregs, and lower frequency of CD80+ eTregs were associated with a shorter OS. The BSC group may have best reflected the natural course of CCA because there was no treatment in this group. A higher frequency of eTregs in peripheral blood suggests a higher frequency of eTregs in tumors. In addition, eTregs are known to have potent immunosuppressive properties among Tregs¹⁵. A high frequency of eTregs is associated with poor prognoses in other types of cancer because of their potent immunosuppressive mechanism¹⁶, and eTregs likely reduce the OS by a similar mechanism in CCA.

In the chemotherapy group, a lower frequency of CD25+ nTregs was suggested to be associated with a shorter OS. nTregs are Tregs originating from the thymus that have high-affinity T cell receptors and weak immunosuppressive properties¹⁵. CD25 is known to activate both effector T cells and Tregs¹⁷. In this

study, CD25+ nTregs were more abundant in HV than in CCA. Effector T cells may be activated predominantly over Tregs by a higher frequency of CD25+ nTregs in HV. On the other hand, Tregs may be activated predominantly over effector T cells by a lower frequency of CD25+ nTregs in CCA, which may reduce the anti-tumor immunity, thereby reducing the OS in CCA.

In the surgery group, a lower frequency of OX40+ helper T cells, CD25+ CD8+ T cells, and OX40+ CD8+ T cells was associated with a shorter OS. Among these immune cells associated with OS, the immune cells that significantly differed between CCA and HV, and markedly differed between pre- and post- surgery may be more closely associated with OS. CD25+ CD8+ T cells was identified as immune cells that meet these conditions. CD25+ CD8+ T cells are considered to be immunosuppressive-related immune cells similar to Tregs and have been reported to affect the disease progression of colon cancer¹⁸. Therefore, it is highly possible that CD25+ CD8+ T cells also affect the disease progression of CCA and are associated with OS.

To further examine whether ICP can function as a prognostic factor, we investigated the relationship between ICP and recurrence in the surgery group. As the ICP became similar to those of HV postoperatively, it is highly likely that ICP before surgery are associated with recurrence. We found that a higher frequency of CD4+ T cells and nTregs (CCR6+ and CXCR3+ nTregs), and a lower frequency of helper T cells (PD-1+ and OX40+ helper T cells), CD8+ T cells, and CTLA-4+ CD8+ T cells were factors for recurrence. Recurrence is likely to occur because a lower frequency of helper T cells (OX40+ helper T cells) and CD8+ T cells increases the suppressive function of Tregs¹⁹ and reduces the cytotoxicity against cancer cells^{7,8}, and a higher frequency of nTregs (CCR6+ and CXCR3+ nTregs) induces the immunosuppressive mechanism and reduces the function of CD8+ T cells against cancer cells^{20,21}. In general, CD4+ T cells help to induce an immune response of CD8+ T cells⁸, and a higher frequency of CD4+ T cells leads to a good prognosis in patients with cancer²². However, a higher frequency of CD4+ T cells led to a poor prognosis in this study. The feedback mechanism may increase CD4+ T cells because CD8+ T cells are suppressed by the above mechanism of Tregs¹⁰⁻¹³. As a result, a higher frequency of CD4+ T cells may be an alert of recurrence in CCA.

In conclusion, ICP of CCA, which differ from those of HV, may be specifically attributable to cholangiocarcinoma, and may be biomarkers for the therapeutic effects and prognoses of CCA.

Abbreviations

CCA: cholangiocarcinoma patients

CTLs: cytotoxic T lymphocytes

HCC: hepatocellular carcinoma

PBMCs: peripheral blood mononuclear cells

Tregs: regulatory T cells

MDSCs: myeloid-derived suppressor cells

ICP: immune cell profiles

CD: anti-cluster of differentiation

HV: healthy volunteers

HCV SVR12: hepatitis C virus sustained virological response 12

iCCA: intrahepatic cholangiocarcinoma

pCCA: perihilar cholangiocarcinoma

GBCA: gallbladder carcinoma including cystic duct carcinoma

dCCA: distal cholangiocarcinoma

BSC: best supportive care

HBV: hepatitis B virus infection

HCV: HCV infection

DM: diabetes mellitus

UICC: Union Internationale Contre Le Cancer

HLA: human histocompatibility leukocyte antigen

FoxP3: forkhead box P3

CTLA-4: cytotoxic T-lymphocyte antigen-4

PD-1: programmed cell death-1

PD-L1: programmed cell death-1 ligand

CCR: C-C chemokine receptor

CXCR3: C-X-C motif chemokine receptor 3

AST: aspartate transaminase

ALT: alanine transaminase

γGTP: γ-glutamyltranspeptidase

WBC: white blood cell

ALP: alkaline phosphatase

T.bil: total bilirubin

CA19-9: carbohydrate antigen 19-9

nTregs: naïve Tregs

eTregs: effector Tregs

OS: overall survival

CEA: carcinoembryonic antigen

RFS: recurrence-free survival

FSC: forward scatter

SSC: side scatter

pre: pre-operation group

post: post-operation group

NR: non-recurrent cholangiocarcinoma patient group

R: recurrent cholangiocarcinoma patient group

before: before biliary drainage group

after: after biliary drainage group

Freq: frequency

M: male

F: female

D.bil: direct bilirubin,

CRP: C-reactive protein

PSC: primary sclerosing cholangitis

ND: not determined

FCS: heat-inactivated fetal calf serum

Declarations

Funding: This study was supported by research grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (25460984).

Conflict of interest: Akihiko Kida declared that no conflict of interest exists. Eishiro Mizukoshi declared that no conflict of interest exists. Hidenori Kido declared that no conflict of interest exists. Tadashi Toyama declared that no conflict of interest exists. Takeshi Terashima declared that no conflict of interest exists. Kuniaki Arai declared that no conflict of interest exists. Tatsuya Yamashita declared that no conflict of interest exists. Kazumi Fushimi declared that no conflict of interest exists. Taro Yamashita declared that no conflict of interest exists. Yoshio Sakai declared that no conflict of interest exists. Masao Honda declared that no conflict of interest exists. Akio Uchiyama declared that no conflict of interest exists. Akito Sakai declared that no conflict of interest exists. Koichi Shimizu declared that no conflict of interest exists. Shuichi Kaneko declared that no conflict of interest exists.

Ethics approval: This study was approved by the ethics committee of Kanazawa University (No.1237), and all patients provided written informed consent to participate in accordance with the Declaration of Helsinki.

Consent to participate: All patients provided written informed consent to participate in this study.

Consent for publication: Consent for publication was received from the patients.

Availability of data and material: There are no linked research data sets for this submission. The following reason is given: Data will be available on request.

Code availability: Not applicable.

Author's contributions: Conception and design: Kida A, Mizukoshi E, Terashima T, Arai K, Yamashita T, Yamashita T, Sakai Y, Honda M, and Kaneko S. Development of methodology: Mizukoshi E, and Kaneko S. Acquisition of data: Kida A, Uchiyama A, Sakai A, and Shimizu K. Analysis and interpretation of data: Kida A, and Toyama T. Writing and review of the manuscript: Kida A, and Mizukoshi E. Administrative, technical, or material support: Kida A, Kido H and Fushimi K. Study supervision: Kaneko S.

Acknowledgments: The authors thank Taro Kawane, Aya Ito, Michiko Nishino, Saiho Sugimoto, Toshiki Matsuo, Atsuyoshi Mizukami, Masaaki Yano, Fumitaka Arihara, Hironori Hayashi, Koji Amaya, Koichiro Matsuda, Kohei Ogawa, and Mitsuru Matsuda from the Department of Internal Medicine and Surgery of Toyama Prefectural Central Hospital for collecting cholangiocarcinoma samples.

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Tables

Table. 1 Characteristics of cholangiocarcinoma patients and healthy volunteers

	Cholangiocarcinoma patients	Healthy volunteers	<i>p</i>
No. of Patients	41	10	ND
Sex, M/F	29 / 12	3 / 7	0.028
Age, years	75 (48-90)	27 (23-32)	< 0.001
WBC, /μL	6000 (3300-18500)	7450 (4060-9600)	0.525
AST, IU/L	48 (13-323)	16 (13-23)	< 0.001
ALT, IU/L	50 (8-290)	12 (7-17)	< 0.001
ALP, IU/L	711 (142-2660)	ND	ND
γGTP, IU/L	190 (12-1075)	16 (11-17)	< 0.001
T.bil, mg/dL	0.9 (0.4-17.3)	ND	ND
D.bil, mg/dL	0.3 (0.1-12.2)	ND	ND
CRP, mg/dL	0.66 (0.05-7.64)	ND	ND
CEA, ng/mL	3.2 (0.8-137)	ND	ND
CA19-9, U/mL	71 (2-40042)	ND	ND
Types and stage			
iCCA	23 (II (6), IIIB (4), IIIC (3), IV (5), IVA (2), IVB (3))	ND	ND
GBCA	10 (IIA (2), IIB (2), IVB (6))	ND	ND
dCCA	8 (I (2), IIA (2), IIB (2), IIIA (1), IIIB (1))	ND	ND
Pathological diagnosis, yes/ no	32 / 9	ND	ND
Therapy		ND	ND
Surgery	18	ND	ND
Chemotherapy	12	ND	ND
BSC	11	ND	ND
Risk factors for cholangiocarcinoma	PSC (0), hepatolithiasis (0), cirrhosis (2), HBV (3), HCV (1), obesity (9), diabetes mellitus (11), chronic alcohol	ND	ND

Abbreviations; M: male, F: female, WBC: white blood cell, AST: aspartate transaminase, ALT: alanine transaminase,

ALP: alkaline phosphatase, γ GTP: γ -glutamyltranspeptidase, T.bil: total bilirubin, D.bil: direct bilirubin,

CRP: C-reactive protein, CEA: carcinoembryonic antigen, CA19-9: carbohydrate antigen 19-9,

iCCA: intrahepatic and perihilar cholangiocarcinoma, GBCA: gallbladder carcinoma including cystic duct carcinoma,

dCCA: distal cholangiocarcinoma, BSC: best supportive care, PSC: primary sclerosing cholangitis,

HBV: hepatitis B virus infection, HCV: hepatitis C virus infection, ND: not determined.

Table. 2 Characteristics of each treatment group

	BSC group	Chemotherapy group			Surgery group		
No. of Patients	11	12			14*		
Sex, M/F	9 / 2	6 / 6			11 / 3		
Age, years	86 (65-90)	71 (48-85)			74 (67-85)		
		before	after	<i>p</i>	before	after	<i>p</i>
WBC, /μL	5900 (3300-18500)	7250 (3900-15900)	7250 (3900-18300)	0.286	5850 (4700-12100)	5250 (3600-8900)	0.102
AST, IU/L	77 (19-241)	44 (26-323)	32.5 (18-1145)	0.272	45 (13-128)	26 (16-58)	0.079
ALT, IU/L	74 (8-185)	40 (13-205)	26.5 (17-439)	0.480	71.5 (15-254)	23 (14-77)	0.039
ALP, IU/L	1030 (124-1845)	573 (271-2068)	540 (290-3302)	0.638	442 (142-2660)	379.5 (175-883)	0.331
γGTP, IU/L	249 (12-1075)	242.5 (74-733)	168 (41-741)	0.347	145 (22-546)	51.5 (15-499)	0.022
T.bil, mg/dL	1.3 (0.5-17.3)	0.8 (0.4-2.7)	0.7 (0.4-24.1)	0.782	0.75 (0.4-8.3)	0.6 (0.4-2.3)	0.200
D.bil, mg/dL	0.5 (0.2-12.2)	0.4 (0.2-1.1)	0.3 (0.1-17.2)	0.932	0.2 (0.1-0.9)	0.2 (0.2-0.9)	0.281
CRP, mg/dL	1.87 (0.07-7.64)	2.49 (0.09-7.48)	2.23 (0.4-9.84)	0.534	0.45 (0.1-4.64)	0.45 (0.01-3.25)	1.000
CEA, ng/mL	3.2 (1.5-93.6)	3.3 (1-137)	4.8 (0.9-107)	0.625	3.45 (1.3-7.6)	2.8 (0.8-11.3)	0.753
CA19-9, U/mL	692 (2-4819)	72.8 (18.8-40042)	92.5 (24.4-53064)	0.015	28.75 (3.2-5013)	18.15 (5.1-243)	0.221

Types and stage			
iCCA	7 (II (2), IIIB (2), IV (1), IVB (2))	7 (II (1), IIIB (1), IIIC (1), IV (3), IVB (1))	7 (II (3), IIIB (1), IIIC (2), IV (1))
GBCA	1 (IIB (1))	5 (IVB (5))	4 (IIA (2), IIB (1), IVB (1))
dCCA	3 (I (1), IIA (1), IIB (1))	0	3 (I (1), IIB (1), IIIA (1))
Recurrence (stage at recurrence), yes / no	ND	ND	9 (II (2), IIB (1), IIIA(1), IIIB (1), IIIC (2), IV (1), IVB (1)) / 5 (I (1), II (1), IIA (2), IIB (1))

Abbreviations; BSC: best supportive care, M: male, F: female, WBC: white blood cell, AST: aspartate transaminase,

ALT: alanine transaminase, ALP: alkaline phosphatase, γ GTP: γ -glutamyltranspeptidase, T.bil: total bilirubin,

D.bil: direct bilirubin, CRP: C-reactive protein, CEA: carcinoembryonic antigen, CA19-9: carbohydrate antigen 19-9,

iCCA: intrahepatic and perihilar cholangiocarcinoma, GBCA: gallbladder carcinoma including cystic duct carcinoma,

dCCA: distal cholangiocarcinoma, ND: not determined. *: Surgery group consisted of 14 of 18 patients in whom immune

cell profiles was able to be assessed before and after surgery.

Figures

Figure 1

Immune cell profiles between CCA and HV (a) The frequency of lymphocytes, CD3+ T cells, CD4+ T cells, CD8+ T cells, helper T cells, nTregs, and eTregs was measured by FACS analysis using the following antibodies: anti-CD3, CD4, CD8, CD45RA, and FoxP3. Helper T cells were defined as CD3+CD4+CD45RA+/-FoxP3-cells. nTregs were defined as CD3+CD4+CD45RA+FoxP3+lowcells. eTregs were defined as CD3+CD4+CD45RA-FoxP3+high cells. (b) Expression levels of PD-1, CD25, CTLA-4, CXCR3, CCR4, CCR6, CD80, OX40, and 4-1BB were measured in CD8+ T cells. (c-d) The frequency distributions of immune cells in HV and CCA are shown in the box and whisker plots. The lower and upper quartiles are at the ends of the box, the median is indicated by a horizontal line in the interior of the box, and the minimum and maximum values are at the ends of the whiskers. ● indicates patients with a frequency 1.5- to 3-times greater than the interquartile range. * indicates patients with a frequency 3-times greater than the interquartile range. There were significant differences between CCA and HV in the following items: (c) OX40+ helper T cells, PD-1+ eTregs, CD80+ eTregs, OX40+ eTregs, (d) nTregs, CD25+ nTregs, CCR6+ nTregs, CXCR3+ nTregs, and CD25+ CD8+ T cells. Abbreviations: FSC: forward scatter, SSC: side scatter, CD: anti-cluster of differentiation, FoxP3: forkhead box P3, PD-1: programmed cell death-1, CTLA-4: cytotoxic T-lymphocyte antigen-4, CXCR3: C-X-C motif chemokine receptor 3, CCR: C-C

chemokine receptor, HV: healthy volunteers, CCA: cholangiocarcinoma patients, eTregs: effector regulatory T cells, nTregs: naïve regulatory T cells

Figure 2

The changes in immune cell profiles after surgery in CCA compared with HV (a-c) The frequency distributions of immune cells in HV, the pre-surgery group, and the post-surgery group are shown in the box and whisker plots. The immune cells that exhibited a significant difference before surgery compared with HV but became similar to those in HV after surgery were as follows: (a) lymphocyte, PD-1+ CD8+ T cells, (b) CXCR3+ nTregs, CD80+ nTregs, (c) eTregs, CTLA-4+ eTregs, PD-1+ eTregs, and CXCR3+ eTregs. The immune cells with a significantly higher frequency in CCA even after surgery were as follows: (a) CD4+ T cells, helper T cells, CD25+ CD8+ T cells, CD80+ CD8+ T cells, (b) PD-1+ nTregs, CCR4+ nTregs, CCR6+ nTregs, OX40+ nTregs, and (c) OX40+ eTregs. (d) The frequency distributions of immune cells before surgery in NR patients, R patients, and HV are shown in the box and whisker plots. The frequency of CD4+ T cells, PD-1+ nTregs, CCR6+ nTregs, and OX40+ nTregs before surgery was higher in R patients than in HV, whereas it was comparable between NR patients and HV. Abbreviations: pre: pre-surgery group, post: post-surgery group, NR: non-recurrent CCA group, R: recurrent CCA group

Figure 3

The relationship between immune cell profiles and OS in each treatment group (a) Kaplan-Meier plot of OS according to immune cell profiles in peripheral blood in the BSC group. Using the median value as a threshold, the group in which the frequency of the immune cell was no less than the median is shown as a solid line, and the group in which the frequency of the immune cell was less than the median is shown as a dotted line. (b) Kaplan-Meier plot of OS according to immune cell profiles in peripheral blood in the chemotherapy group. Using the median value as a threshold, the group in which the frequency of the immune cell was no less than the median is shown as a solid line, and the group in which the frequency of the immune cell was less than the median is shown as a dotted line. (c) Kaplan-Meier plot of OS according to immune cell profiles in peripheral blood in the surgery group. Using the median value as a threshold, the group in which the frequency of the immune cell was no less than the median is shown as a solid line, and the group in which the frequency of the immune cell was less than the median is shown as a dotted line. Abbreviations: OS: overall survival, BSC: best supportive care, Freq: frequency

Figure 4

The relationship between immune cell profiles and RFS in the surgery group Kaplan-Meier plot of RFS according to immune cell profiles in peripheral blood in the surgery group. Using the median value as a threshold, the group in which the frequency of the immune cell was no less than the median is shown as a solid line, and the group in which the frequency of the immune cell was less than the median is shown as a dotted line. Abbreviations: RFS: recurrence-free survival

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