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Original article

High ratio of programmed cell death protein 1 (PD-1)$^+$/CD8$^+$ tumor-infiltrating lymphocytes identifies a poor prognostic subset of extrahepatic bile duct cancer undergoing surgery plus adjuvant chemoradiotherapy

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ABSTRACT

Background and purpose: This study investigated the prognostic role of PD-L1 expression, PD-1$^+$ tumor-infiltrating lymphocytes (TILs), and the ratio of PD-1$^+$/CD8$^+$ TILs in extrahepatic bile duct (EHBD) cancer.

Materials and methods: We analyzed 83 patients with EHBD cancer who underwent curative surgery plus fluoropyrimidine-based chemoradiotherapy (CRT). Expressions of PD-L1, PD-1, and CD8 were assessed by immunohistochemistry.

Results: Fifty-six (68%) patients were PD-L1-positive, and its lower expression level was associated with hilar tumor location ($P = 0.044$). A higher ratio of PD-1$^+$/CD8$^+$ TILs was associated with poorer overall survival (OS) ($P = 0.032$), relapse-free survival (RFS) ($P = 0.024$), and distant metastasis-free survival (DMFS) ($P = 0.039$) in Kaplan–Meier analyses, but survival differences were not observed according to the PD-L1 expression level. With Cox proportional hazards models, the ratio of PD-1$^+$/CD8$^+$ TILs was the independent prognostic factor in OS (HR 2.47, 95% CI 1.04–5.86), RFS (HR 2.41, 95% CI 1.08–5.41), and DMFS (HR 2.67, 95% CI 1.00–7.11) after adjusting for other significant clinicopathologic variables.

Conclusion: A strong survival impact of the ratio of PD-1$^+$/CD8$^+$ TILs was observed in EHBD cancer. In the poor prognostic subgroup, the blockade of the immune checkpoint in combination with conventional multimodality treatment needs to be considered.

Extrahepatic bile duct (EHBD) cancer is a relatively rare malignancy with poor prognosis. In most cases, the pathologic diagnosis is classified as an adenocarcinoma with mutated epithelial cells arising from the bile ducts [1]. The 5-year survival rates recently reported for EHBD cancer range from 18% to 40% [2,3]. No randomized phase III trials have evaluated the role of adjuvant treatment in the disease entity, but some retrospective studies have suggested that postoperative chemoradiotherapy (CRT) has advantages [4,5]. In a recent multicenter analysis, the use of adjuvant CRT resulted in survival benefits in patients with N1 stage biliary tract cancer [6].

There has been increasing evidence that anti-tumor immune responses are helpful in eliminating residual tumors and enhancing the cytotoxic effect of conventional chemotherapy or radiotherapy [7]. Several molecular markers of immune cells were expected to be prognostic factors for oncologic treatment outcomes [8,9], but few therapeutic applications have been established in clinical practices. In cancer immunology, the balance of co-stimulatory or inhibitory responses is an important underlying mechanism in the function of T cell-mediated anti-cancer immunity [10].

The programmed cell death protein 1 (PD-1)/programmed cell death protein 1 ligand 1 (PD-L1) axis is one of the prominent immune checkpoints, which are known as the inhibitory mechanisms of immune responses [11]. The PD-1/PD-L1 pathway

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downregulates the inflammatory reactions of infection or autoimmunity under normal physiologic conditions, and this function can be translated into resistance to the immunologic killing of tumor cells [12]. PD-1 is a cell surface receptor molecule on the activated tumor-infiltrating lymphocytes (TILs), which interact with tumor cells and are involved in tumor-specific immune responses [13]. PD-L1, the main ligand of PD-1, is expressed in peripheral tissues, including the tumor. In proinflammatory conditions, the molecular interaction between PD-1 and PD-L1 induces a co-inhibitory signal and decreases the level of cytokines, which subsequently results in the anergy and apoptosis of the effector T lymphocytes [14]. Therefore, the blockade of the immunoinhibitory mechanism has been considered a promising treatment approach [15]. Based on the therapeutic implications of immunomodulation, there have been ongoing clinical trials to evaluate the tumor-suppressive effect of the PD-1/PD-L1 blockade [16,17].

Considering the clinical application of the PD-1/PD-L1 axis, its expression level and prognostic role in a variety of malignancies have been investigated. Several studies have demonstrated that the activity of PD-1 and/or PD-L1 was associated with patients' prognoses [18–20], but others could not validate the significance [21,22]. Until now, in bile duct cancer, two published studies and one abstract have analyzed the immunohistochemical results of PD-1 and/or PD-L1 [23–25]. However, the role of the immune checkpoint in patients' prognoses is not known yet.

This study analyzed PD-1 and PD-L1 expressions in patients with EHBD cancer who underwent curative surgery followed by adjuvant CRT. The survival impacts of the immune checkpoint markers were evaluated from the immunohistochemistry of PD-L1 in tumor tissues and PD-1$^+$ or CD8$^+$ TILs.

Materials and methods

Patients and treatment

This study analyzed a total of 83 patients with EHBD cancer who were treated with surgery followed by CRT between August 2000 and August 2006. With the approval of the Institutional Review Board, the patients gave informed consent prior to treatment.

All of the enrolled patients underwent curative surgical resection followed by adjuvant CRT. In 64 patients, a total of 40 Gy was delivered with a fraction size of 2 Gy (5 days/week) with a planned rest of 2 weeks after receiving 20 Gy. Another continuous course of RT was delivered to 19 patients, and its total dose ranged from 50 Gy to 56 Gy with conventional fractionation. Concurrent chemotherapy with intravenous bolus 5-fluorouracil (5-FU, 500 mg/m^2 per day) was administered for the first three days of every 2 weeks of RT in most cases, except in the cases of two patients who received oral capecitabine. After the completion of adjuvant CRT, 62 patients were treated with maintenance chemotherapy. The chemotherapy regimen consisted of 5-FU (n = 31), 5-FU and leucovorin (n = 21), enteric-coated tegafur–uracil (n = 4), enteric-coated tegafur–uracil and leucovorin (n = 3), and oral capecitabine (n = 3).

Tissue microarray and immunohistochemistry

All of the cases were diagnosed with adenocarcinoma of the EHBDs. Each hematoxylin and eosin-stained slide was reviewed and confirmed as adenocarcinoma. A tissue microarray (TMA) was made from 4-mm cores derived from representative formalin-fixed paraffin-embedded (FFPE) tissue blocks.

In the immunohistochemistry of PD-L1, there have been controversies about the quality of staining results [21,26]. It was suggested that positive membranous staining is important in interpreting the expression level and is consistent with the biological function of PD-L1 [27]. Using the rabbit anti-PD-L1 monoclonal antibody (mAb) (clone E1L3 N, Cell Signaling Technology, Danvers, MA, USA; 1:100), we obtained the appropriate membranous staining pattern. In PD-1 and CD8, rabbit anti-PD-1 mAb (clone EPR4877 [2], Abcam, Cambridge, MA, USA; 1:300) and rabbit anti-CD8 mAb (clone SP16, Thermo Fisher Scientific, Fremont, CA, USA; 1:100) were used, respectively. Immunohistochemical staining was performed with the Ventana BenchMark XT autostainer (Ventana Medical Systems, Tucson, AZ, USA).

Histologic examination

Evaluation of PD-L1

The PD-L1 expression on the tumor cell membrane was evaluated semi-quantitatively on a 0–2 scale; 0 indicated no expression; 1 indicated weak to moderate expression; and 2 indicated strong expression (Fig. 1A). Considering the tumor heterogeneity of PD-L1 expression, the H-score was calculated using the formula of the representative intensity of each case multiplied by the percentage of expressed tumor cells. A case was considered positive when the H-score was more than 5. A single pathologist (J.K.) reviewed the stained slides without any knowledge on the clinical information and survival outcomes.

Automatic enumeration of PD-1 and CD8

PD-1 and CD8 slides were scanned for each TMA using the Aperio ScanScope (Aperio Technologies, Vista, CA, USA). We chose the corresponding tumor region of the PD-1 and CD8 slides. Aperio ImageScope software (Aperio Technologies) used Aperio nuclear IHC algorithms for PD-1 and CD8 staining. These algorithms are based on the spectral differentiation between brown (positive) and blue (counter) staining. Total percentage positivity (1+ through 3+) was recorded for each case. The density of PD-1$^+$ or CD8$^+$ TILs was the total number of 1+ and 3+ scored cells divided by the total area (mm^2). Fig. 1B and C shows the representative slide views of PD-1$^+$ and CD8$^+$ TILs according to the ratio of PD-1$^+$/CD8$^+$ TILs.

Statistical analysis

The clinicopathologic variables were categorized, and their associations with the expression levels of PD-L1, PD-1, CD8, and the ratio of PD-1$^+$/CD8$^+$ TILs were calculated with the Mann–Whitney U test. The relationships between the expression level of PD-L1, PD-1, and CD8 were assessed using Spearman's rank correlation coefficient (ρ). In the survival analyses, the positive or negative expression of PD-L1, high or low density of PD-1$^+$ TILs, and high or low ratio of PD-1$^+$/CD8$^+$ TILs were applied. The cut-off values were determined by the maximal chi-square method. Overall survival (OS), relapse-free survival (RFS), and distant metastasis-free survival (DMFS) were defined as the time interval between the date of surgical resection and specific events (deaths, overall tumor recurrences, and distant metastases, respectively). A Kaplan–Meier analysis and log-rank test were conducted to compare patients' survival outcomes. Cox proportional hazards models were used to evaluate independent prognostic factors in OS, RFS, and DMFS. Two-sided P-values of less than 0.05 were considered statistically significant. All of the statistical analyses were performed using SPSS (IBM, Armonk, NY, USA) and R software version 2.8.1 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Table 1 lists the baseline patient characteristics. The median duration of follow-up for all patients was 27 months and

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