Epithelial–Mesenchymal Transition Phenotype and Peritumoral Immune Cell Infiltration in Advanced Biliary Tract Cancer

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Abstract

We evaluated the clinical implications of epithelial–mesenchymal transition (EMT) markers and peritumoral immune cell infiltration in patients with biliary tract cancer (BTC) treated with gemcitabine plus cisplatin (GemCis). Forty-five patients with advanced BTC who received GemCis were included as the study population. We conducted multiplex immunohistochemistry and examined EMT markers and their correlations with immune cell infiltrate at the invasive tumor margin. Study population was subdivided into two groups: twenty-four patients with short-term survival (SS) and 21 with long-term survival (LS). The density of tumor cells expressing epithelial marker E-cadherin (E-cadherin⁺ CK⁺) at the invasive tumor margin tended to be higher in the LS group than in the SS group ($p = 0.065$). The density of tumor cells expressing mesenchymal marker vimentin (vimentin⁺ CK⁺) was significantly higher in the SS group than in the LS group ($p = 0.021$). Accordingly, the density of E-cadherin[−] vimentin⁺ CK⁺ cells was also significantly higher in the SS group ($p = 0.020$). The density of vimentin⁺ CK⁺ cells was positively correlated with FoxP3⁺ CD4⁺ regulatory T-cells ($r = 0.29$, $p = 0.047$). EMT-related features were enriched in BTC patients with poor survival outcomes and were associated with the immunosuppressive tumor microenvironment.

Introduction

Biliary tract cancer (BTC), accounting for about 3% of all gastrointestinal cancers, is a heterogeneous group of malignancies originating from the bile ducts, including gallbladder cancer (GBC), intrahepatic cholangiocarcinoma (IHCCA), and extrahepatic cholangiocarcinoma (EHCCA) [1, 2]. In the early stage, surgery is the mainstay of the treatment of BTC. However, post-surgical relapse frequently occurs, and most patients are diagnosed at an advanced stage. Although gemcitabine-cisplatin (GemCis) chemotherapy has been the standard of care in patients with advanced BTC, their median survival remains poor with less than one year [3]. Thus, understanding the mechanisms that lead to cancer dissemination in patients with BTC is currently an important issue to establish novel therapeutic strategies.

During the past decade, epithelial–mesenchymal transition (EMT) was found to be a crucial step in cancer progression and metastasis. EMT is a cellular process in which epithelial cells lose their epithelial characteristics, including cell polarity and intercellular adhesions, and acquire mesenchymal properties associated with enhancing cell motility and invasiveness [4]. During the EMT process, the loss of epithelial markers, such as E-cadherin, and activation of mesenchymal markers including N-cadherin, vimentin, and fibronectin occurs [5]. Recently, emerging evidence suggested that inflammatory cells in the tumor microenvironment promote EMT, thereby allowing tumor cells to inhibit the antitumor immune response by acquiring mesenchymal traits [6, 7]. Given that the tumor margin is the major site for active infiltration of immune cells [8], the interconnected relationship between tumor and immune cells at the invasive tumor margin may provide insights into understanding tumor progression and immune evasion.

Although previous studies have already revealed that mesenchymal marker expression of tumor cells is associated with poor clinical outcomes in patients with several types of malignancies [9–11], the clinicopathological significance of EMT marker expression and its association with immune cell infiltration in advanced BTC patients remain unclear. This study investigated the expression of EMT markers and peritumoral immune cell infiltration using multiplex immunohistochemistry (IHC) in advanced BTC patients treated with GemCis.

Materials And Methods

Study population

The patients were identified from our previously reported retrospective cohort of advanced BTC who received first-line palliative GemCis treatment between April 2010 and June 2015 at Asan Medical Center, Seoul, Korea [12]. Clinical information was collected from medical record review, including patient demographics, performance status (PS), baseline carbohydrate antigen (CA) 19-9 level, location of tumors, metastatic sites, and survival data. Patients who had adequate surgical specimens were eligible for this study. We excluded patients who had tumor samples with inappropriate for multiplex IHC or incomplete clinical data. This study was approved by the Institutional Review Board (IRB No: 2018-0716) of Asan Medical Center, Seoul, Korea.

Multiplex IHC

Multiplex IHC analysis was conducted using Opal multiplex immunofluorescence on Leica Bond RX Automated Stainer (Leica Biosystems, Newcastle, UK) with antibodies against the following: E-cadherin, vimentin, ZEB1, N-cadherin, and cytokeratin (CK) in EMT marker panel, CD3, OX40 (CD134), interleukin-17 (IL-17), major histocompatibility complex (MHC) class I and II in immune marker panel, and CD4, CD8, and FoxP3 for T-cell marker panel. Each antibody is paired with an individual Opal fluorophore for visualization. All fluorescently labeled slides were scanned on the Vectra Polaris Quantitative Pathology Imaging System (Perkin Elmer, Boston, MA). We subdivided the tumor regions into three areas: tumor center, tumor margin, and stroma. Regions of interest (ROIs) representing each subdivision were carefully selected by a hepatobiliary pathologist based on hematoxylin and eosin (H and E) slides. We selected 7 to 16 ROIs for each subsection. As the invasive margin of the tumor is considered a site where dynamic interactions between tumor cells and the surrounding stroma occur [8], we focused on the tumor margin and examined the tumor and immune cells in this area.

Statistical analysis

Overall survival (OS) was calculated as the time from the beginning of treatment to death from any cause. The survival curve for OS was estimated using the Kaplan–Meier method and compared by log-rank test. Additionally, we assessed the differences in continuous variables between two or three groups

using the Mann–Whitney U test or Kruskal–Wallis test, respectively. Finally, Spearman’s correlation was used to evaluate the degree of relationships between two variables. A two-sided p -value less than 0.05 was considered statistically significant. All statistical analyses were conducted using IBM SPSS Statistics for Windows v.21.0 (IBM Corp., Armonk, NY, USA).

Results

Patients

In this study, 45 patients were analyzed. To investigate the EMT and immune-related features according to distinct survival outcomes, we subdivided patients into two subgroups: 24 patients who died within ten months from the start of GemCis treatment (short-term survivor group, SS), and 21 remained alive for longer than 20 months from the treatment (long-term survivor group, LS). Figure 1A represents the Kaplan–Meier curve for the OS of these two groups of patients. Baseline characteristics of both groups are shown in Table 1. There was no significant difference in age, sex, primary tumor location, or initial extent of disease between the SS and LS groups, while PS was poorer in the SS group ($p = 0.033$). Additionally, the baseline level of CA 19–9 was significantly higher in the SS than in the LS group ($p = 0.032$).

Table 1
Baseline characteristics of study patients

Characteristics	Short-term survivor group (n = 24)	Long-term survivor group (n = 21)	p-value
Age (median, range)	59 (39–77)	60 (35–73)	0.673
Sex (%)			0.632
Male	12 (50.0)	12 (57.1)	
Female	12 (50)	9 (42.9)	
ECOG performance status (%)			0.033
0	9 (37.5)	15 (71.4)	
1	11 (45.8)	6 (28.6)	
2	4 (16.7)	0 (0.0)	
Primary tumor location (%)			0.644
Intrahepatic CCA	6 (25.0)	7 (33.3)	
Extrahepatic CCA	10 (41.7)	6 (28.6)	
GB cancer	8 (33.3)	8 (38.1)	
Initial CA 19-9 level (median, IQR)	139.5 (41.3–328.0)	22.8 (9.9–222.6)	0.032
Disease setting at presentation (%)			0.380
Locally advanced	1 (4.2)	3 (14.3)	
Metastatic	6 (25.0)	3 (14.3)	
Recurrent	17 (70.8)	15 (71.4)	
ECOG, Eastern Cooperative Oncology Group; CCA, cholangiocarcinoma; GB, gallbladder; CA, carbohydrate antigen; IQR, interquartile range			

EMT marker expression of tumor cells by multiplex IHC in both groups

Between the two groups, the density of tumor cells expressing EMT-associated protein at the invasive tumor margin based on IHC was compared; E-cadherin as a marker for epithelial characteristics and vimentin, ZEB1, and N-cadherin as markers for the mesenchymal phenotype. The density of E-cadherin

expressing tumor cells (E-cadherin⁺ CK⁺) was lower in the SS group than the LS group, but the difference was not statistically significant (median; 489 vs. 624/mm³, $p = 0.065$, Figure 1B). The density of vimentin-stained tumor cells (vimentin⁺ CK⁺) was significantly higher in the SS group (median; 70 vs. 30/mm³, $p = 0.021$, Figure 1C). The number of tumor cells expressing ZEB1 (ZEB1⁺ CK⁺) or N-cadherin (N-cadherin⁺ CK⁺) was also higher in the SS group than the LS group, but the difference was not statistically significant (median; 3.4 vs. 3.0 mm³, $p = 0.560$ and median; 24 vs. 20 mm³, $p = 0.774$, respectively). The density of tumor cells negative for E-cadherin and positive for vimentin (E-cadherin⁻ vimentin⁺ CK⁺) was significantly higher in the SS group compared with the LS group (median; 64 vs. 25 mm³, $p = 0.020$, Figure 1D).

Immune marker expression at the invasive margin of the tumor in both groups

Expression levels of CD3, OX40, IL-17, MHC class I and II at the tumor margin were examined as markers of immune-related parameters. The density of cells positive for CD3⁺ T-cells was significantly higher in the LS group than the SS group (median; 261 vs. 179 mm³, $p = 0.045$, Figure 2A). In contrast, the density of cells expressing OX40 was significantly higher in the SS group than in the LS group (median; 279 vs. 65 mm³, $p = 0.006$, Figure 2B). The density of cells expressing MHC class I, MHC class II, and IL-17, was comparable between the two groups. There was no significant difference in the density of FoxP3⁺ CD4⁺ T-cells (*Treg* cells) between the two groups (median; 10 vs. 11 mm³, $p = 0.626$).

Correlation between expression levels of EMT markers and immune cell infiltrates

The association between mesenchymal marker expression of tumor cells and infiltrating immune cells at the tumor margin was evaluated. A weak but significant positive correlation was noted between the level of vimentin⁺ CK⁺ cells and that of *Treg* cells ($r = 0.29$, $p = 0.047$, Figure 3A). In contrast, the density of vimentin⁺ CK⁺ cells had no significant association with either the density of CD8⁺ cytotoxic T-cells ($r = 0.019$, $p = 0.905$) or that of FoxP3⁻ CD4⁺ helper T-cells ($r = -0.279$, $p = 0.067$). The density of OX40⁺ cells had a significant positive correlation with that of vimentin⁺ CK⁺ cells ($r = 0.48$, $p < 0.001$, Figure 3B).

Expression of EMT- and immune-related markers according to primary tumor location

EMT marker expression of tumor cells and immune marker expression of peritumoral cells according to the primary tumor location was compared. There was no significant difference in E-cadherin⁺ CK⁺ cells between the patients with GBC, IHCCA, and EHCCA (median; 495 vs. 638 vs. 529 mm³, $p = 0.838$, Figure

4A). The density of vimentin⁺ CK⁺ cells did not differ significantly by primary tumor location (median; 48 vs. 18 vs. 59 mm³, $p = 0.248$, Figure 4B). CD3⁺ cells (median; 185 vs. 171 vs. 240 mm³; $p = 0.418$) and OX40⁺ cells (median; 154 vs. 82 vs. 224 mm³, $p = 0.315$) at the invasive tumor margin were comparable between patients with GBC, IHCCA, and EHCCA, respectively.

Discussion

Current study investigated the clinical implications of the EMT markers and immune cell infiltrates in the tumor microenvironment of BTC patients. Mesenchymal marker vimentin expressing tumor cells was enriched in BTC patients with a poorer survival rate, whereas the level of epithelial marker expression was relatively lower in these patients. We also found that EMT marker expression of tumor cells was significantly associated with the immune microenvironment.

Mesenchymal marker expression of tumor cells was reportedly associated with poor prognostic factors such as lymph node metastasis, portal vein invasion, and advanced stage in patients with BTC [13]. In addition to increased invasiveness and metastatic activity, EMT has been associated with resistance mechanisms against chemotherapy in previous research [14]. Yamada *et al.* demonstrated that gemcitabine-resistant CCA cells exhibited decreased expression of E-cadherin and elevated expression of vimentin and N-cadherin [14]. Our study results show that vimentin expression of tumor cells by multiplex IHC was significantly higher in BTC patients with dismal survival outcomes who received GemCis, which conforms to prior studies [9, 13].

A notable finding in this study is that the density of vimentin expressing tumor cells was significantly correlated with *Treg* cells. As an important part of the peritumoral microenvironment, *Treg* cells play a critical role in immunosuppression and tumor progression through interaction with other immune cell subsets and tumor cells [15]. Oh *et al.* have previously shown that contact between melanoma and *Treg* cells was integral to the elevation of TGF- β expression and subsequent induction of EMT in melanoma cells [16]. Several studies have also suggested that the infiltration of *Treg* cells triggers the TGF- β 1 signaling pathway and promotes EMT of cancer cells during hematogenous dissemination in hepatocellular carcinoma [17, 18]. Our results, showing a significant relationship between *Treg* cell infiltration and vimentin expressing tumor cells, agree with previous research findings. Thus, tumor cells and adjacent *Treg* cells may interact reciprocally, and the upregulation of *Treg* cells in the peritumoral microenvironment may be one of the most critical steps in promoting the EMT pathway. Cancer cells acquire their motility and invasiveness due to this process [6, 7, 15]. In contrast, the density of peritumoral helper T-cells associated with the prognosis of patients with advanced BTC in our previous report was not correlated with the density of EMT marker-expressing tumor cells in the current study [8].

EMT is a complex and dynamic process, which may be an adaptation mechanism of tumor cells responding to their hostile microenvironment [19]. Recently, experimental data have demonstrated that EMT can be reversed by targeting the specific signaling pathways related to this process [20]. In this context, it is expected that the reversal of EMT can be a novel therapeutic strategy for anticancer

treatment. Furthermore, the potential involvement of the EMT process and Treg cells in resistance to immune checkpoint inhibitors (ICIs) has been reported [21, 22]. Therefore, results on the association between mesenchymal marker expression and Treg cells and OX40, which is preferentially expressed on Treg cells, suggest that this interconnected process explains the limited response of BTC to ICIs. Therefore, further investigations should explore the immunological and clinical impact of targeting these in BTC.

This study showed that OX40 was significantly highly expressed in patients with worse survival rates. OX40 is one of the co-stimulatory molecules expressed by effector T-lymphocytes and enhances immune responses [23]. Nevertheless, there have been contradictory results about the prognostic implication of OX40, indicating a favorable prognosis in ovarian and lung cancer but poor prognosis in hepatocellular carcinoma and head and neck squamous cell carcinoma [24–27]. However, several previous studies have shown that Treg cells preferentially express OX40 compared with conventional T-cells, although OX40 is transiently expressed by effector CD4⁺ and CD8⁺ T-cells following T-cell receptor ligation [28, 29]. We also found that the peritumoral cellular density of OX40 expression was significantly associated with the mesenchymal phenotype of tumor cells. This finding conforms to prior studies demonstrating that OX40 was one of the key genes coexpressed with the EMT signature in various cancers [30]. Altogether, our results suggest that OX40 is involved in regulating the antitumor immune response, which is closely related to the EMT pathway, and contributes to cancer progression, leading to poor survival outcomes in BTC.

Limitations of this study include selection bias caused by its retrospective nature and heterogeneous study population in terms of the primary tumor, disease extent, and clinical settings. Our study is also limited by the relatively small number of EMTs, and immune markers were analyzed. Additionally, OX40 expression by each T-cell subset could not be evaluated because the immune marker panel was stained separately from T-cell markers, including CD4, CD8, and FoxP3. Nevertheless, our results show a meaningful interaction between EMT signature and tumor microenvironment may provide valuable insight into potential biomarkers or relevant targets associated with the EMT pathway treating advanced BTC.

Conclusions

Conclusively, the expression of EMT-associated markers in tumor cells, closely related to the immunosuppressive tumor microenvironment, serves as an adverse prognostic factor and may be a potential therapeutic target for treating patients with advanced BTC.

Abbreviations

BTC: Biliary tract cancer, CA: carbohydrate antigen, CCA: cholangiocarcinoma, CK: cytokeratin, EHCCA: extrahepatic cholangiocarcinoma, EMT: epithelial–mesenchymal transition, GBC: gallbladder cancer, GemCis: gemcitabine plus cisplatin, ICI: immune checkpoint inhibitor, IHC: immunohistochemistry, IHCCA:

intrahepatic cholangiocarcinoma, LS: long-term survival group, MHC: major histocompatibility, OS: overall survival, PS: performance status, ROI: region of interest, SS: short-term survival group

Declarations

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Author contributions: CRO and CY participated in the study design and writing the manuscript. HDK, Y-MR, SL, DK, DSL, JHJ, H-MC, B-YR, KK, MK, S-YK, and CY were involved in data acquisition. CRO, HDK, YMR, S-YK, and CY contributed to performing data analysis and interpretation.

Competing interests: The authors declare no competing interests.

Data availability statement: The data are not publicly available due to privacy and ethical concerns.

Ethics declarations: All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008. Ethical approval was obtained from institutional review board (IRB No: 2018-0716).

Consent for publication: Informed consent was obtained from all patients for being included in the study.

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Figures

Figure 1

Survival of patients and expression of EMT markers by multiplex IHC in both groups (A) Overall survival of patients in both groups (B) E-cadherin⁺ tumor cells (E-cadherin⁺ CK⁺) (C) Vimentin⁺ tumor cells (vimentin⁺ CK⁺) (D) E-cadherin⁻ vimentin⁺ tumor cells (E-cadherin⁻ vimentin⁺ CK⁺) EMT, epithelial-mesenchymal transition IHC, immunohistochemistry CK, cytokeratin SS, short-term survival group LS, long-term survival group

A. CD3⁺

B. OX40⁺

Figure 2

Immune markers at the invasive margin of tumor in both groups (A) CD3⁺ (B) OX40⁺ SS, short-term survival group LS, long-term survival group

A.

B.

Figure 3

Correlation between vimentin-expressing tumor cells and the peritumoral microenvironment (A) FoxP3+ CD4+ (B) OX40+

A. E-cadherin⁺ CK⁺

A. Vimentin⁺ CK⁺

Figure 4

EMT marker expression of tumor cells by primary tumor location (A) E-cadherin+ tumor cells (E-cadherin+ CK +) (B) Vimentin+ tumor cells (vimentin+ CK +) EMT, epithelial-mesenchymal transition CK, cytokeratin GBC, gallbladder cancer IHCCA, intrahepatic cholangiocarcinoma EHCCA, extrahepatic cholangiocarcinoma