Multiplexed immunohistochemical analysis of the immune microenvironment of biliary tract cancers pre- & post-neoadjuvant chemotherapy: case series

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Background: Neoadjuvant chemotherapy (NACT) is increasingly being used in the management of locally advanced biliary tract cancer (BTC). The evidence suggests a contributing role of tumor infiltrating immune cells in the prognosis and response. We set out to characterize immune modulation of tumor immune microenvironment in BTC following NACT.

Case Description: Patients with BTC who underwent diagnostic biopsy, then NACT then resection between 2014–2018 were identified. Multiplexed immunohistochemical consecutive staining on single slide (MICSSS) analysis was performed with a series of immune markers to characterize T-cells, immune checkpoints etc. on pre- & post-NACT tumor tissue. Density was calculated for each marker. The final analysis included five patients. Median age was 48 (range, 41–56) years, with 4 female, 4 intrahepatic cholangiocarcinoma and 1 gallbladder. All patients received gemcitabine/cisplatin as NACT (median of 5 cycles). Median time from diagnosis to surgery was 4.3 (range, 1.4–7.8) months. All patients were mismatch repair proficient (pMMR). NACT on average produced a depletion of all immune markers. Given small sample size, each patient was considered their own control and changes in mean cell densities post-NACT were calculated. Patient #2 with a 40-fold increase in PD-L1 expression & 5-fold decrease in CD8:FOXP3 ratio after NACT notably had the shortest disease-free interval (DFI). Patient #3 with the longest DFI had the largest increase in CD8:FOXP3 by about 8-fold with a decrease in PD-L1.

Conclusions: Preliminary results suggest NACT may differentially modulate various compartments of the immune tumor contexture despite overall cell depletion. Future studies should focus on strategies to expand immune modulation of tumor microenvironment, including immune-oncology agents to augment the effects of chemotherapy.

Keywords: Cholangiocarcinoma; multiplex immunohistochemistry (multiplex IHC); microenvironment; case series; immune modulation

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Introduction

Biliary tract cancers (BTC) represent a group of highly lethal adenocarcinomas arising from the biliary tree and include cholangiocarcinoma (intrahepatic, perihilar, or distal) and gallbladder cancer. Though their incidence varies from rare malignancies in developed countries to a major public health hazard in endemic areas, clinical outcomes remain dismal with a 5-year survival under 10% (1). The standard of care for patients with resectable BTC is surgery followed by adjuvant chemotherapy or chemoradiation although this strategy has not conclusively been shown to improve survival (2). Over 50% of these patients still succumb to the disease due to recurrence, a majority of which are distant recurrences attributable to micro-metastatic disease (3,4).

Recently, a paradigm shift in sequencing therapies has evolved in favor of a neoadjuvant approach across disease types, particularly in pancreas and rectal cancers. This approach has the potential benefit of mitigating early occult micro-metastatic disease, tumor downstaging, augmenting R0 resection rates, and enhancing patient selection for surgery. Furthermore, it provides an opportunity to test novel therapeutic approaches at an early stage in the disease management (5). In BTC, the role of neoadjuvant chemotherapy (NACT) is limited to downstaging unresectable disease with a goal of conversion to resectability and is therefore rarely employed. However, small but compelling reports suggest improved outcomes in BTC treated with NACT (6,7). In a report of 45 patients with BTCs, 12 of whom were treated with neoadjuvant chemoradiation, the 5-year survival was improved to 53% in the neoadjuvant group *vs.* 23% in the adjuvant group, despite having more advanced disease at presentation (8). Gemcitabine combined with cisplatin chemotherapy has been established as the standard of care for the treatment of BTCs in both locally advanced and metastatic disease states (9).

Today, it is widely acknowledged that the tumor immune microenvironment (TIME) of neoplasms plays a pivotal role in tumor development and progression and holds substantial implications for therapeutic strategies (10,11). In BTCs, the TIME contexture has not been well characterized to date. Limited data in intrahepatic cholangiocarcinoma (iCCA) suggest that lymphocytic infiltrate is present in all intrahepatic tumors (10). Nonetheless, the composition and organization of immune cells in these tumors as well as expression of immunomodulatory markers such as PD-1 and PD-L1 has yet to be extensively characterized.

The role of chemotherapy in modulation of the TIME is multifold and has been shown to induce tumor-associated neoantigen release, increase dendritic cells while depleting myeloid-derived suppressor cells (MDSCs) and increase infiltration of CD8+ve T cells and CD20+ve B cells (12-15). The proportion of effector to suppressive immune cells in the TIME has also been shown to predict outcomes in various cancers (16-18). Preclinical studies suggest that chemotherapy can induce T cell modulation, and in mice with pancreatic neoplasms, can counteract resistance to checkpoint blockade with PD-1 and CTLA-4 antibodies leading to tumor regression (19,20). In one study, investigators demonstrated that treatment of *in-vitro* cholangiocarcinoma cancer cells with gemcitabine and 5-fluorouracil induced immunogenic modulation by up-regulating the expression of PD-L1 and other immune markers (21).

Highlight box

Key findings

- We performed a novel multiplexed immunohistochemical consecutive staining on single slide analysis to characterizing the immune modulation of the tumor microenvironment pre- and post-neoadjuvant chemotherapy (NACT) in biliary tract cancer patients.

What is known and what is new?

- Chemotherapy leads to an overall depletion of immune cell infiltration in the tumor microenvironment.
- Our study found differential effects on various immune markers, with some patients showing increases in certain markers while others showed decrease. These also correlated with clinical outcomes.

What is the implication, and what should change now?

- The study highlights the potential importance of immune modulation in the response to NACT. Future studies are recommended to focus on strategies to expand immune modulation of the tumor microenvironment, including the use of immune-oncology agents to augment the effects of chemotherapy.

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In this case series, we set out to understand the landscape of TIME of BTCs and the impact of cytotoxic cisplatin combined with gemcitabine chemotherapy delivered *in-vivo*, by performing a multiplexed immunohistochemical (IHC) consecutive staining on single slide (MICSSS) analysis, on paired pre- and post-NACT tumor specimens in patients who underwent NACT followed by resection of their tumors. We present this case series in accordance with the AME Case Series reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-23-1928/rc).

Case presentation

Patients

The case series was performed in accordance with the Declaration of Helsinki (as revised in 2013). Ethical approval to conduct this study was granted following review of the study protocol by The Mount Sinai Health System Institutional Review Board (IRB; HS#: 18-01371; GCO#1: 18-2764). All study participants, or their legal guardians, provided informed written consent prior to study enrollment. A copy of the written consent is available for review by the editorial office of this journal. Patients were identified through a retrospective chart review. A total of 125 patients were screened. Patients with locally advanced BTCs who underwent a diagnostic biopsy, either by fine or core needle aspiration, then NACT followed by surgical resection between July 2014 & November 2018 at the Mount Sinai Hospital were identified. Mount Sinai Hospital is a large academic tertiary-care hospital located in New York City. Nine patients met the aforementioned inclusion criteria. Patients with adequate tissue for analysis from both pre- and post-NACT specimens were included in the final analysis. MICSSS was performed on paired tissue obtained from biopsy samples prior to NACT and at the time of surgery.

Multiplex IHC staining and analysis

MICSSS, a sample-sparing chromogenic consecutive multiplex tissue staining method, was performed as described (22) with a series of immune markers (CD3, FOXP3, CD20, CD68, CD163, DC-LAMP, PD-1 & PD-L1), to characterize T cell subsets, B cells, macrophages, mature dendritic cells, and immune checkpoints on pre- & post-NACT formalin-fixed paraffin-embedded tumor tissue sections (*Figure 1*; Table S1). Image analysis was performed using the open-source software QuPath (23). Briefly, color deconvolution into three separate channels [hematoxylin, 3-amino-9-ethylcarbazole (AEC) chromogen, and residual] and stain vectors were determined to ensure the accurate cell segmentation and positive cell detection. Then, whole tissue annotations were performed in batch on all sections by a pathologist to identify areas of interest, including tumor, stromal tumor-infiltrating lymphocytes (sTILs) & tumor TILs (tTILs) infiltrating lymphocyte-enriched tumor and non-tumor areas such as glandular normal tissues, cirrhosis (fibrotic and sclerotic tissues), and necrosis without tumor cells (*Figure 2*). Intratumoral TILs were defined as lymphocytes residing within tumor nests, exhibiting direct cell-to-cell contact without intervening stroma, and engaging in interactions with carcinoma cells. Conversely, stromal TILs are situated in a dispersed manner within the stromal spaces amidst carcinoma cells, lacking direct contact with carcinoma cells (*Figure 3*). After cell segmentation using StarDist (24,25), an algorithm using a pre-trained deep learning model to detect hematoxylin-stained nuclei, and cytoplasmic expansion, detection of positive cells was done in QuPath using random forest machine learning approach to train classifiers based on features like AEC intensity and cell shape, after manual selection of both positive and negative cells. This was then repeated for every individual image. Density was calculated for each marker (positive cells/mm^2) following annotation of tissues by area. Cell densities analysis was performed in sTILs, tTILs and tumor tissue. Normal liver, cirrhotic, and necrotic/fibrotic liver tissues were excluded from analysis.

Statistical analysis

Descriptive statistics were used to summarize baseline status, including demographics, disease characteristics, and treatment characteristics. Descriptive statistics were also used to illustrate immune marker density changes before and after chemotherapy as well as to depict differences in clinical outcomes and immune marker expression between patients. Values reported for analysis indicate the $\log_2$ fold change in the combined densities in sTILs, tTILs and tumor tissue areas post-NACT compared to pre-NACT. In this case series, inferential statistical methods were not utilized due to the limited number of patients in the sample.

Nine patients were enrolled. Final analysis included five patients with adequate pre- and post-NACT tissue for MICSS. It may be noted that only core needle specimens were used for analysis, as they had preserved architecture that

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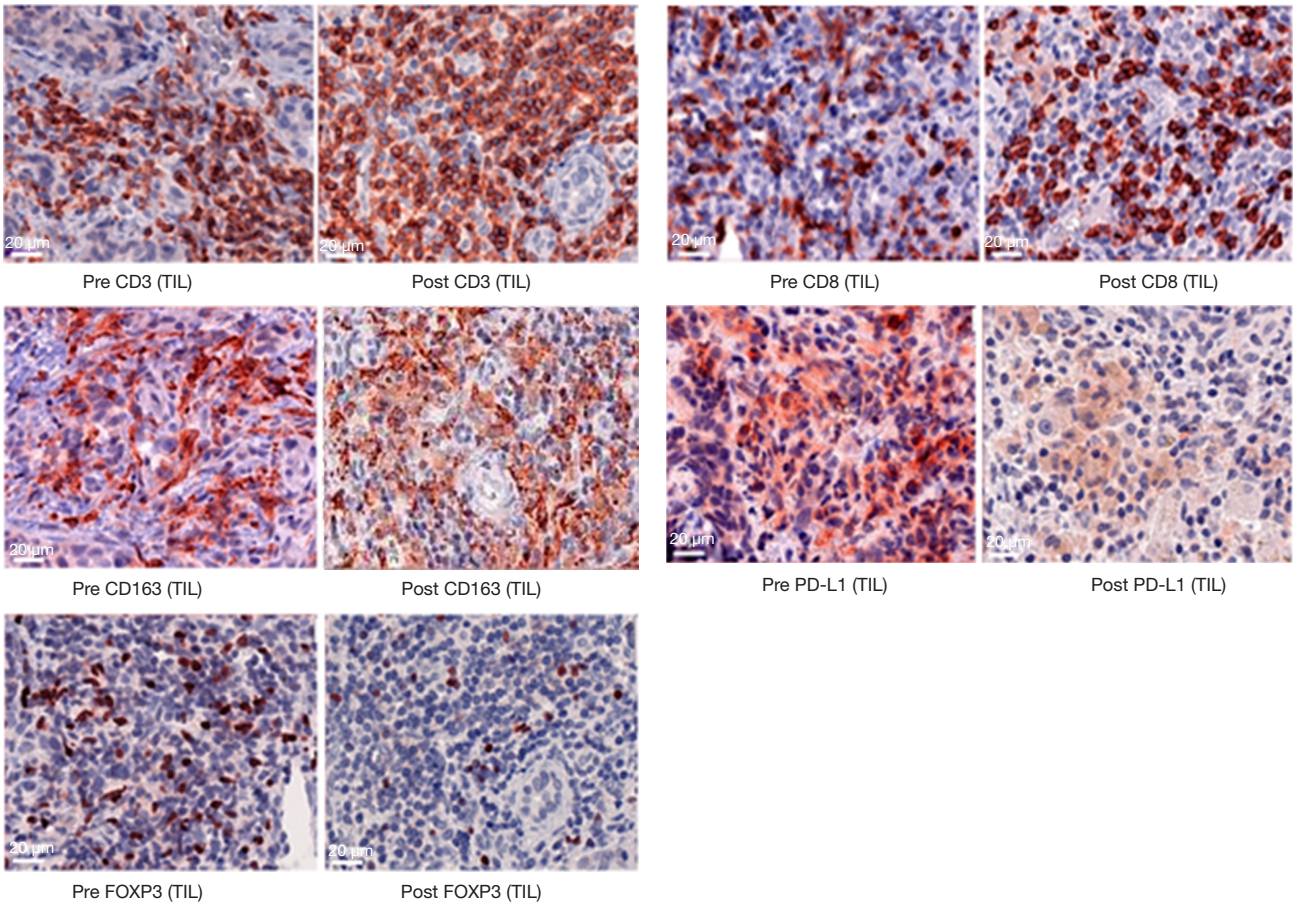

Figure 1 Representative examples of immunostaining for CD3, CD8, CD163, PD-L1, and FOXP3 in patient #3 pre- and post-NACT in BTC. Pictures demonstrate an increase in the expression of CD3, CD8, CD163 and a decrease in PD-L1, FOXP3 post-NACT at 40× magnification using MICSSS, refer to methods section for details. TIL, tumor infiltrating lymphocyte; NACT, neoadjuvant chemotherapy; BTC, biliary tract cancer; MICSSS, multiplexed immunohistochemical consecutive staining on single slide.

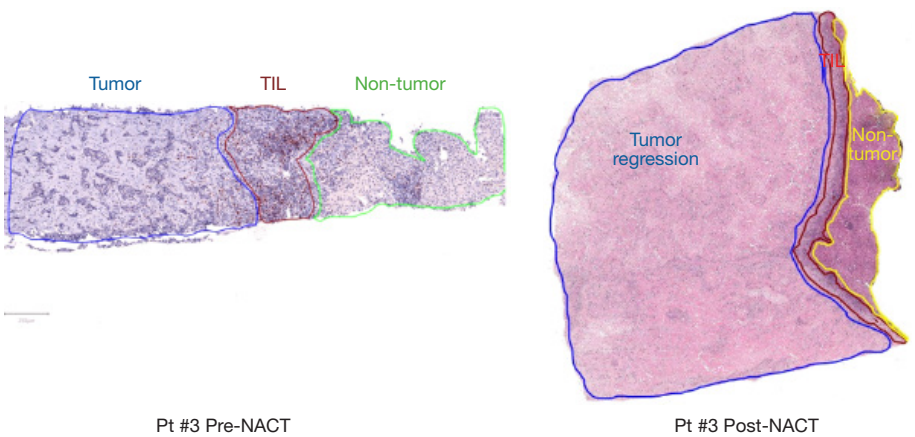

Figure 2 Low magnification at 10× of pre- and post-NACT BTC with their annotations as indicated representing TIL enriched tumor and non-tumor areas in Pt #3 using MICSSS (refer to methods section for details). Pt, patient; TIL, tumor infiltrating lymphocyte; NACT, neoadjuvant chemotherapy; BTC, biliary tract cancer; MICSSS, multiplexed immunohistochemical consecutive staining on single slide.

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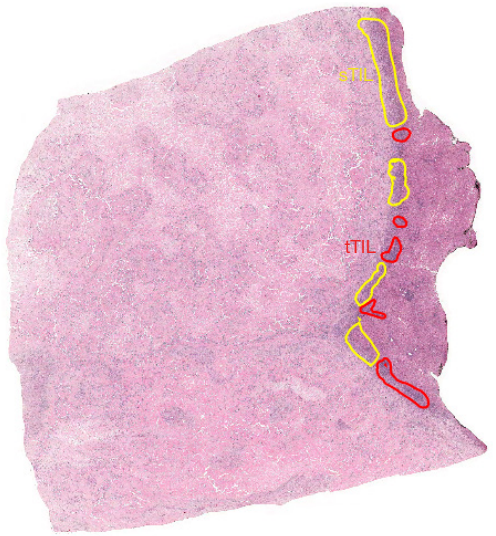

Figure 3 Identification of sTILs & tTILs in patient #3 at 5× magnification in post-NACT in BTC with their annotations as indicated. sTIL, stromal TIL; tTIL, tumor TIL; TIL, tumor infiltrating lymphocyte; NACT, neoadjuvant chemotherapy; BTC, biliary tract cancer.

was adequate for the study. Median age was 48 (range, 41–56) years with 4 (80%) being female. Four iCCA & 1 gallbladder cancer were included. All patients received gemcitabine combined with cisplatin as NACT with a median of 5 (range, 4–7) cycles. Median time from diagnosis to surgery was 4.3 (range, 1.4–7.8) months and last cycle to surgery was 0.9 (range, 0.6–1.5) months. All patients had margin negative (R0) resections. All patients were mismatch repair (MMR) proficient. One patient was Her2 positive by IHC (patient #5). Patient #2 had a tumor with an FGFR amplification and fusion and patient #3 with an FGFR rearrangement. *Table 1* describes patient characteristics in detail.

NACT, on average, produced a depletion of all immune markers. Among the five patients, however, the median CD8:FOXP3 ratio increased from pre-chemotherapy to post-chemotherapy analysis. *Table 2* describes the immune marker density changes from pre- and post-chemotherapy measurements.

The mean time to recurrence for the five patients described in this analysis was 285 days. Patient #3 had the longest time to recurrence of 598 days. Of note,

Table 1 Patient characteristics

Variables	Pt 1	Pt 2	Pt 3	Pt 4	Pt 5
Age (years)	42	43	52	57	48
Sex	F	M	F	F	F
Type of CCA	iCCA	iCCA	iCCA	iCCA	Gallbladder
NACT	Gem/Cis	Gem/Cis	Gem/Cis	Gem/Cis	Gem/Cis
Cycles, n	4	4	7	5	5
Stage by path	pT1N0	ympT2B N0 M1	ypT1N0	ypT1N0	ypT3N1Mx
MMR	Proficient	Proficient	Proficient	Proficient	Proficient
HER2 (by IHC)	Negative	Negative	Negative	Negative	Positive
Next-generation sequencing	Not performed	FGFR2 amp	TMB-4	ROS1 mutation	CDK4 ERBB3
		FGFR2-RABGAP1L fusion	FGFR2 rearrangement	BRCA2 VUS	ATM
		MEN1	IKBKE amp		GLI1
		MCL1 amp	MCL1 amp		
		ZNF703 amp	MDM3 amp		
			PIK3c2B amp		
Response by RECIST 1.1	PR	SD	PR	SD	SD
Time to recurrence (days)	204	111	598	267	243

Pt, patient; M, male; F, female; CCA, cholangiocarcinoma; iCCA, intrahepatic CCA; NACT, neoadjuvant chemotherapy; Gem/Cis, gemcitabine/cisplatin; MMR, mismatch repair; IHC, immunohistochemistry; FGFR, fibroblast growth factor receptor; amp, amplification; TMB, tumor mutational burden; PR, partial response; SD, stable disease.

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Table 2 MICSSS marker density reported as log_2 fold change post-NACT compared to pre-NACT in tumor + tTIL + sTIL

IHC stain	Marker of	Average	Pt 1	Pt 2	Pt 3	Pt 4	Pt 5
CD20	B lymphocytes	–1.7	–1.1	2.6	–0.2	–1.4	–5.9
CD3	T lymphocytes	–0.1	–0.6	–1.2	1	–0.7	–0.8
CD8	Cytotoxic T lymphocytes	–0.2	0.3	–0.9	0.7	–1.6	–2.5
FOXP3	Treg lymphocytes	–1.7	–1.4	0.5	–1.8	–1.4	–2.9
PD-1	Treg and T lymphocytes	0.1	0.2	0.8	1.3	–4.4	–3.8
PD-L1	Macrophages & tumor cells	–1.4	–2.2	5.3	–1.2	–3.7	–4
CD68	Monocytes/macrophages	–0.1	–0.2	–0.6	0.8	–1.3	–0.1
CD163	Monocytes/macrophages	–0.5	–1.1	–0.9	0.9	–0.6	–1.8
DC-LAMP	Mature dendritic cells	–2.8	–1.9	4.2	–2.2	–0.1	–6.5
CD8:FOXP3 ratio	–	0.4	3.2	–5.3	7.8	–0.7	0.4
Time to recurrence (days)	–	285	204	111	598	267	243

MICSSS, multiplexed immunohistochemical consecutive staining on single slide; NACT, neoadjuvant chemotherapy; TIL, tumor infiltrating lymphocyte; tTIL, tumor TIL; sTIL, stromal TIL; Pt, patient; Treg, regulatory T cell.

patient #3 with the longest time to recurrence is the only patient whose tumor was observed to have an increase in CD3, CD8, CD68 and CD163 markers and a decrease in expression of CD20, PD-L1 and DC-LAMP. Patient #3 also mounted the largest increase in CD8:FOXP3 ratio among the five patients. Conversely patient #2 who had the shortest interval to recurrence (111 days), was observed to have the largest decrease in CD8:FOXP3 ratio as well as the largest increase in PD-L1 and DC-LAMP. It may be noted that patient #2 underwent an adrenal metastasectomy along with resection of the primary iCCA after NACT.

Discussion

The TIME with its innate immune cells such as MDSCs, natural killer (NK) cells and adaptive immune cells, particularly the T and B lymphocytes play a pivotal role in shaping tumorigenesis, disease progression and response to therapy (26). The predictive value of these immune cells has been well described in various tumor types, including in iCCA (27). The impact of PD-1 and PD-L1 expression as well as subsets of tumor infiltrating immune cells may also correlate with prognosis in iCCA (28-30). The overall picture, however, is a complex interplay of various pro and anti-tumor elements and it is likely that no single marker alone correlates with clinical outcomes. In this modest case series, we aimed to begin to analyze and comprehend the impact of cytotoxic chemotherapy on TIME in bile duct cancers.

Our rationale for selection of the IHC stains was based on their known significance in tumorigenesis and their notable role in the TIME (*Table 2*). $CD4^+$ and $CD8^+$ tumor infiltrating T lymphocytes form the backbone of anticancer immune response. T-cells exhibit antigen-specific recognition of tumor cells, resulting in a cascade of immunological responses including cytokine secretion, release of cytotoxic effector molecules such as perforin and granzyme, and direct cell mediated cytotoxicity (31,32). $CD20^+$ B lymphocytes exert antitumor effect by producing tumor-specific antibodies, supporting T cell responses, and maintaining the structure and function of tertiary lymphoid structures within the TIME (33). $FOXP3^+$, expressed on regulatory T cell (Tregs) plays a pivotal role in maintaining the immune suppressive function of Tregs via transcriptional regulation, epigenetic modification, and downstream target protein expression modulation (34). Tumor-associated macrophages (TAMs; $CD68^+$, $CD163^+$) recruited and activated in the TIME have been shown to have a protumor effect in iCCA exerted by stimulating angiogenesis, inhibiting cytotoxic T/NK cells and remodeling the extracellular matrix to promote tumor cell invasion and metastasis (35,36). Similarly, the PD-1/PD-L1 axis represents the primary bottleneck in the anti-cancer immune response across various tumor types and has thus emerged as a widely pursued therapeutic target in oncology (37,38).

The analysis of our cohort showed that despite overall

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immune marker depletion by cytotoxic chemotherapy, some of the tumors exhibited an increase in a subset of immune markers after NACT. Of interest is patient #2 with the shortest time to recurrence, who had the highest increase in PD-L1 expression after cytotoxic chemotherapy. PD-L1 expressing cells in the TIME cause inhibitory downstream signaling on engaging with the PD-1 ligand on T cells and thereby decrease T-cell cytotoxic capacity, resulting in a protumor effect (39). It is conceivable that the addition of an immune checkpoint inhibitor could have improved the prognosis of this patient. The TOPAZ-1 trial recently reported a modest improvement in overall survival in BTC with the addition of immune checkpoint inhibitor durvalumab to standard chemotherapy with cisplatin and gemcitabine in treatment of advanced BTC (40). The trial reported the tumor area positivity (TAP) score defined as the proportion of tumor and/or immune cells with PD-L1 staining at any intensity. In the subgroup analysis of the TOPAZ-1 the TAP score of >1% *vs.* TAP score <1% did not seem to impact survival. However, the TAP score did not account for potential change in PD-1 and PD-L1 expression with therapy. Our findings prompt inquiry into the possibility of reevaluating the sequencing of cytotoxic chemotherapy and immunotherapy in the treatment algorithm of BTCs, as opposed to the current conventional approach of concurrent administration of chemoimmunotherapy.

Our analysis found that patient #3 had the longest time to recurrence. Interestingly, this patient also had the largest increase in CD8:FOXP3 ratio, a marker of the interactions between immune promotive $CD8^+$ TILs and regulatory $FOXP3^+$ TILs. Conversely, patient #2 was noted with the largest decrease in the ratio. An elevated CD8:FOXP3 ratio has been identified as a positive prognostic marker in various tumor types including breast and esophageal cancers (41-44). However, the upregulation of this ratio with chemotherapy is suggestive of the benefits of NACT in a subset of patients that need to be identified in order to tailor individual treatments. Patient #3's tumor was also noted to harbor an FGFR2 rearrangement (45,46). Preclinical analyses suggest that fibroblast growth factor pathways may impact TIME, especially MDSC and tumor immune evasion mechanisms (47,48). This may also have contributed to a more favorable prognosis, further reiterating the need for personalized therapeutic strategies and potential benefit of assessing changes in TIME with various chemotherapeutic, targeted and immunotherapy treatments. It is imperative to underscore, however, that due to the limited size of our patient cohort, a comprehensive exploration of the nuanced impact of these markers remains unattainable within the scope of our study.

Novel therapeutic targets aimed at components of the TIME are presently under investigation. One such approach involves utilizing antibody-drug conjugates (ADCs) composed of anti-CD163 immunoliposomes loaded with doxorubicin, which have been demonstrated to deplete immunosuppressive TAM subsets, leading to heightened recruitment of $CD4^+$ and $CD8^+$ T cells that promote tumor regression in mouse models of melanoma and ovarian cancer (49,50). Adoptive T cell therapy, including the use of CD8-T-LP-BMS-202, is another promising approach (51). This liposomal immune regulator has been conjugated with effector OT-1 $CD8^+$ T cells to boost their anti-tumor activity. In a mouse model, this approach resulted in a 20-fold reduction of polymorphonuclear (PMN)-MDSCs in the TIME, leading to a 2-fold increase in the frequency of cytotoxic $CD8^+$ TILs. These findings suggest that CD8-T-LP-BMS-202 has the potential to enhance the effectiveness of adoptive T cell therapy for cancer (52). These strategies offer new and promising opportunities to overcome some of the limitations of current anti-cancer therapies.

Our study, being a retrospective case series, has several limitations including its small sample size, and the tumor heterogeneity. The small number of patients that were included in this series was a result of limited financial support and a scarcity of cases with adequate pathologic tissue, leading to the exclusion of four patients who had consented. In the presentation of the case series data, we contemplated various statistical tests, such as Pearson's correlation and paired sample *t*-test. However, acknowledging the limited sample size of 5, a collective decision was made to opt for the presentation of descriptive statistics only. This strategic choice aims to mitigate the potential for drawing incorrect conclusions by refraining from statistical analyses that could be compromised by the constraints of the small sample size. Prior studies in BTC have predominantly examined intra-tumoral cell densities without accounting for non-tumoral compartments of TIL infiltration such as sTILs (11). Emerging literature suggests that a combined evaluation of TILs within both tumoral and extra-tumoral compartments can offer significant prognostic insight (53,54). In our analysis, we examined alterations in cell densities of both tTILs and sTILs, providing a more comprehensive understanding of the immune dynamics at play in BTCs.

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Conclusions

Our sample size precludes definitive conclusions, but it underscores the significance of investigating *in-vivo* changes in immune contexture of bile duct cancers in the neoadjuvant period and can therefore be considered hypothesis-generating. Window-of-opportunity trials provide a platform to study mechanisms of disease and test novel immunotherapeutic strategies prior to surgery (55). It is imperative to design additional trials in this area to gain a deeper understanding of the immunomodulatory alterations induced by novel systemic therapies particularly in BTCs where the prognosis remains dismal.

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Footnote

Reporting Checklist: The authors have completed the AME Case Series reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-23-1928/rc

Peer Review File: Available at https://atm.amegroups.com/article/view/10.21037/atm-23-1928/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-23-1928/coif). G.A. is currently working as a Director at Merck & Co., Inc. in the Department of Molecular Pathology, Translational Molecular Biomarkers Kenilworth, New Jersey, United States and also owns Merck & Co., Inc. stocks. M.E.S. reports consulting or advisory role in Eisai and Genentech; research funding from Bristol-Myers Squibb. D.J.C. reports consulting or advisory role in Taiho Oncology; research funding from Bristol-Myers Squibb, Eisai, and Merck (all payments were made to institution). S.G. reports research funding from Boehringer Ingelheim, Bristol-Myers Squibb/Medarex, Celgene, EMD Serono, Janssen, Regeneron and Takeda (all payments were made to institution); patents, royalties, other intellectual property: patent on GM-CSF autoantibodies; patent on Multiplex Immunohistochemistry with Chromogen Staining on a Single Slide (MICSSS); patents related to immune composition of NY-E; patents related to immune composition of NY-ESO-1 and peptides, SO-1 and peptides; provisional patent on ELLA cytokines. S.P. reports that this work was funded by the Cholangiocarcinoma Foundation, and consulting or advisory role in Tesaro. The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are 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. The case series was performed in accordance with the Declaration of Helsinki (as revised in 2013). Ethical approval to conduct this study was granted following review of the study protocol by The Mount Sinai Health System Institutional Review Board (IRB; HS#: 18-01371; GCO#1: 18-2764). All study participants, or their legal guardians, provided informed written consent prior to study enrollment. A copy of the written consent is available for review by the editorial office of this journal.

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