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Signature:

_____ _____

Jessica M. Keilson, MD Date

Thesis Approval

Galectin-9 Expression and its Role in Biliary Tract Cancers

By

Jessica M. Keilson, MD
Master of Science
Clinical Research

_____

Gregory B. Lesinski, PhD, MPH
Advisor

_____

Shishir K. Maithel, MD
Advisor

_____

Amita Manatunga, PhD
Committee Member

_____

Brian S. Robinson, MD, PhD
Committee Member

Accepted:

_____

Kimberly Jacob Arriola, PhD, MPH
Dean of the James T. Laney School of Graduate Studies

_____

Date

Galectin-9 Expression and its Role in Biliary Tract Cancers

By

Jessica M. Keilson
Doctor of Medicine

Advisors: Gregory B. Lesinski, PhD, MPH
Shishir K. Maithel, MD

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ABSTRACT

Galectin-9 Expression and its Role in Biliary Tract Cancers

By

Jessica M. Keilson, MD

Biliary tract cancers (BTCs) are a group of rare and aggressive malignancies often refractory to chemotherapeutic or targeted therapies, carrying a poor prognosis and high mortality. Identifying prognostic biomarkers and effective therapeutic targets for this disease therefore remains a high priority. TIM-3 is a negative regulatory immune checkpoint receptor known to induce immune tolerance and inhibit T-cell antitumor immunity. Its ligand, galectin-9 (gal-9), plays a paradoxical role in tumorigenesis in numerous disease states. However, the role of gal-9 in the setting of BTCs is not completely understood.

Membrane-bound and soluble gal-9 expression were examined in a unique panel of six human BTC cells. Membrane-bound gal-9 is differentially expressed across all BTC cell lines *in vitro*; however, only some cell lines also secrete gal-9. There does not seem to be a correlation between membrane-bound and soluble gal-9 expression levels among cell lines that express both forms. BTC cell viability is unaffected by antibody-mediated gal-9 neutralization.

Baseline plasma was isolated from 74 patients with metastatic BTC (NCI10139) and soluble gal-9 expression was measured. Patients were dichotomized by low and high soluble gal-9 expression, a cut point that was identified using cox proportional hazards modeling for differences in overall survival. Age, sex, and line of treatment were similar between groups (all $p>0.6$). A greater proportion of patients with intrahepatic cholangiocarcinoma (65%, n=26) and extrahepatic cholangiocarcinoma (87%, n=13) had low soluble gal-9 expression, whereas patients with gallbladder cancer were nearly equally distributed between groups ($p=0.05$). No clinicopathologic variables were associated with an increased odds of high soluble gal-9 expression.

On Kaplan-Meier analysis, high soluble gal-9 expression was associated with worse overall survival ($p=0.013$). When controlling for site of disease, high sGal-9 expression remained significantly associated with a higher hazard rate of death compared to low sGal-9 expression (HR 1.782, 95% CI 1.067-2.977, $p=0.027$). Thus, soluble galectin-9 expression may be related to a more aggressive disease state.

Additional work is needed to better inform the mechanistic role of the galectin-9 on disease progression and how best to leverage the galectin-9/TIM-3 pathway as a therapeutic target in the management of biliary tract cancers.

Galectin-9 Expression and its Role in Biliary Tract Cancers

By

Jessica M. Keilson
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Mentors
Gregory B. Lesinski, PhD, MPH
Shishir K. Maithel, MD

Thesis Committee
Brian S. Robinson, MD, PhD – Thesis Reader
Amita Manatunga, PhD – Thesis Advisor

Lesinski Laboratory, Department of Hematology & Medical Oncology, Winship Cancer Institute
Dejah Blake
Batoul Farran, PhD
Emily Greene, MS
Jacklyn Hammons
Isaac Karpovsky
Jayden Kim
Maggie Philips, MS
Amanda Ruggieri, BS
Former:
Cameron Herting, PhD
Michael Ware, PhD

Olson Laboratory, Department of Hematology & Medical Oncology, Winship Cancer Institute
Brian Olson, PhD

Division of Surgical Oncology, Department of Surgery, Emory University
Kenneth Cardona, MD
David Kooby, MD
Shishir M. Maithel, MD
Maria C. Russell, MD
Juan Sarmiento, MD
Charles A. Staley, MD

Surgical Oncology Research Team
Caroline R. Medin, MD
Michael K. Turgeon, MD
Maryam Z. Ahmad, MPH

Department of Pathology, Emory University
Alyssa Krasinkas, MD
Brian Robinson, MD, PhD

Department of Pediatrics, Winship Cancer Institute
Curtis Henry, PhD

Winship Cancer Institute
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Introduction

Biliary tract cancers (BTCs) represent a rare and heterogenous group of cancers that are classified as intrahepatic, perihilar, or distal cholangiocarcinomas, or gallbladder cancer, based on their location within the biliary tree [1]. Incidence rates range from 0.24-1.49 per 100,000 persons each year in the United States, with higher rates reported globally among Asian and Middle Eastern regions [2 3]. Chronic inflammatory states, including primary sclerosing cholangitis, hepatitis, liver fluke infection, and cholelithiasis are the strongest predisposing factors for the development of BTCs. Distinct gene mutations profiles specific to each site of disease also play a role in BTC pathogenesis [4].

BTCs are aggressive cancers most often identified in late stages due to lack of early clinical features and established prognostic markers, carrying an overall poor prognosis and high mortality. Currently, limited effective therapeutic options are available for the management of this disease. Surgery with complete resection remains the only potential for cure; however, even after complete resection, 5-year survival rates are only 10-40% and the majority of patients (70-80%) are diagnosed at advanced stages with unresectable tumors, at which point palliative measures are pursued [5 6]. Further, even among the minority of patients who undergo resection, >53% have disease recurrence within 5 years [7-10]. In the advanced setting, the current mainstay of treatment includes a combination cytotoxic chemotherapy regimen of gemcitabine and cisplatin [11]. Studies are currently underway evaluating the addition of nab-paclitaxel to standard of care gemcitabine and cisplatin, which demonstrated improved progression-free survival and overall survival compared to historical controls in a phase II randomized study [12]. However, there are limited data exploring chemotherapeutic options

beyond the first line setting. A number of clinical trials are currently exploring targeted therapies directed at known genetic mutations among BTCs, including fibroblast growth factor receptor (FGFR), isocitrate dehydrogenase (IDH) 1 and 2, HER2/neu, BRAF, among others; however, these remain in the early phase of investigation. Still, given the heterogenous nature of these malignancies, limited therapeutic options, and overall poor prognosis, identifying novel and effective treatment approaches for BTC, both for resectable and metastatic disease, remains a priority.

Therapies targeting the immune system have shown considerable success in the treatment of advanced solid tumors. These treatment modalities exploit normal immune function by redirecting those efforts toward the identification and elimination of cancer cells. But human cancer cells have developed mechanisms which allow them to dampen anti-cancer immune responses and even escape immunosurveillance. One such mechanism involves increasing expression of immune checkpoint receptors, which safeguard against autoimmunity under normal physiologic conditions. Blocking these pathways attempts to resolve a major challenge of tumor-induced immune suppression, releasing the brake on autoimmune regulation and tipping the balance in favor of anti-tumor response. One such pathway is the galectin-9/TIM-3 immune checkpoint pathway.

T cell immunoglobulin and mucin domain-containing protein 3 (TIM-3) is an immune checkpoint receptor that is expressed on a variety of immune cells, including effector CD4+ and CD8+ T cells and regulatory T cells within the tumor microenvironment. Galectin-9 (gal-9), the canonical ligand to TIM-3, belongs to a family of β-galactoside-binding proteins that interact with glycoconjugates on the cell surface and extracellular matrix. Gal-9 is known to exist in a

membrane-bound form on a number of different cell types, including tumor cells, and in a soluble, or secreted form. Activation of the gal-9/TIM-3 axis has been shown to induce immunosuppression and promote cancer immune escape [13]. Gal-9, independent of its function with TIM-3, has demonstrated mixed roles. In several solid organ cancers, gal-9 plays a favorable role in preventing disease progression; however, secreted gal-9 promotes anti-cancer immune suppression by impairing the activity of important effector immune cells [13].

Targeting the gal-9/TIM-3 complex has seen significant promise in early phase clinical trials in a variety of solid tumors [14-17]. However, our understanding of the role of gal-9 in BTC tumorigenesis is limited to only two *in vitro* studies employing extrinsic gal-9 in the context of only two BTC cell lines, thus failing to capture the true heterogeneity of this disease [18 19]. The aim of our study was to enhance our understanding of the role of gal-9 on BTC tumorigenesis. More specifically, we sought to: 1) define the expression patterns of gal-9 in human BTC cell lines; 2) characterize the effect of gal-9 neutralization on human BTC cell viability; and 3) evaluate the association between soluble gal-9 expression and overall survival in patients with advanced BTCs.

Aims 1 & 2

To define the expression patterns of galectin-9 in human biliary tract cancer cell lines.

We hypothesize that galectin-9 is widely expressed in human biliary tract cancer cell lines in vitro.

To characterize the effect of galectin-9 neutralization on human biliary tract cancer cell viability.

We hypothesize that galectin-9 neutralization will affect human biliary tract cancer cell viability in vitro.

Methods

Cell culture

We utilized a unique panel of six human BTC cell lines representative of each site of disease (Figure 1A). Human BTC cell lines HuCCT-1, HuH28, and WITT were a gift from Dr. Tushar Patel (Mayo Clinic, Jacksonville, FL); human SNU-478 cell line was purchased from the Korean Cell Line Bank (Seoul, Korea); human SNU1196 cell line was purchased from American Type Culture Collection (ATCC; Manassas, VA), and human MzChA1 was a gift from Dr. Shannon Glaser (Texas A&M Health Sciences Center, Bryan, TX). A normal human biliary tract cell line, MMNK1, was also included and was purchased from the Japanese Collection of Research Bioresources Cell Bank. SNU478, SNU1196, HuCCT-1, HuH28, and MMNK1 cell lines were cultured in RPMI-1640 media (Gibco, Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS), 10 mM L-glutamine, and antibiotic-antimycotic (ThermoFisher Scientific, Waltham, MA, USA). WITT and MzChA1 cells were cultured in DMEM-media (Gibco) containing 10% FBS, 10 mM L-glutamine, and antibiotic-antimycotic.

Jurkat cells are an immortalized line of human T lymphocytes used as a negative control for gal-9 expression. Cells were purchased from ATCC and cultured in RPMI-1640 media (Gibco) containing 10% FBS, 10 mM Hepes buffer (Gibco), and antibiotic-antimycotic (ThermoFisher Scientific). THP-1 cells are a human monocyte line used as a positive control for gal-9 expression. Cells were purchased from &&& and cultured in RPMI-1640 media (Gibco) containing 10% FBS, 10 mM L-glutamine, and antibiotic-antimycotic (ThermoFisher Scientific).

All cell lines were cultured at 37°C with 5% CO_2.

Immunoblot

Immunoblots were created to measure membrane-bound gal-9 expression (Figure 1B-1). Cell lines were cultured at a concentration of $1x10^6$ cells per plate in their respective growth media at 37°C with 5% CO_2. Cell lysates were prepared using RIPA buffer, 1/100 phosphatase inhibitor (catalog no. 78420; ThermoFisher Scientific), and 1/100 protease inhibitor (catalog no. 78430; ThermoFisher Scientific). Pierce BCA Protein Assay Kit (catalog no. 23225; ThermoFisher Scientific) was used to determine total protein concentrations. Immunoblots were prepared as described (REF) using antibodies for gal-9 (ab69630; Abcam) and β-actin (4967S; Cell Signaling Technology, Danvers, MA, USA). Following incubation with horseradish peroxidase-linked secondary antibody (7074S; Cell Signaling Technology), immune complexes were detected via SuperSignal® West Pico Chemiluminescent Substrate (catalog no. 34579; ThermoFisher Scientific). β-actin is a protein that is ubiquitously expressed in all cells and was used as a loading control to ensure that the same amount of total protein was included for each cell line.

Densitometry

Using Fiji, an open access image process software, band densities for gal-9 and β-actin were measured for each cell line. The density of each band corresponds to the protein concentration for each cell line. Band densities are reported as gal-9 band density relative to the β-actin loading control for each respective cell line.

Enzyme-linked immunosorbent assay (ELISA)

An enzyme-linked immunosorbent assay was performed to measure soluble gal-9 expression (Figure 1B-1). Cell lines were cultured at a concentration of $1x10^6$ cells per plate in their respective growth media at 37°C in 5% CO_2. Supernatants were then collected for each cell

line. Gal-9 concentration was quantified using ELISA kit (R&D Systems, Minneapolis, MN, USA) according to manufacturer's protocol. Supernatants for each cell line were added to plates pre-treated with gal-9 capture antibody. Detection antibody was then added, and a streptavidin-horseradish peroxidase solution was then added. Assays included protein standards at known concentrations. Absorbance was measured at 450nm.

A standard curve was created using protein standards at known concentrations. The absorbance of each cell line was then to fit the standard curve to determine gal-9 protein concentration for each cell line.

Cell viability assay

Cells were grown in a 96 well plate at 5,000 cells per well and incubated overnight at 37°C with 5% CO_2. Cells were subsequently treated with 10µg/mL gal-9 neutralizing antibody (clone MOPC-21; BioLegend, San Diego, CA, USA) or left untreated for 48 or 72 hours. Cell viability was then measured using a commercially available MTS colorimetric assay kit (ab197010, Abcam) (Figure 1B-2). At treatment endpoint, cells were incubated with 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide for 3 hours and absorbance was measured at 595nm according to manufacturer's protocol.

Statistical analysis

Data from cell lines were compared for overall difference across groups via ANOVA. Pairwise group differences will be further tested by using Mann-Whitney non-parametric test when the overall difference is significant at a level of 0.05. All analyses have been designed with our collaborating biostatisticians in the Winship Biostatistics and Bioinformatics Shared Resource. Statistical analyses were conducted using SPSS 26.0 software® (IBM Inc., Armonk, NY).

Results

Aim 1:

Our panel of human BTC cell lines included: intrahepatic cholangiocarcinoma (HuCCT-1 and HuH28), perihilar cholangiocarcinoma (SNU1196), distal cholangiocarcinoma (SNU478 and WITT), and gallbladder cancer (MzChA1) (Figure 1A). Total membrane-bound and soluble gal-9 expression were examined via immunoblot and enzyme-linked immunosorbent assay, respectively (Figure 1B-1).

Qualitatively, based on the presence of a protein band for each cell line, membrane-bound gal-9 is expressed across all BTC lines *in vitro* (upper blot, Figure 2A). β-actin was used as a loading control (lower blot, Figure 2A). Immunoblots for intrahepatic (HuCCT-1 and HuH28) and extrahepatic (SNU478, distal; and SNU1196, perihilar) cholangiocarcinoma cell lines contain more than one band, suggesting that these cell lines express more than one isoform of gal-9. Normal biliary tract cells (MMNK-1) also express membrane-bound gal-9.

Membrane-bound gal-9 expression is reported quantitatively in Figure 2B. Band density for gal-9 expression was compared relative to the β-actin loading control to create a standardized measure of comparison across groups. Data is reported as relative units of gal-9/β-actin. HuCCT-1 (0.8273 relative units gal-9/β-actin) demonstrated the highest expression of membrane-bound gal-9, significantly higher compared to SNU1196 (0.3625), WITT (0.3258), and MzChA1 (0.3553) cell lines (all $p=0.03$). Normal biliary tract cells (MMNK-1) express membrane-bound gal-9 (0.9095) to a degree comparable to the highest expressing BTC cell line ($p=0.63$).

Soluble gal-9 was measured by ELISA in all BTC cell lines as well as a normal biliary tract cell line (MMNK-1) (Figure 2C). THP-1 cells and Jurkat cells were included as control for gal-9.

Soluble gal-9 is expressed in some BTC cell lines: intrahepatic cholangiocarcinoma (HuCCT-1 123.1 pg/dL (95% CI 18.7-205.4) and HuH28 151.3 pg/dL (95% CI 66.1-237.1)); distal cholangiocarcinoma (WITT 131.8 pg/dL (95% CI 37.9-215.1)); and gallbladder cancer (MzChA1 193.6 pg/dL (95% CI 113.1-284.6)) (Figure 2C). However, one distal cholangiocarcinoma cell line (SNU478) and perihilar cholangiocarcinoma cell line (SNU1196) do not express soluble gal-9. Among the cell lines that express both membrane-bound and soluble gal-9, there is no correlation between membrane-bound and soluble gal-9 levels among cells lines that express both forms. Normal biliary tract cells (MMNK-1) do not express soluble gal-9.

Aim 2:

Cell metabolic activity as a surrogate for cell viability was assessed in all BTC cell lines following antibody-mediated gal-9 neutralization for 48 and 72 hours. Percent viability was determined by comparing the number of viable cells following treatment relative to untreated cells. After 48 hours of treatment, all cell lines, including normal bile duct, demonstrated nearly 100% cell viability (median range 93.4-105.3%) (Figure 3). No difference in cell viability across cell lines was noted after 72 hours of treatment with gal-9 neutralization compared to 48 hours of treatment (all $p>0.1$).

Discussion

Our findings suggest that biliary tract cancer cells differentially express membrane-bound and soluble gal-9 in vitro. Gal-9 belongs to a family of galectin proteins that exist intracellularly and can bind β-galactoside carbohydrate chains on glycoconjugates within the cell membrane as membrane-bound proteins. Gal-9 expression has been documented in a variety of cancer cell types and has been shown to play opposing roles. In breast cancer cells, cytoplasmic Gal-9 inhibits cancer cell aggregation and invasion [20]. Studies in liver and gastric cancers suggest that Gal-9 induces apoptosis and inhibits tumor cell growth, thereby preventing disease progression [21 22]. These findings conflict with early studies in acute myeloid leukemia, which showed that the gal-9/TIM-3 pathway promoted tumor cell immune evasion [13].

Within our panel of human BTC cell lines, all cell lines expressed membrane-bound gal-9. Intrahepatic (HuH28), perihilar (SNU1196), and distal (WITT, SNU478) cholangiocarcinoma cell lines, and our gallbladder cancer (MzChA1) cell line express similar levels of gal-9. HuCCT-1, an intrahepatic cholangiocarcinoma cell line, expressed significantly higher levels of membrane-bound gal-9 compared to our second intrahepatic cholangiocarcinoma cell line and cells lines from other sites of disease. Interestingly, HuCCT-1 cells are derived from metastatic lesions of intrahepatic cholangiocarcinoma origin. The remainder of our cells lines within this panel originate from the primary tumor site, suggesting that this significantly higher level of expression within our metastatic cell line may be reflect changes to the tumor-immune landscape that occur between localized and advanced disease settings.

BTC cell lines not only demonstrate varied levels of total membrane-bound gal-9 expression, they also differentially express gal-9 isoforms. Gal-9 has three primary isoforms,

characterized by a long (L), medium (M), or short (S) linker peptide (gal-9L, gal-9M, and gal-9S). Expression patterns of these unique isoforms is not uniform across cell types. For example, T cells are known to express high levels of Gal-9M and Gal-9L but not Gal-9S isoforms [23]. Looking specifically at cancer cells, certain cancer types such as non-small cell lung cancer cells express only one isoform of gal-9, whereas others such as acute myeloid leukemia, colon and breast cancer cells express all three isoforms [24]. The underlying function of gal-9 isoforms has not been completely elucidated; however, studies have shown that these distinct isoforms play distinctive roles in cell adhesion and proliferation [25 26]. Our findings demonstrate that intrahepatic (HuCCT-1 and HuH28) and perihilar (SNU1196), and cholangiocarcinoma cell lines, in addition to normal biliary tract cells, express more than one gal-9 isoform. Interestingly, only one of the two distal cholangiocarcinoma cell lines (SNU478) expresses more than one isoform. Given that all cell lines express membrane-bound gal-9, differences in gal-9 isoform expression may be related to variations in the underlying genetic landscape of these disease sites. Variations in isoform expression even within site-specific disease further reflects the heterogeneity of this disease.

While gal-9 is known to exist in a soluble form, the mechanism by which gal-9 is secreted by cells is poorly understood. Gal-9, like other galectins, lacks a secretory domain and thus cannot be secreted independently. Several non-classical secretory pathways for galectins have been suggested, including direct transport through pore proteins in the cell membrane and membrane translocation via extracellular vesicles and subsequent cleavage from the cell membrane [27 28]. Interestingly, gal-9, in the context of viral infection, is secreted into the extracellular environment as a damage-associated molecular pattern induced by a cellular stress response [29]. This same

mechanism of gal-9 secretion may be elicited as a stress response to the cellular transformation into cancer. We demonstrated that some cell lines secrete gal-9; however, secretion of gal-9 does not appear to be consistent within disease-sites and we did not identify any correlation between degree of membrane-bound and soluble expression within cell lines that express both forms. Additionally, as gal-9 function is dependent on cellular localization, secretion is tightly regulated and is likely dependent on immune interactions in the setting of the tumor microenvironment. Further work is needed to evaluate gal-9 expression in the context of its receptor, TIM-3, and an intact immune microenvironment and better understand the tumor-immune crosstalk and what role gal-9 plays in this context.

Lastly, to investigate the cell intrinsic role of gal-9, BTC cell lines were treated with a gal-9 neutralizing antibody and assessed for cellular metabolic activity as a surrogate for cell viability. Cell viability was unaffected by gal-9 neutralization after 48 or 72 hours of treatment. Based on our understanding of gal-9 as a potent immunomodulator, blocking gal-9 should not directly affect cancer cell viability in isolation from immune cells. Interpretation of this data, however, is limited. It is unclear whether gal-9 neutralization in this context blocks both membrane-bound and soluble forms of gal-9 or if all isoforms are equally targeted. Additionally, antibody-mediated gal-9 neutralization would not neutralize intracellular gal-9, which has distinct immunomodulatory functions [30]. Additional work is needed to better elucidate whether both membrane-bound and soluble gal-9 were indeed neutralized and whether all three isoforms are equally targeted by this antibody.

Conclusions

Human biliary tract cancer cells differentially express membrane-bound gal-9. Not all cell lines secrete gal-9 and there does not seem to be any correlation between membrane-bound and soluble gal-9 expression among cell lines that express gal-9 in both forms. Further, antibody-mediated gal-9 neutralization does not affect biliary tract cancer cell viability *in vitro*.

Aim 3

To evaluate the association between soluble galectin-9 expression and overall survival in patients with advanced biliary tract cancers.

We hypothesize that galectin-9 expression is associated with more aggressive disease states and worse outcomes in biliary tract cancers.

Methods

Study population

Peripheral blood was collected from patients enrolled in a multicenter, randomized phase 2 clinical trial through the National Cancer Institute's Cancer Therapy Evaluation Program (CTEP) at multiple Experimental Therapeutics Clinical Trials Network (ETCTN) sites in the United States from February 2018 to October 2018. Eligibility criteria included: age greater than or equal to 18 years, ECOG performance status score of 0 or 1, pathologically confirmed biliary tract cancer (intrahepatic cholangiocarcinoma, extrahepatic cholangiocarcinoma, or gallbladder cancer), receipt of 1 or 2 prior lines of systemic therapy in the unresectable or metastatic setting, and measurable disease according to RECIST 1.1 criteria [31].

Demographic, clinicopathologic, and long-term outcomes data were collected for each patient. At study completion, patient allocation was documented.

Baseline Plasma Patient Samples

Peripheral blood specimens from enrolled patients were collected prior to receipt of study drug (Figure 1B-3). Whole blood specimens were centrifuged at 2000rpm for 10 minutes. Plasma was separated into a separate vial for each patient and stored at -4°C.

Enzyme-linked immunosorbent assay (ELISA)

Soluble gal-9 was measured by ELISA as described above.

Statistical analysis

An exploratory analysis was performed to identify an appropriate for soluble gal-9 levels at which there is a difference in overall survival using cox proportional hazards regression. Based on this analysis, patients were dichotomized into low or soluble gal-9, where concentrations in

the lower and middle tertile for soluble gal-9 were recoded as low soluble gal-9 and concentrations in the upper tertile for soluble gal-9 were recorded as high soluble gal-9.

Descriptive statistics were then performed for the entire study cohort dichotomized by low and high soluble gal-9 as previously described. For univariate analyses, a chi-squared test was used for categorical variables and an unpaired t-test or Mann-Whitney test was used for continuous variables, where indicated. Soluble gal-9 expression was tested for Gaussian distribution using the D'Agostino-Pearson normality test. A Kruskal-Wallis test followed by selected comparison by Dunn's multiple comparison tests with multiple comparison correction was used to compare soluble gal-9 expression by disease site.

Binary logistic regression analysis was performed to evaluate the association between clinicopathologic variables and the odds of having high soluble gal-9 expression. Kaplan-Meier models were used to assess the association between low or high soluble gal-9 expression and overall survival. Log-rank test was used to test differences in overall survival between cohorts. Univariate cox proportional hazards analyses were performed to evaluate the association between study covariates and overall survival. Covariates that demonstrated a significant association were included in multivariable model.

A subset analysis was performed evaluating patients for distribution across low and high soluble gal-9 expression as well as overall survival by study allocation. Statistical significance was predefined as $p<0.05$, using two-tailed tests for all analyses. Statistical analysis was conducted using SPSS 26.0 software ® (IBM Inc., Armonk, NY).

Results

Baseline peripheral blood was collected from 74 patients and plasma was isolated for measurement of soluble-gal-9 expression by enzyme-linked immunosorbent assay. A total of 74 patients were included in the final analysis. Median age was 63 years (IQR 55-70) and 38% of patients were male (Table 1). The majority of patients had intrahepatic cholangiocarcinoma (n=40, 54%). Twenty percent had extrahepatic cholangiocarcinoma (n=15) and 19% had gallbladder cancer (n=26). Most patients received one prior line of systemic therapy (60%). Seventy eight percent of patients had disease progression as study endpoint (n=58) with a median progression-free survival of 1.9 months (IQR1.3-3.8). Overall survival was 5.3 months (IQR 3.1-11.8). Median soluble gal-9 expression was significantly higher among patients with gallbladder cancer compared to intrahepatic and extrahepatic cholangiocarcinoma (977.9 pg/dL (IQR 631.0-1280.0) vs. 602.6 pg/dL (IQR 402.9-1017.0) and 496.8 pg/dL (IQR 286.8-845.9), respectively; p=0.02) (Figure 4A).

An exploratory analysis was performed using cox proportional hazards modeling to identify a cut point for soluble gal-9 at which there is a difference in overall survival (Table 2). Soluble gal-9 was evaluated as a continuous variable, dichotomized at the 50^{th} percentile, and as a three-level variable by tertile. The upper tertile of soluble gal-9 expression was associated with a higher hazard rate of death compared to the lower tertile on univariate analysis (HR 1.846, 95% CI 1.010-3.373, p=0.046). Based on this finding, a dichotomous variable was created: expression levels in the lower and middle tertiles were recorded as low soluble gal-9 and expression levels in the upper tertile were recorded as high soluble gal-9. With this new two-level variable, high

soluble gal-9 was associated with a higher hazard rate of death compared to low soluble gal-9 (HR 1.745, 95% CI 1.047-2.908, p=0.033).

Patients were dichotomized by low (n=48, 65%) and high (n=26, 35%) soluble gal-9 (Table 3). Age, sex, and line of treatment were similar between groups (all p>0.04). A greater proportion of patients with intrahepatic cholangiocarcinoma (65%, n=26) and extrahepatic cholangiocarcinoma (87%, n=13) had low soluble gal-9 expression whereas patients with gallbladder cancer were nearly equally distributed between groups (p=0.05). Disease progression (82% for low soluble gal-9 and 71% for high soluble gal-9) and progression-free survival (2.1 months for low soluble gal-9 and 1.8 months for high soluble gal-9) was equivalent between groups (p>0.3). Patients with high soluble gal-9 had worse overall survival compared to patients with low soluble gal-9 (5.8 months vs. 6.9 months, respectively; p=0.047). This difference in overall survival was expected based on how patients were dichotomized.

Binary logistic regression was performed to identify an association between clinicopathologic variables and high soluble gal-9 expression (Table 4). Age (HR 0.999, 95% CI 0.951-1.051), male sex (HR 1.337, 95% CI 0.503-3.554), site of disease (relative to intrahepatic cholangiocarcinoma; extrahepatic cholangiocarcinoma, HR 0.286, 95% CI 0.056-1.450; gallbladder cancer, HR 2.063, 95% CI 0.680-6.264), line of treatment (relative to second line treatment; third line, HR 1.429, 95% CI 0.542-3.759), and disease progression (HR 0.450, 95% CI 0.146-1.389) were not significantly associated with a higher odds of high soluble gal-9 expression (all p>0.1).

On Kaplan-Meier analysis for overall survival, high soluble gal-9 expression was associated with worse overall survival compared to low soluble gal-9 expression (5.8 months vs. 8.8 months;

p=0.031) (Figure 4). On univariate cox proportional hazards modeling for overall survival, extrahepatic disease was associated with a higher hazard rate of disease compared to intrahepatic disease (HR 2.117, 95% CI 1.132-3.959, p=0.019) (Table5). The hazard rate of death among patients with high soluble gal-9 expression was 1.745 times that of patients with low soluble gal-9 expression (95% CI 1.047-2.908, p=0.033). On multivariable modeling, when controlling for site of disease, high soluble gal-9 expression remained significantly associated with a higher hazards rate of death compared to low soluble gal-9 expression (HR 1.782, 95% CI 1.067-2.977, p=0.0279).

As a result of patient randomization, the distribution of disease site was equivalent between treatment allocation groups (intrahepatic cholangiocarcinoma, 50% and 58%; extrahepatic cholangiocarcinoma, 19% and 21%; gallbladder cancer, 31% and 21%; p=0.64). Similarly, patients were equally distributed between treatment allocation arms with respect to soluble gal-9 (sGal-9) expression (low sGal-9, 69% in arm A, 61% in arm B; high sGal-9, 31% in arm A, 39% in arm B; p=0.58). As expected, based on findings reported by Yarchoan and colleagues, there was no difference in overall survival between treatment arms (5.2 months vs. 5.8 months, p=0.66) (Suppl. Table 1).

Discussion

Among patients with advanced BTCs, high soluble gal-9 is associated with worse overall survival, even when controlling for site of disease. BTCs are highly aggressive malignancies with poor survival. Disease diagnosis is often delayed due to lack of early clinical features and diagnostic markers. Therapeutic options remain limited, and there are currently no establish prognostic markers to help guide disease management.

In pancreatic and ampullary cancer, increased cytoplasmic gal-9 expression was associated with improved cancer-specific survival and prognostic of relevant clinicopathologic characteristics [32]. Similarly, low galectin-9 expression correlates with more aggressive clinicopathologic characteristics and worse cancer-specific survival in urothelial carcinoma of the bladder [16]. In patients with gastric, breast, and hepatocellular carcinomas, gal-9 expression correlates with tumor histopathologic grade and invasiveness, as well as metastatic potential and increased expression was associated with improved survival [33-35]. We report that high gal-9 expression is associated with worse overall survival in patients with metastatic BTCs. One major distinction from our findings is that these studies primarily focus on gal-9 expression specific to the tumor membrane and tumor-infiltrating lymphocytes, whereas we evaluated soluble levels of gal-9. These key differences may suggest distinct functions of cytoplasmic, membrane-bound, and soluble forms of gal-9. Cleavage of gal-9 from the membrane surface or induced secretion may be induced in more aggressive or advanced disease states. Additional work is currently underway evaluating tumor membrane-bound gal-9 expression via immunohistochemistry in tumor specimens from patients who underwent resection for BTCs. These data will serve as

important correlates to our current work examining soluble gal-9 expression in patients with metastatic disease and may shed light on the underlying physiologic triggers for gal-9 secretion.

Interestingly, among patients with hepatitis B-associated hepatocellular carcinoma, high TIM-3 expression is associated with decreased survival [36]. Further, in a meta-analysis evaluating TIM-3 expression patterns in solid tumors, Zhang et al. reported that high TIM-3 expression is associated with advanced tumor stage and worse overall survival [37]. We did not evaluate TIM-3 expression in this context; however, looking at receptor expression in the context of metastatic BTCs could provide key information on the tumor-immune crosstalk and how the gal-9/TIM-3 pathway is regulated.

Soluble gal-9 expression did not correlate with available clinicopathologic data. While median soluble gal-9 expression was significantly higher among patients with gallbladder cancer, this is likely owing to a limited sample size, where patients with gallbladder cancer only accounted for 26% of the overall population. Clinicopathologic data, including tumor genetic markers and tumor differentiation, were not available; however, this information would provide a critical correlate to better understand the relevance of sGal-9 expression in disease prognosis and the underlying role of sGal-9 expression in tumorigenesis and disease progression.

Management of BTCs remains a significant challenge due to delayed diagnosis and our inability to screen for disease and predict both long-term outcomes and response to therapies. This is the first study to examine soluble gal-9 in patients with BTCs. The methods used to stratify patients by soluble gal-9 expression was strictly exploratory, as soluble gal-9 has not been studied previously in this disease state. In clinical practice, expression levels denoted as ‘low’ or ‘high’ are convenient, more easily interpretable, and translatable. These data provide initial evidence

that soluble gal-9 expression may confer worse survival among patients with metastatic BTC and could serve as an important prognostic marker of disease.

Conclusions

Among patients with metastatic biliary tract cancers, higher plasma levels of soluble gal-9 are association with worse overall survival, suggesting gal-9 expression may be related to a more aggressive disease state. Additional work is currently underway evaluating membrane-bound galectin-9 expression among patients who have undergone resection and its association with clinicopathologic characteristics and survival outcomes as well as employing *in vivo* mouse models to evaluate tumor growth and disease progression following Gal-9/TIM-3 blockade. If successful, this pathway has the potential to transition into the clinical trial setting as a therapeutic target for the management of biliary tract cancers.

Strengths and Limitations: Aims 1 & 2

Our study had a several limitations. Relevant to **aim 1**, our assays evaluating gal-9 expression, devoid of immune cells and thus independent of its receptor TIM3. We cannot speak to whether gal-9 expression is increased or decreased in biliary tract cancer cell lines in the presence of its receptor on immune cells or whether the presence of an intact tumor microenvironment regulates gal-9 expression, both membrane-bound and soluble forms. In evaluating soluble gal-9 expression, it is unclear from our current assay whether measured soluble gal-9 is in an activated form able to exert immunomodulatory effects.

Specific to **aim 2**, cell viability in the setting of gal-9 neutralization was similarly performed in an environment devoid of immune cells, therefore it is not known whether the presence of immune cells could affect cancer cell viability with gal-9 blockade. Further, it is unclear whether the specific gal-9 neutralizing antibody used targets membrane-bound gal-9, soluble gal-9, or both, and what effect targeting one form or the other has on cell viability. Additionally, we do not know whether this antibody is able to discriminate between the three known gal-9 isoforms and what affect targeting a specific isoform has given their distinct functions.

Additional work is currently underway co-culturing our BTC cell lines with immune cells to evaluate gal-9 expression and its effect on cell viability in an environment that more closely mimics the tumor-immune microenvironment.

Strengths and Limitations: Aim 3

For **aim 3**, we had limited demographic and clinicopathologic data for our patient population. This restricted our ability to perform more robust modeling to account for clinicopathologic variables relevant to this disease and survival outcomes or perform competing

risk analyses. Most patients included in the analysis were missing relevant tumor genetic markers and pathologic differentiation, which influence disease aggressiveness and overall prognosis. However, study enrollment for clinical trials requires strict inclusion/exclusion criteria, which creates allows us to assume a nearly homogenous population. This study population included patients with confirmed advanced biliary tract cancers that did not undergo resection, suggesting similar stages of disease at trial enrollment. Further, this study included only patients who had optimal ECOG status, suggesting similar co-morbid conditions and overall functional status, which are important factors that influence survival.

Survival analyses relative to baseline soluble gal-9 expression were conducted for all patients irrespective of their treatment allocation, confounding the interpretation of these associations. To mitigate this, subset analyses were performed to evaluate the distribution of patients into low and high soluble gal-9 expression by treatment allocation. Patients in each allocation group were nearly equally distributed across soluble gal-9 expression and the association with worse overall survival among patients with high soluble gal-9 expression persisted, irrespective of treatment allocation. Further, the study concluded that there was no difference in overall survival between treatment groups [38].

Finally, this study was the first to evaluate gal-9 expression in human clinical samples of biliary tract cancers.

Conclusions

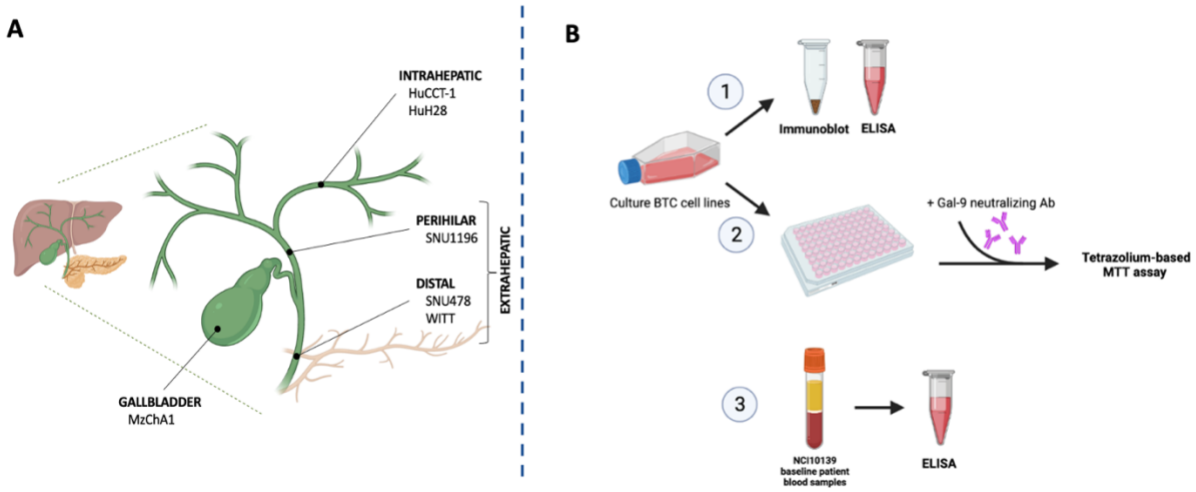
Human biliary tract cancer cells differentially express membrane-bound galectin-9. Some, but not all, cell lines secrete galectin-9; however, there does not seem to be any correlation between membrane-bound and soluble galectin-9 expression among cell lines that express galectin-9 in both forms. Further, galectin-9 neutralization does not affect biliary tract cancer cell viability *in vitro*.

Among patients with metastatic biliary tract cancers, higher plasma levels of soluble galectin-9 are association with worse overall survival, suggesting galectin-9 expression may be related to a more aggressive disease state.

Additional work is needed to better inform the mechanistic role of the gal-9/TIM-3 axis on disease progression and how best to leverage this pathway as a therapeutic target in the management of biliary tract cancers.

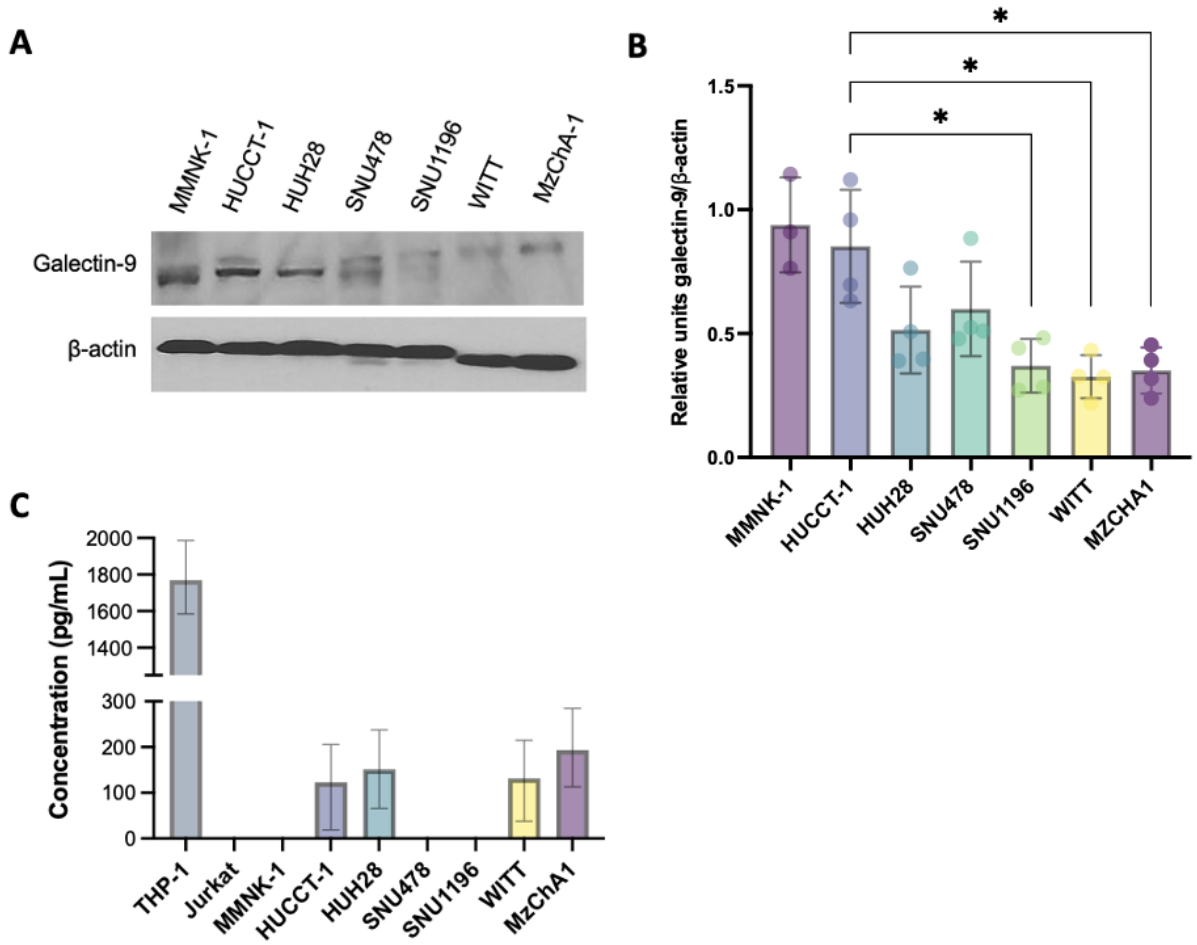
Figures

Figure 1. *Human biliary tract cancer cell line site of origin diagram and methods schema*



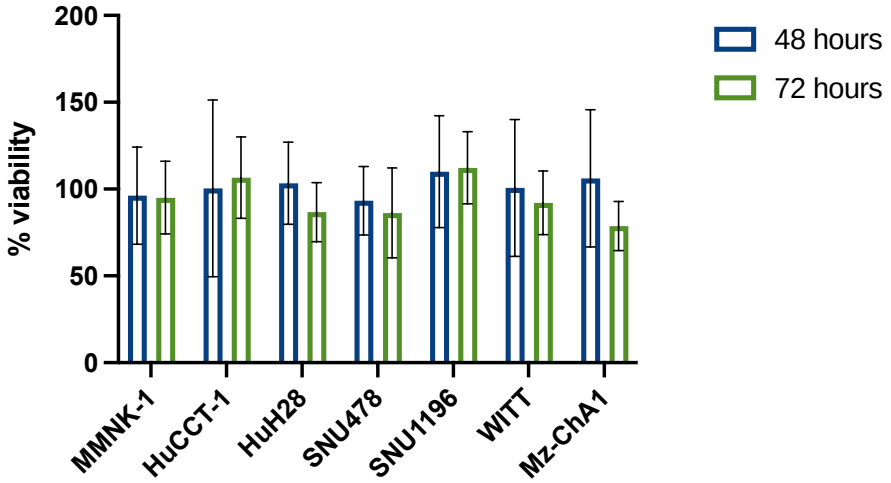
(A) Human biliary tract cancer (BTC) cell line site of origin diagram. A total of 6 human BTC cell lines were used for *in vitro* studies (HuCCT-1 and HuH28, intrahepatic cholangiocarcinoma; SNU1196, perihilar cholangiocarcinoma; SNU478 and WITT, distal cholangiocarcinoma; MzChA1, gallbladder cancer). (B) Methods schema. 1. Human BTC cell lines were cultured and examined for membrane-bound galectin-9 (gal-9) via immunoblot and soluble gal-9 via enzyme-linked immunoassay (ELISA). 2. Human BTC cell lines were treated with gal-9 neutralizing antibody and evaluated for cell metabolic activity as a surrogate for cell viability via a tetrazolium-based MTT assay. 3. Baseline peripheral blood samples from patients with metastatic BTCs (NCI10139) were collected and plasma was isolated to measure soluble gal-9 expression via ELISA. Created with BioRender.com.

Figure 2. Human biliary tract cancer membrane-bound and soluble gal-9 expression in vitro



(A) Membrane-bound gal-9 expression by immunoblot analysis in human BTC cell lines. MMNK-1 cells are normal biliary tract cells. β-Actin is included as a loading control. Data shown are representative of n=3 biological replicates. (B) Quantitative representation of membrane-bound gal-9 expression by densitometry. Expression reported as gal-9 band density relative to β-actin band density on immunoblot. Bars represent mean expression with error bars for 95% confidence interval. Statistical significance was predefined as $p<0.05$, using two-tailed tests for all analyses. Human BTC cell lines demonstrate differential expression levels of membrane-bound gal-9. (C) Soluble gal-9 expression measured by enzyme-linked immunosorbent assay (ELISA) in human BTC lines after 48 hours of culture. Positive (THP-1) and negative (Jurkat) controls included. Error bars indicate standard deviation from triplicate experiments. Some (HuCCT-1 and HuH28, intrahepatic cholangiocarcinoma; WITT, distal cholangiocarcinoma; MzChA1, gallbladder cancer) but not all human biliary tract cancer cell lines express soluble gal-9.

Figure 3. *Human biliary tract cancer cell viability following antibody-mediated gal-9 neutralization*



Tetrazolium-based MTS assay for cell viability following antibody-mediated gal-9 neutralization (clone 9M1-3, BioLegend) for 48 and 72 hours. Error bars indicate standard deviation from triplicate experiments. Percent viability reported as the number of viable cells following treatment with the gal-9 neutralizing antibody relative to an untreated control. Gal-9 neutralization does not alter cell viability.

Figure 4. Site-specific soluble gal-9 expression and Kaplan-Meier analysis for overall survival

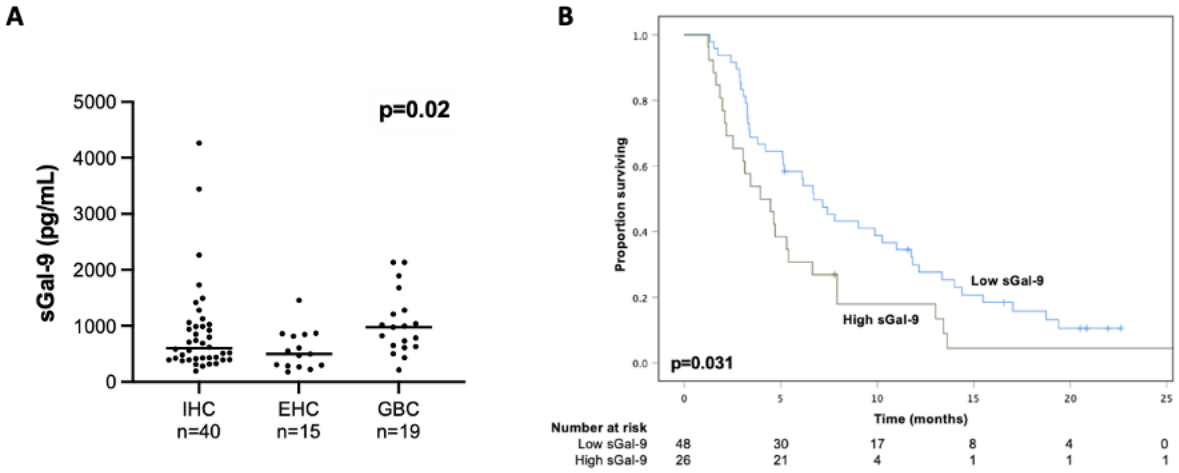


Figure 4. (A) Soluble gal-9 (sGal-9) concentration for each patient characterized by disease site (intrahepatic cholangiocarcinoma (IHC), n=40; extrahepatic cholangiocarcinoma (EHC), n=15; gallbladder cancer (GBC), n=19). Scatter plot by disease site for each patient included in the analysis with a horizontal bar representative of median sGal-9 concentration in pg/mL. sGal-9 is not significantly different across disease sites (IHC median 602.6 (IQR 402.9-1017.5), EHC median 496.8 (IQR 286.8-845.89), GBC median 977.9 (631.0-1279.9); p=0.14. (B) Kaplan-Meier analysis for the association between sGal-9 expression and overall survival. High baseline plasma levels of sGal-9 were associated with worse overall survival among patients with advanced BTCs (p=0.031).

Tables

Table 1. Demographic and clinicopathologic factors for all patients

Variable	All patients (n=74)
Age (years), median (IQR)	63 (55-70)
Male, n (%)	28 (38)
Site of disease, n (%)	
Intrahepatic cholangiocarcinoma	40 (54)
Extrahepatic cholangiocarcinoma	15 (20)
Gallbladder cancer	19 (26)
Line of treatment, n (%)	
Second line	44 (60)
Third line	30 (40)
Disease progression, n (%)	58 (78)
Progression-free survival (months), median (IQR)	1.9 (1.3-3.8)
Overall survival (months), median (IQR)	5.3 (3.1-11.8)

Number, n; interquartile range, IQR

Table 2. Exploratory analysis using cox proportional hazards modeling to identify an optimal cut-point for soluble galectin-9 (sGal-9) expression and a difference in overall survival

Variable	OR (95% CI)	p-value
sGal-9 (continuous)	1.000 (1.000-1.001)	0.076
50th percentile sGal-9		
Below	Reference	Reference
Above	1.523 (0.929-2.497)	0.095
Tertile sGal-9		
Lower	Reference	Reference
Middle	1.119 (0.604-2.073)	0.722
Upper	1.846 (1.010-3.373)	0.046
sGal-9 (by tertile)		
Low sGal-9 (lower/middle tertiles)	Reference	Reference
High sGal-9 (upper tertile)	1.745 (1.047-2.908)	0.033

Soluble galectin-9, sGal-9; odds ratio, OR; confidence interval, CI

Table 3. Demographic and clinicopathologic factors for all patients, stratified by low soluble galectin-9 (sGal-9) and high sGal-9

Variable	All patients (n=74)	Low sGal-9 (n=48)	High sGal-9 (n=26)	p-value
Age (years), median (IQR)	63 (55-70)	63 (56-70)	62 (55-71)	0.98
Sex, n (%)				0.74
Female	46 (62)	31 (67)	15 (33)	
Male	28 (38)	17 (61)	11 (39)	0.47
Site of disease, n (%)				0.05
Intrahepatic cholangiocarcinoma	40 (54)	26 (65)	14 (35)	
Extrahepatic cholangiocarcinoma	15 (20)	13 (87)	2 (13)	
Gallbladder cancer	19 (26)	9 (47)	10 (53)	
Line of treatment, n (%)				0.63
Second line	44 (60)	30 (68)	14 (32)	
Third line	30 (40)	18 (60)	12 (40)	
Disease progression, n (%)	58 (78)	41 (82)	17 (71)	0.43
Progression-free survival (months), median (IQR)	1.9 (1.3-3.8)	2.1 (1.4-3.9)	1.8 (0.9-2.4)	0.32
Overall survival (months), median (IQR)	5.3 (3.1-11.8)	6.9 (3.3-13.1)	5.8 (2.1-7.8)	0.047

Soluble galectin-9, sGal-9; number, n; interquartile range, IQR

Table 4. Univariate binary logistic regression for the association between clinicopathologic variables and high soluble galectin-9 (sGal-9) expression

Variable	OR (95% CI)	p-value
Age	0.999 (0.951-1.051)	0.982
Sex		
Female	Reference	Reference
Male	1.337 (0.503-3.554)	0.560
Site of disease		
Intrahepatic cholangiocarcinoma	Reference	Reference
Extrahepatic cholangiocarcinoma	0.286 (0.056-1.450)	0.131
Gallbladder cancer	2.063 (0.680-6.264)	0.201
Line of treatment		
Second line	Reference	Reference
Third line	1.429 (0.542-3.759)	0.470
Disease progression	0.450 (0.146-1.389)	0.165

Odds ratio, OR; confidence interval, CI

Table 5. Univariate and multivariable cox proportional hazards regression models for overall survival

Variable	Univariate		Multivariable	
	HR (95% CI)	**p-value**	**HR (95% CI)**	**p-value**
Age	1.017 (0.992-1.042)	0.176		
Sex				
Female	Reference	Reference		
Male	1.002 (0.607-1.654)	0.994		
Site of disease				
IHC	Reference	Reference	Reference	Reference
EHC	2.117 (1.132-3.958)	**0.019**	2.119 (1.177-4.108)	**0.013**
GBC	1.450 (0.806-2.610)	0.215	1.782 (1.067-2.977)	0.282
Line of treatment				
Second line	Reference	Reference		
Third line	1.557 (0.946-2.562)	0.082		
Disease progression	0.774 (0.428-1.401)	0.397		
sGal9				
Low sGal9	Reference	Reference	Reference	Reference
High sGal9	1.745 (1.047-2.908)	**0.033**	1.782 (1.067-2.977)	**0.027**

Intrahepatic cholangiocarcinoma, IHC; extrahepatic cholangiocarcinoma, EHC; gallbladder cancer, GBC; soluble galectin-9, sGal-9; hazard rate, HR; confidence interval, CI

Supplementary Table 1. Subset analysis of all patients, stratified by treatment allocation

Variable	Arm A (n=36)	Arm B (n=38)	p-value
Site of disease, n (%)			0.64
Intrahepatic cholangiocarcinoma	18 (50)	22 (58)	
Extrahepatic cholangiocarcinoma	7 (19)	8 (21)	
Gallbladder cancer	11 (31)	8 (21)	
Overall survival months, median (IQR)	5.2 (3.0-10.2)	5.8 (3.0-12.1)	0.66
sGal9, n (%)			0.58
Low sGal9	25 (69)	23 (61)	
High sGal9	11 (31)	15 (39)	

Soluble galectin-9, sGal-9; interquartile range, IQR

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